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Distribution of β-lactamase genes of *Klebsiella pneumoniae* isolates in Zhejiang province, China, and regulation of gene expression

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Abstract: *Klebsiella pneumoniae* is a common causative agent of nosocomial infections with a high level of resistance toward β-lactam antibiotics. Our previous study showed that TEM-1 and SHV-11 are the predominant β-lactamase-encoding genes of *K. pneumoniae* isolates in the Zhejiang area, China. In this study, more clinical *K. pneumoniae* isolates were collected for detecting their β-lactamase-encoding gene profiles by PCR and sequencing. qRT-PCR was then performed to determine the role of cefotaxime or penicillin in low concentrations to induce the β-lactamase gene expression of *K. pneumoniae* isolates. Moreover, the *K. pneumoniae* isolates were pretreated with closantel (CLO), a histidine kinase inhibitor, before antibiotic treatment, and qRT-PCR and the β-lactamase phenotype confirmatory test were then applied to determine the effect of CLO on the expression of the β-lactamase genes. The results showed that, except for KPC-2, the 1/4 MIC cefotaxime or penicillin induced significant mRNA elevation of the TEM-1, CTX-M-14, SHV-11 and OXA-1 β-lactamase genes, but this induction could be inhibited by CLO. After pretreatment with CLO, 78.4~81.4% of the β-lactam-resistant isolates became sensitive and the positive rate of the β-lactamase production phenotype in the isolates was decreased from 100% to 27.1%. The data indicate that TEM-1 (70.7%), SHV-11 (64.2%) and CTX-M-14 (40.5%) are the predominant β-lactamase genes of the *K. pneumoniae* isolates in Zhejiang and sublethal dosage of β-lactam antibiotics can induce the β-lactamase gene expression of *K. pneumoniae* through histidine kinase-mediated two-component signaling systems.

Keywords: *Klebsiella pneumonia*; resistance; β-lactamases; gene expression; closantel

INTRODUCTION

Klebsiella pneumoniae, an important member of the *Enterobacteriaceae* family, is present as a saprophyte in the human gastrointestinal tract, nasopharynx and skin [1]. However, as an opportunistic pathogen, *K. pneumoniae* is frequently involved in nosocomial infections [2], causing many different diseases, such as pneumonia, urinary and biliary tract infections, wound and soft tissue infection, osteomyelitis and septicemia [3]. Cephalosporins, a group of β-lactam antibiotics, are the most valuable and frequently used drugs for treatment of *Klebsiella* infection. However, *K. pneumoniae* isolates commonly show high resistance rates toward β-lactam antibiotics, leading to inefficient antibiotic therapy and prolonged hospital stay and greater hospital charges for patients [4-6]. The most important resistance mechanism against β-lactam antibiotics is the production of β-lactamases [7-9]. Many different β-lactamases such as extended-spectrum β-lactamases (ESBLs) and carbapenemases from *K. pneumoniae* isolates have been identified, among which TEM, CTX-M, SHV and OXA are the most common ESBLs, and KPC is also frequently detected [10-12]. However, there is diversity between the predominant ESBLs and carbapenemase genes in *K. pneumonia* isolates from different areas [13-15].

Previous studies revealed that antibiotics can trigger specific transcriptional changes in several bacteria [16-18], including upregulation of the expression of genes responsible for resistance [18]. Our previous study also found that antibiotics used at lower concentrations can act as an environmental inducer to

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upregulate the expression of enzymes to inactivate different antibiotics in *S. aureus*, *E. coli* and *A. baumannii* [19], and two-component signaling systems (TCSS) are involved in the regulation of the gene expression [20]. TCSS, usually composed of a transmembrane histidine kinase (HK) and an intracellular response regulator (RR), are frequently administered as the drug targets of antimicrobials [21]. Therefore, inhibitors of TCSS, such as closantel (CLO), have a potential for developing novel antibacterial drugs [22].

In our previous study, we detected the β-lactamase gene profile of 118 β-lactam antibiotic-resistant *K. pneumoniae* isolates in Zhejiang province, and TEM-1 and SHV-11 were the predominant β-lactamase genes in these isolates [23]. In the present study, another 118 *K. pneumoniae* isolates were collected for detecting their β-lactamase gene profiles. To avoid possible interference among different β-lactamase genes, the isolates that only carry a single β-lactamase gene were subjected to 1/4 MIC β-lactam antibiotic treatment to determine the induction of sublethal dosage of β-lactam antibiotics on the expression of β-lactamase genes. Moreover, CLO, an HK inhibitor, was applied to understand the probable inhibitory effect of HK-based TCSS-mediated β-lactamase gene expression.

MATERIALS AND METHODS

Source and identification of *K. pneumoniae* isolates

In our previous study, we obtained 118 β-lactam antibiotic-resistant *K. pneumoniae* isolates [23]. In the present study, 272 *K. pneumoniae* isolates were collected. All the isolates were isolated by fractional cultivation on Columbia blood plates from sputum, pleural effusion, urine, peripheral blood of patients with pneumonia, upper respiratory tract infection, pleurisy, urethritis, bacteremia or septicemia from eight hospitals in Zhejiang province, and subsequently identified using the VITEK 2 Compact Automatic Microbial identification system plus GNI bacterial verification card (BioMérieux, France). Duplicates of each of the specimens were collected at the same time from the same sampling sites.

Drug sensitive tests

Susceptibility of the 272 *K. pneumoniae* isolates to penicillin (CPN), ampicillin (AMP), cefoxitin (CFX), cefotaxime (CTX) and ceftazidime (CAZ) was determined using the microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) of USA [24]. To obtain the accurate minimal inhibitory concentration (MIC) values of CTX and CPN against the β-lactam antibiotic-resistant isolates, E-tests were performed using CTX and CPN strips (BioMérieux). In these tests, *Escherichia coli* ATCC25922 and *K. pneumoniae* ATCC700603 were used as control strains.

Phenotypic detection of β-lactamases

Activities of ESBLs of the β-lactam antibiotic-resistant *K. pneumoniae* isolates were detected by double paper disk synergy screening test as recommended by the CLSI [24]. Briefly, the isolates were spread on Mueller Hinton (MH) agar plates (BioMérieux) and then CAZ and CAZ-clavulanic acid disks, and CTX and CTX-clavulanic acid disks (Oxoid, England) were placed on the seeded agar plates and the plates were incubated at 37°C for 24 h. Diameters exceeding 5 mm between the inhibition zones of the inhibitor compound antibiotic disks and single antibiotic disks were considered to be positive for β-lactamase. In the test, *E. coli* ATCC25922 and the *K. pneumoniae* ATCC700603 were used as the control strains. Furthermore, the Hodge test was applied to detect carbapenemase activity of the β-lactam antibiotic-resistant *K. pneumoniae* isolates from this study and the 118 β-lactam antibiotic-resistant *K. pneumoniae* isolates from our previous study using meropenem paper disks (Oxoid) according to the CLSI protocol [23,24].

Detection of β-lactamase genes

According to the reported consensus primer sequences [25-28], the PCR primers for amplification of the ESBL TEM, CTX-M, SHV and OXA genes and carbapenemase KPC, IMP and VIM genes were synthesized by Invitrogen Co. in Shanghai, China (Table 1). Several PCRs were performed to detect these genes in the β-lactam antibiotic-resistant *K. pneumoniae* isolates, and the PCR products were then sequenced

Table 1. Sequences of the primers used in PCR or qRT-PCR.

Primer	Sequence (5′ to 3′)	Target	Size (bp)
KPC-1	F: GCTACACCTAGCTCCACCTTC	detection of entire KPC gene	1050
	R: TCAGTGCTCTACAGAAAACC		
KPC-2	F: AGCCGCCAAAGTCCTGTTC	detection of partial KPC-mRNA	169
	R: ATTGCTACACCTAGCTCCACCTTC		
IMP	F: CTACCGCAGCAGAGTCTTTG	detection of entire IMP gene	587
	R: AACCAGTTTTGCCTTACCAT		
VIM	F: AGTGGTGAGTATCCGACAG	detection of entire VIM gene	261
	R: ATGAAAGTGCGTGGAGAC		
TEM-1	F: TCGGGGAAATGTGCG	detection of entire TEM gene	972
	R: TGCTTAATCAGTGAGGCACC		
TEM-2	F: CCCAGAAACGCTGGTGAAA	detection of partial TEM-mRNA	109
	R: GGGGCGAAAACTCTCAAGG		
SHV-1	F: GCCGGGTTATTTTATTTGTCGC	detection of entire SHV gene	1015
	R: TCTTTCCGATGCCGCCGCCAGTCA		
SHV-2	F: TTGATCCGCTCCGTGCT	detection of partial SHV-mRNA	121
	R: CCACAATCCGCTCTGCTTT		
CTX-M-1	F: GTTACAGCCCTTCGGCGATGATTC	detection of entire CTX-M gene	877
	R:GCGCATGGTGACAAAGAGAGTGCA		
CTX-M-2	F:TGCGGCTGGGTAAAATAGGT	detection of partial CTX-M-mRNA	120
	R:GTCGTGGACTGTGGGTGATAAG		
OXA-1	F:CTGTTGTTTGGGTTTCGCAAG	detection of entire OXA gene	440
	R:CTTGGCTTTTATGCTTGATG		
OXA-2	F:TATGGCATTTGATGCGGAAA	detection of partial OXA-mRNA	134
	R:GCGAAACCCAAACAACAGAAA		
16S RNA	F:CGGTCTGTCAAGTCGGATGT	internal reference in qRT-PCR	197
	R:TTTGCTCCCCACGCTTTC		

F – forward primer; R – reverse primer

by Invitrogen Co. [23]. The obtained sequencing data were compared to the corresponding sequences of β-lactamase genes of *K. pneumoniae* in GenBank using BLAST software.

Measurement of β-lactamase gene-mRNA levels

To avoid possible interference among different β-lactamase genes, 68 *K. pneumoniae* isolates carrying a single β-lactamase gene (of which 32 isolates are from our previous study and 36 isolates are from the present study) were selected for treatment with 1/4 MIC of CTX or CPN for 0.5, 1, 2 or 4 h at 37°C, and then the TEM, CTX-M, SHV, OXA and KPC mRNAs were detected by real-time fluorescent quantitative RT-PCR (qRT-PCR). Briefly, total bacterial RNAs were extracted with TRIzol (Sigma, USA), and then the cDNAs were synthesized from the total RNAs using a PrimeScript™ RT reagent kit (TaKaRa). Using the cDNAs as templates, the target gene mRNA levels were assessed by qRT-PCR using a SYBR® Premix Ex-Taq™ II Kit (TaKaRa) on a LightCycler 480 Real-Time PCR System (Roche, Germany). The primers used in the qRT-PCR were designed by Primer Premier 6.0 Software and synthesized by Invitrogen Co. (Table 1). In the qRT-PCR, 16S rRNA of *K. pneumoniae* was used as the internal control. The qRT-PCR data were analyzed using the ΔΔCT model and randomization test in REST2005 software [29].

Detection of CLO toxicity against *K. pneumoniae*

One hundred isolates were randomly selected from the *K. pneumoniae* isolates for treatment with 25, 50, 100, 250 or 500 μg/mL CLO (Sigma) at 37°C for 30 min [30], and then the MICs of CLO against the isolates were detected using the microdilution method as described above. Subsequently, 0.1 mL of each of the CLO-treated bacterial suspensions was inoculated on MH agar plates (BioMérieux) for a 24-h incubation

at 37°C. The bacterial growth was observed to determine the minimal bactericidal concentration (MBC) of CLO against the isolates [24]. In the detections, the CLO-untreated isolates were used as controls.

Detection of β-lactamase gene-mRNA levels after CLO treatment

The 68 *K. pneumoniae* isolates carrying a single β-lactamase gene were treated with 25, 50, 100, 250 or 500 μg/mL CLO (Sigma) at 37°C for 30 min, and then CTX or CPN with 1/4 MIC was added for a 60-min incubation period at 37°C. The TEM, CTX-M, SHV or OXA mRNA were detected by qRT-PCR as described above. In the assay, the CLO-untreated isolates were used as the controls.

Detection of β-lactamase activity after CLO treatment

One hundred μg/mL CLO was used to pretreat the 68 *K. pneumoniae* isolates carrying a single β-lactamase gene at 37C for 30 min. The change in β-lactamase activity in the isolates or the MICs of CPN, AMP, CFX, CTX and CAZ against the isolates were detected by the double paper disk synergy screening test or microdilution method as described above. In the assay, the CLO-untreated isolates were used as the controls.

Data analysis

Data from a minimum of three experiments were averaged and presented as means±standard deviation (SD). The χ^2 test and *t*-test were used to determine significant differences. Statistical significance was defined as $p<0.05$.

RESULTS

Resistance rate and β-lactamase phenotypes of the *K. pneumoniae* isolates

In this study, 118 of the 272 *K. pneumoniae* isolates were resistant to CPN, AMP, CFX, CTX and CAZ, with a resistance rate of 43.4% (118/272). The E-test result showed that the MICs of CTX and CPN against theβ-lactam antibiotic-resistant *K. pneumoniae* isolates were 4~64μg/mL. The phenotype confirmatory tests showed that all the β-lactam antibiotic-resistant isolates were phenotypically positive for ESBL production, but only 23 of the isolates were phenotypically positive for carbapenemase production.

Table 2. β-lactamase gene profiles of the 215 *K. pneumoniae* isolates.

β-lactamase genes and combination	Strains (n)	Percentage (%)
KPC-2	2	0.9
TEM-1	30	14.0
CTX-M-14	19	8.8
SHV-11	16	7.4
OXA-1	1	0.5
KPC-2+CTX-M-14	2	0.9
KPC-2+SHV-11	2	0.9
TEM-1+CTX-M-14	17	7.9
TEM-1+SHV-11	66	30.7
CTX-M-14+SHV-11	14	6.5
OXA-1+SHV-11	4	1.9
KPC-2+TEM-1+CTX-M-14	6	2.8
KPC-2+TEM-1+SHV-11	7	3.3
KPC-2+CTX-M-14+SHV-11	3	1.4
TEM-1+CTX-M-14+SHV-11	25	11.6
KPC-2+CTX-M-14+TEM-1+SHV-11	1	0.5
Total	215	100

Predominant β-lactamase genes of the *K. pneumoniae* isolates

Consistent with our previous study [23], the PCR and sequencing data showed that 91.5% of the 118 β-lactam antibiotic-resistant *K. pneumoniae* isolates in this study were positive for TEM-1, CTX-M-14, SHV-11, OXA-1 and/or KPC-2 genes according to a comparison with the reported β-lactamase gene sequences in GenBank (Accession No.: TEM-1/NC_009651.1, CTX-M-14/NC_016839.1, SHV-11/NC_016845.1, OXA-1/JQ235810.1, KPC-2/NC_016846.1). A combination of this result with the β-lactamase gene detection data from our previous study (107 in the 118 isolates) revealed that 68.4% of the β-lactam antibiotic-resistant *K. pneumoniae* isolates (147/215) possess more than two β-lactamase genes (Table 2), with TEM-1 (70.7%, 152/215) and SHV-11 (64.2%, 138/215) and CTX-M-14 (40.5%, 87/215) as the predominant genes of ESBLs. TEM-1 plus SHV-11 was the common combination of β-lactamase encoding genes carried by the isolates (30.7%, 66/215) (Table 2).

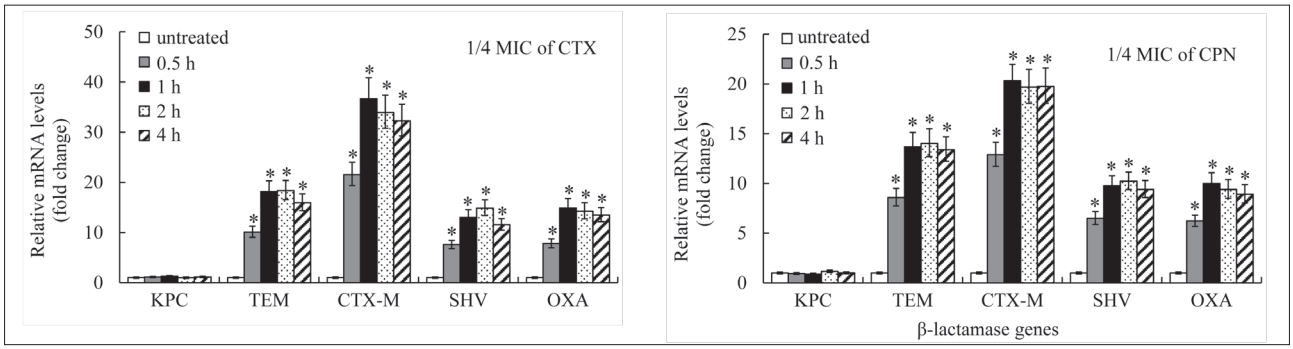

Fig. 1. Effect of CTX and CPN on upregulation of β-lactamase-mRNA levels. A total of 68 *K. pneumoniae* isolates carrying a single β-lactamase gene were subjected to 1/4 MIC of CTX or CPN treatment for the indicated times and the mRNA levels of KPC (2 isolates), TEM (30 isolates), CTX-M (19 isolates), SHV (16 isolates) and OXA (1 isolate) were detected by qRT-PCR. Bars show the means±SD of three independent experiments. *p<0.01 vs. the β-lactamase-mRNA levels in the CTX- or CPN-untreated isolates.

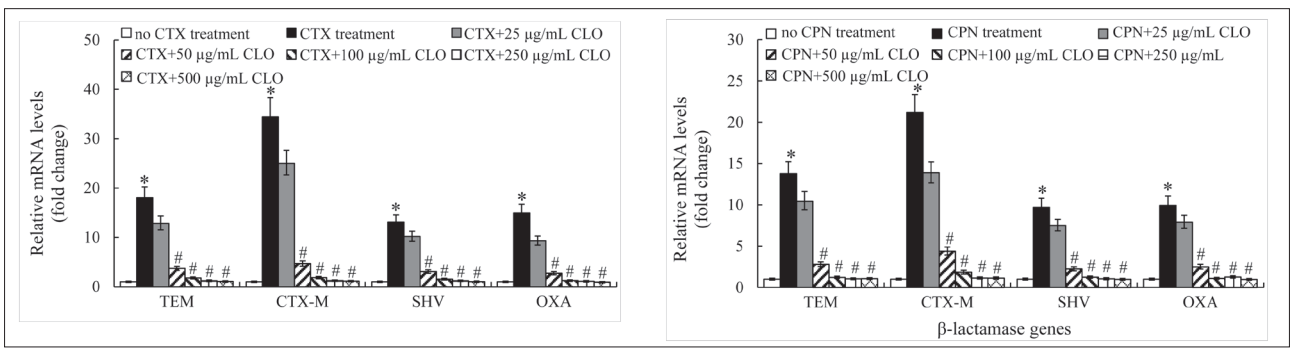

Fig. 2. Effect of CLO on inhibition of CTX- or CPN-induced β-lactamase-mRNA level elevation. The 68 *K. pneumoniae* isolates carrying a single β-lactamase gene were pretreated with different concentrations of CLO for 30 min, followed by treatment of 1/4 MIC of CTX or CPN for 1 h, and the TEM, CTX-M, SHV and OXA mRNA levels were detected by qRT-PCR. Bars show the means±SD of three independent experiments. *p<0.01 vs. the β-lactamase-mRNA levels in the CTX- or CPN-untreated isolates; #p<0.01 vs. the β-lactamase-mRNA levels in the CLO-untreated but CTX- or CPN-treated isolates.

Table 3. Changes of β-lactam antibiotic-resistance rate in CLO-treated *K. pneumoniae* isolates.

100 μg/mL CLO	Resistant isolates / sensitive isolates / sensitive rate (%)				
	CPN	AMP	CFX	CTX	CAZ
Before treatment	236/0/0	236/0/0	236/0/0	236/0/0	236/0/0
After treatment	51/185/78.4*	46/190/80.5*	49/187/79.2*	45/191/80.9*	44/192/81.4*

* p<0.01 vs. the isolates before CLO treatment

Increase of β-lactamase gene-mRNA levels after antibiotic treatment

In the total of 68 β-lactam antibiotic-resistant *K. pneumoniae* isolates carrying a single β-lactamase gene, the mRNA levels of TEM-1 (30 isolates), CTX-M-14 (19 isolates), SHV-11 (16 isolates) and OXA-1 (one isolate) genes, but not that of KPC-2 genes (2 isolates), were rapidly increased after treatment with 1/4 MIC of CTX or CPN at different time intervals (Fig. 1).

Inhibition of CLO on antibiotic-induced β-lactamase gene-mRNA level elevation

The toxicity test showed that 25~500 μg/mL CLO neither inhibited nor killed the *K. pneumoniae* isolates. When the 68 β-lactam antibiotic-resistant *K. pneumoniae* isolates carrying a single β-lactamase gene were pretreated with 50~500 μg/mL CLO, the 1/4 MIC of CTX- or CPN-induced elevation of the TEM-1, CTX-M-14, SHV-11 or OXA-1 mRNA levels was significantly inhibited in a dose-dependent manner (Fig. 2).

Changes of antibiotic resistance and ESBL phenotype after CLO treatment

When the 236 β-lactam antibiotic-resistant *K. pneumoniae* isolates from our previous study and the present study were pretreated with 100 μg/mL CLO as above, a great portion of the resistant isolates became sensitive to CPN, AMP, CFX, CTX or CAZ, resulting in the following percentages of sensitized isolates of 78.4% (185/236), 80.5% (190/236), 79.2% (187/236), 80.9% (191/236) and 81.4% (192/236) (Table 3). In addition, the rate of phenotypically positive ESBLs in the CLO-pretreated isolates was decreased from 100% (236/236) to 27.1% (64/236).

DISCUSSION

Drug resistance of bacteria is a global major public health challenge, which has resulted in the inefficacy of antibiotic treatments for infectious diseases caused by bacteria, including *K. pneumonia* [31]. Recent data announced by the CHINET bacterial resistance surveillance network showed that more than 70% of bacterial infectious diseases were caused by infection of Gram-negative bacteria in China, of which *K. pneumonia* infection accounted for 16.1% [32]. The CHINET resistance data revealed that the positive rate of different β-lactamase genes in *K. pneumoniae* isolates was 43.6%, while 31.8% of the isolates contained detectable ESBL genes [32]. Therefore, *K. pneumoniae* is a prominent causative agent of infectious diseases with a high rate of antibiotic resistance in China.

β-lactam antibiotics are commonly used for the treatment of bacterial infectious diseases. However, many bacteria, including *K. pneumoniae*, can resist β-lactam antibiotics through the production of different β-lactamases [33]. The most frequently found β-lactamases in clinical isolates of β-lactam antibiotic-resistant bacteria includes three major genetic groups: TEM, SHV and CTX-M types [10-12]. Recently, KPC- or OXA-positive *K. pneumoniae* isolates were also frequently reported [12,34]. However, distribution of the bacteria with different β-lactamase genes presents a geographical or local diversity. In North America and Western Europe, TEM and SHV types still dominate [12], while in Eastern Europe, Asia and South America, the CTX-M type has replaced TEM and SHV as the predominant ESBL [13,35-37]. In particular, CTX-M-9, CTX-M-14 and CTX-M-15 are the predominant CTX-M subtypes in Japan, China and India, respectively [38-40]. The diversity of ESBLs in bacteria was also found in different areas of China. For example, CTX-M is predominant in bacteria from Guangdong province but TEM is the most common ESBL in bacteria from Shanxi province [41,42]. Our previous study revealed the prevalence of TEM-1 and SHV-11 in *K. pneumoniae* isolates from Zhejiang province [23]. In this study, more *K. pneumoniae* isolates were collected and the high prevalence of both TEM-1 and SHV-11 in the isolates from Zhejiang province was further confirmed, which differed from the epidemiology of β-lactamase genes in Guangdong or Shanxi province [41,42]. Moreover, our results also showed that the multiple β-lactamase gene carrying rate in the isolates was significantly higher than the single gene carrying rate ($p<0.05$), where TEM-1 plus SHV-11 was the most common carrying mode.

Currently, a combination of β-lactam antibiotics with β-lactamase inhibitors, such as sulbactam, tazobactam and clavulanic acid, is the most common strategy to treat β-lactam antibiotic-resistant Gram-negative infectious diseases [43]. The β-lactamase inhibitors can inactivate class A β-lactamases, including CTX-M and the ESBL derivatives of TEM-1, TEM-2, and SHV-1, but they cannot inhibit the expression of β-lactamase genes [44]. TCSS is the sensor and effector of bacteria in adaptation to environments [45]. In different bacterial TCSSs, the sensor, histidine kinase (HK), accepts environmental signals and activates the response regulator (RR) by phosphorylation, while the activated RR regulates the expression of target genes [45,46]. Closantel (CLO), a salicylanilide anthelmintic, has been confirmed as an inhibitor of bacterial HK through enzymatic allosterism [48,49]. In this study, we chose cefotaxime (CTX) and penicillin (CPN) as the representatives of β-lactam antibiotics. To avoid the possible interference between different β-lactamase genes, a total of 68 *K. pneumoniae* isolates carrying a single β-lactamase gene were screened to detect the role of CTX or CPN with sublethal dosage (1/4 MIC) to induce the transcription of β-lactamase genes as well as the effect of CLO to inhibit the elevation of transcription. The results showed that, except for the KPC gene, 1/4 MIC of CTX or CPN caused a rapid and significant increase in the TEM-

1, CTX-M-14, SHV-11 or OXA-1 mRNA level, but it was inhibited by the administration of CLO. The data indicated that sublethal dosage of CTX or CPN acts as an inducer for the expression of the four ESBL genes in the *K. pneumonia* isolates probably through HK-related TCSS.

To eliminate the possible toxicity of CLO to the *K. pneumoniae* isolates that also can inhibit the elevation of antibiotic-induced ESBL-mRNAs, both MIC and MBC of CLO against the isolates were determined. The results showed that 25~500 μg/mL CLO could not present any effects to inhibit or kill the isolates tested, indicating that inhibition of HK but not its toxicity is involved in the role of CLO on the inhibition of the CTX- or CPN-induced ESBL-mRNA elevation. In particular, the addition of 100 μg/mL CLO caused the majority of the β-lactam antibiotic-resistant *K. pneumoniae* isolates to become sensitive to the five β-lactam antibiotics tested, and the phenotype of ESBLs in most of the CLO-pretreated isolates to disappear. Since HK-related TCSS is absent in eukaryotes [46,49], CLO may have a potential as a candidate for developing a novel drug against ESBL-producing bacteria.

CONCLUSIONS

TEM-1, SHV-11 and CTX-M-14 are the predominant β-lactamase genes in the β-lactam antibiotic-resistant *K. pneumoniae* isolates from Zhejiang province of China, and TEM-1 plus SHV-11 is the predominant combination of β-lactamase genes carried by the isolates. Sublethal concentrations of CTX or CPN act as an extrinsic inducer to upregulate the expression of TEM-1, SHV-11, CTX-M-14 and OXA-1 genes in the isolates, but it can be inhibited by CLO, a bacterial histidine kinase inhibitor. CLO was also capable of changing the sensitivity to β-lactam antibiotics and to limit the phenotype of ESBLs in the β-lactam antibiotic-resistant isolates.

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