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ISOLATION OF THE FIRST IMP-4 METALLO-B-LACTAMASE PRODUCING *KLEBSIELLA PNEUMONIAE* IN TIANJIN, CHINA

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ABSTRACT

This study shows for the first time the mechanism of carbapenem resistance of a *Klebsiella pneumoniae* clinical isolate TJ8 recovered from Tianjin Medical University General Hospital ,China. The modified Hodge test and EDTA synergy test were performed for the screening of carbapenemases and metallo-β-lactamases (MBLs), respectively. Polymerase chain reactions and DNA sequencing confirmed that the strain carried IMP-4 metallo-β-lactamase (MBL) , SHV-11 and TEM-1 β-lactamase.

Class Ⅰ integron was positive and gave a 3.0-kb PCR amplicon .IMP-4 was located in Class Ⅰ integron 5'CS. The gene determinants were organized in the order of bla_{IMP-4}-*orf*II-*orf*III.In all, the results show that IMP-4 MBL production caused the TJ8 resistance to carbapenems.

Key words: *Klebsiella pneumoniae*, metallo-β-lactamases, integron

INTRODUCTION

Carbapenems are relatively stable agents and commonly used to treat serious infections caused by multi-resistant gram-negative bacilli, especially strains producing high-level of AmpC cephalosporinases or extended-spetrum β-lactamases (ESBLs). However, the occurrence and spread of genes encoding metallo-β-lactamases (MBLs) including IMP, VIM, SPM and GIM have been reported in a variety of clinical isolates of *Enterobacteriaceae* from 28 countries (24).The metallo-β-lactamases emergence is becoming a therapeutic challenge. The class B enzymes, MBLs are the most clinically threatening carbapenems for their ability to hydrolyse almost all β-lactams except monobactams, and they are not susceptible to therapeutic β-lactams inhibitors such as clavulanate, sulbactam and tazobactam.

The bla_{IMP-4} gene ,which encodes IMP-4 MBL, was first identified in the late 1990s in *Acinetobacter* spp. from Hong Kong (2) and *Citrobacter youngae* from Guangzhou in the People's Republic of China (PRC) (7), was recognized in Australian *Enterobacteriaceae* isolates on a road-host-range conjugative plasmid in Sydney, Australia, in 2003 (4) and caused a simultaneous outbreak in Melbourne, Australia (15). In China, there were only three reports on the production of IMP in *Klebsiella pneumoniae*. An IMP-4-producing *K. pneumoniae* from Wuhan in 2008 (13) and an IMP-4-producing *K. pneumoniae* from Shanghai in 2009 (9).

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In this study, an ertapenem-resistant *K. pneumoniae* strain was collected from our hospital and its mechanisms of carbapenem resistance were analyzed. To our knowledge, this is the first report of a clinical isolate of *K. pneumoniae* producing a MBL in Tianjin district of China. A further study revealed that this *K. pneumoniae* strain carried integron-borne bla_{IMP-4}.

MATERIALS AND METHODS

Bacterial strains and reagents

In November 2009, a *K. pneumoniae* strain (TJ8) was isolated from sputum sample from a patient in cadre ward in Tianjin Medical University General Hospital ,China. The strain isolated was identified as *K. pneumoniae* by using the Vitek2 Compact system (bioMe´rieux, France).

Antimicrobial susceptibility testing

Susceptibility testing was performed by using the Vitek2 Compact system and the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (3) .

Carbapenemase assays

A modified Hodge test was performed to screen carbapenemase production (3). A suspension of *E. coli* ATCC 25922, which was adjusted to the turbidity of 0.5 McFarland standard, and inoculated evenly on a Mueller Hinton agar plate. Then, a meropenem disk (10μg) was placed at the center of the plate. Test strains were streaked heavily from the edge of the disk to the periphery of the plate. The presence of a distorted inhibition zone after 16 to 18 hours of incubation at 35ºC interpreted as a positive modified Hodge test. *Escherichia coli* ATCC 25922 and *Klebsiella pnenmoniae* producing KPC-2 carbapenemase were used as reference strains.

An EDTA-disk synergy test was used for the screening of metallo-β-lactamase production. The test strain was adjusted to a turbidity of 0.5 McFarland standard and spread on the surface of a MHA plate. A meropenem disk (10μg) and a sterilized blank filter paper disk were placed 15 mm apart from edge to edge on the agar plate. Four μL of EDTA solution (0.5 M) was applied to the blank disk. After 16 to 18 hours of incubation at 35ºC, the presence of an enlarged zone of inhibition was interpreted as EDTA-synergy test positive (10).

Polymerase chain reaction (PCR) amplification and DNA sequence analysis of β-lactamase genes and Class 1 integrons

Escherichia coli ATCC 25922 was used as negative control, the TJ8 isolate was used as test strain. Prepared bacterial DNA of the TJ8 and *Escherichia coli* ATCC 25922 were used as templates .The primers used to amplify bla_{CTX-M} (5), bla_{SHV} (16), bla_{TEM} (14), bla_{KPC} (13), bla_{IMP} (20), bla_{VIM} (23), bla_{SME} (18) , bla_{DHA} , bla_{ACT} (17), Class 1 integrase gene(8) and Class 1 integrons (11) were described previously. The primers used for detecting resistance gene cassettes and associated integrons are listed in Table 1. Reaction mixtures (25uL) contained 2.5uL of 10XEasyTaq Buffer, 0.2mM of dNTPs, 2.5 U of EasyTaq DNA Polymerase, 0.2 uM of each primer, and 2 uL of the bacterial DNA template. The reaction was conducted in a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer Co. USA). PCR was performed under the following conditions: initial denaturation at 94ºC for 5 minutes, 35cycles of 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 1 minute, and a final elongation at 72ºC for 10 minutes. PCR amplification products were purified and sequenced using an ABI PRISM 3730XL sequencer analyzer (ABI Co. USA). Sequences were compared with the GenBank database via the BLAST network service.

Table 1. List of the primers used in this study

Primer	sequence（5´-3´）	PCR product (bp)	Reference
bla_{KPC}	F: GCTACACCTAGCTCCACCTTC	920	13
	R:ACAGTGGTTGGTAATCCATGC		
bla_{IMP}	F: CTACCGCAGCAGAGTCTTTG	587	20
	R:AACCAGTTTTGCCTTACCAT		
bla_{VIM}	F: AGTGGTGAGTATCCGACAG	261	23
	R:ATGAAAGTGCGTGGAGAC		
bla_{SME}	F: AACGGCTTCATTTTTGTTTAG	830	18
	R: GCTTCCGCAATAGTTTTATCA		
bla_{CTX-M}	F: TTTGCGATGTGCAGTACCAGTAA	544	5
	R:CGATATCGTTGGTGGTGCCATA		
bla_{SHV}	F: TGGTTATGCGTTATATTCGCC	870	16
	R:GGTTAGGGTTGCCAGTGCT		
bla_{TEM}	F: GTATGGATCCTCAACATTTCCGTGTCG	862	14
	R:ACCAAAGCTTAATCAGTGAGGCA		
bla_{DHA}	F: AACTTTCACAGGTGTGCTGGGT	405	17
	R:CCGTACGCATACTGGCTTTGC		
bla_{ACT}	F:TCGGTAAAGCCGATGTTGCGC	302	17
	R:CTTCCACTGCGGCTGCCAGTT		
Class 1	F:CAGTGGACATAAGCCTGTTC	160	8
integrase gene	R: CCCGAGGCATAGACTGTA		
Class 1 integrons	F: GGCATCCAAGCAGCAAGC	various	11
	R: AAGCAGACTTGACCTGA		

RESULTS

Antibiotics susceptibility

MIC method showed reduced susceptibility to imipenem and meropenem with MICs of 2 and 4 μg/ml, and resistance to ertapenem with MIC of 16 μg/ml. The isolate was highly resistant to β-lactams except piperacillin/tazobactam ,quinolones ,aminoglycosides and aztreonam. These results suggest the production of MBL. However, K-B method showed resistance to imipenem and meropenem with zone of 13 and 10 mm. (Table 2).

Table 2. Susceptibility tests and mechanisms of resistance of *K. pneumoniae* TJ8

Antibiotic	Disk diffusion test(mm)	MIC(ug/ml)	Resistance mechanism	Integron
Ertapenem	ND/ND	4/I		
Imipenem	13/R	2/S	IMP-4 SHV-11 TEM-1	IMP-4-*orf*II-*orf*III
Meropenem	10/R	ND/ND		

S, susceptible; I, intermediate; R, resistant; ND, not determined.

Carbapenemase assays

The isolates was positive for carbapenemase by modified Hodge test (Fig. 1). EDTA-disk synergy test was positive (Fig. 2) which indicated the isolate probably produced metallo-β-lactamase.

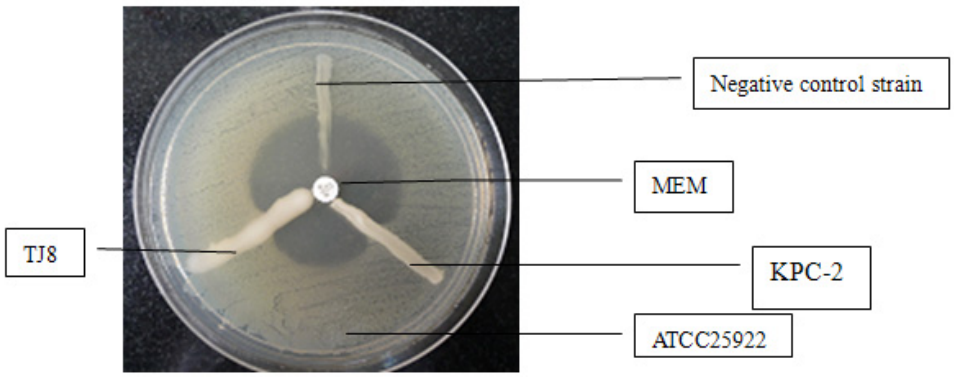

Figure 1. Modified Hodge Test. *E.coli* ATCC 25922 was spread on the surface of Müeller-Hinton agar and a meropenem disk (10μg) was placed at the center of the plate.KPC-2 positive control; negative control and *K. pneumoniae* TJ8 clinical isolate.

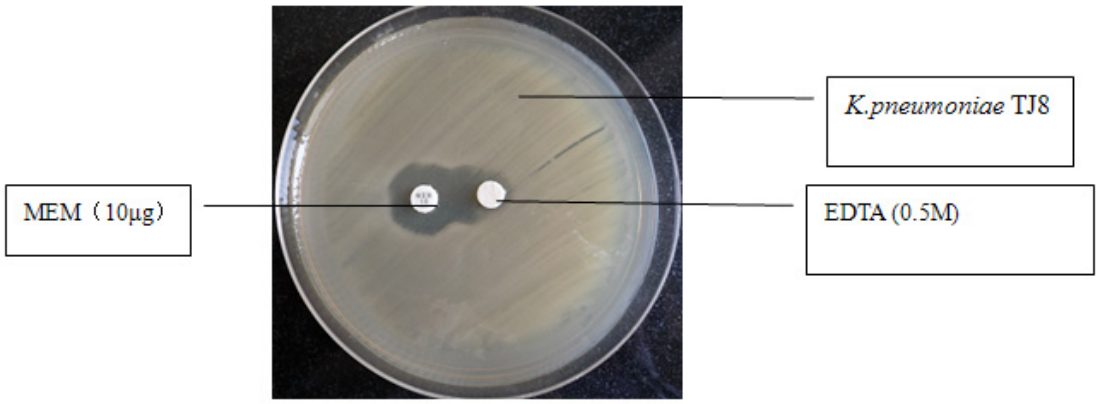

Figure 2. Inhibition test for detection of MBL producer by use of EDTA

PCRs and DNA sequence analysis

PCR amplification from DNA preparations of TJ8 yielded corresponding products for *int1*, bla_{IMP}, bla_{SHV}, bla_{TEM}, but not for bla_{CTX-M}, bla_{KPC}, bla_{SME} , bla_{VIM} , bla_{DHA} or bla_{ACT}. (Figure 3 showed an amplified product of bla_{IMP}).Sequencing of the amplified products revealed the presence of bla_{IMP-4}, bla_{SHV-11} and bla_{TEM-1}. The Class 1 integron was detected by overlapping PCR techniques. *K. pneumoniae* strain TJ8 gave a 3.0-kb PCR amplicon for class 1 integron that contained bla_{IMP-4}. The gene determinants were organized in the order of bla_{IMP-4}-*orf*II-*orf*III.

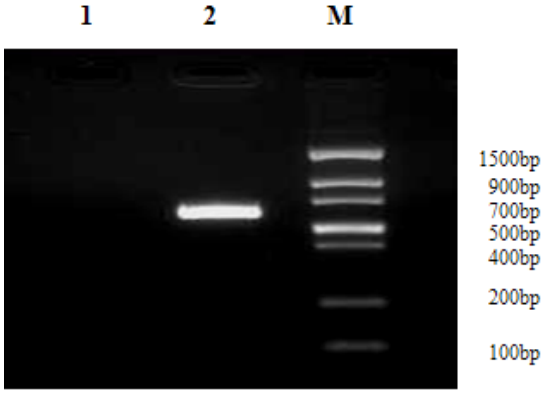

Figure 3. PCR of bla_{IMP-4} gene. Lanes: 1-negative control; 2-amplified product of bla_{IMP-4} gene; M-1500 bp DNA Ladder

DISCUSSION

IMP-type MBLs were most frequently detected among gram-negative nonfermenters. IMP-producing *Enterobacteriaceae* were mainly reported from Japan, Australia, and Taiwan region of China. Production of IMP in *Enterobacteriaceae* was rare in mainland China. Moreover, MBL genes are usually found as gene cassettes in integrons, mostly in class 1 integrons (21). Integrons are not self-transferred elements, but they can capture and carry genes, particularly antibiotic-resistance genes, by site-specific recombination(1, 6, 22). Therefore, the metallo-β-lactamases emergence is becoming dangerous. Five classes of integron are known to play a role in the dissemination of antibiotic resistance, and class 1 integrons are the most extensively studied(12). Typical class 1 integrons contain two conserved segments (CSs), the 5'- CS and 3'-CS. The 5'-CS includes the intI1 gene encoding integrase, the attI site for addition of the inserted gene cassette and a promoter(s). The 3'-CS is composed of the qacED1 and sul1 genes, which are responsible for resistance to quaternary ammonium compounds and sulphonamides, respectively. In this study, we detected $bla_{\text{IMP-4}}$ and class 1 integrons in a clinical isolate of ertapenem-resistant *K. pneumoniae* strain. In addition, further studies revealed a variable region of class 1 integron where $bla_{\text{IMP-4}}$ gene was inserted.

Similar to results in previous reports (13, 9), the *K. pneumoniae* isolate in this study showed "susceptible" results to IMP and MEM by using automated instrumentation, and in the mean-time, the strain showed resistant results to IMP and MEM by using K-B method. As is reported by some researchers, automated instrumentation may fail to detect the resistance of carbapenem strains against carbapenem antibiotics. The main cause is that carbapenem strains have clear inoculation effect, which affects the MIC of imipemen.

IMP-4 metallo-β-lactamase producing *K. pneumoniae* emergence represents a serious diagnostic challenge for microbiology laboratories, as well as challenges for infection control and antimicrobial drug management. IMP might spread without attracting attention. Microbiologists should be aware of suspicious gram-negative bacteria with reduced susceptibility to carbapenems, and examine their carbapenemase activity.

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