academicJournals

Vol. 9(4), pp. 239-247, 28 January, 2015
DOI: 10.5897/AJMR2014.7289
Article Number: C20B2A250422
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Molecular characterization and phenotypic analysis of multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates from a tertiary-care hospital in Yunnan Province, China

Tan, H. L.[1#], Wang, Y.[2,3#], Cheng, X. Q.[2#], Huang, Y. M.[1] and Zhang, L. J.[2]*

[1]Department of Clinical Microbiology Laboratory, The Third People's Hospital of Yunnan Province, Kunming , People's Republic of China.
[2]The National Institute for Communicable Disease Control and Prevention, China CDC, Beijing, People's Republic of China.
[3]College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang Province, People's Republic of China.

Received 20 November, 2011; Accepted 19 January, 2015

Due to the increasing number of multidrug-resistant (MDR) isolates of *Escherichia coli* and *Klebsiella pneumoniae* reported from the Third People's Hospital of Yunnan Province, an investigation was conducted to better understand the phenotype and molecular characterization of the local isolates. Twenty three non-duplicate *E. coli* isolates and nine *K. pneumoniae* isolates were recovered from hospitalized patients and identified and tested for antimicrobial susceptibility using the VITEKw2 system. Drug-resistant genes were amplified and sequenced, and pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) analyses were performed on the tested isolates. All of the isolates, except for one extensively drug-resistant (XDR) *K. pneumoniae* isolate, were demonstrated to be MDR, and 100% of the *E. coli* and *K. pneumoniae* were resistant to ampicillin, cefuroxime, cefazolin and ceftriaxone. Of the isolates, 69.6% of *the E. coli* and 100% of the *K. pneumoniae* isolates were bla_{CTX-M} positive, with CTX-M-55 and CTX-M-15 as the leading genotypes. All *K. pneumoniae* isolates shared bla_{SHV} genes with the dominant SHV-11 genotype. A total of 87.5% *E. coli* and 77.8% of *K. pneumoniae* carried the IS*Ecp1*, 91.3% of *E. coli* and 77.8% of *K. pneumoniae* shared the *int1* gene, and 44.4% of *K. pneumoniae* presented the IS*CR1* gene. A large genetic heterogeneity of *K. pneumoniae* and *E. coli* isolates was confirmed by MLST and PFGE analyses. The high frequency of MDR *E. coli* and *K. pneumoniae* in local areas may be a substantial challenge for infection control.

Key words: *Klebsiella pneumonia*, *Escherichia coli*, molecular characterization, multidrug-resistant.

INTRODUCTION

Multidrug-resistant gram-negative bacteria (GNB) have increased globally in recent years (Adams-Sapper et al., 2012; Drees et al., 2014; Tacconelli et al., 2014). These drug-resistant GNB can not only cause outbreaks in community settings (Smith et al., 2008; Wei et al., 2005) but also spread throughout the world in many ways, including by person-to-person transmission following foreign travel and through widely distributed food

products contaminated with drug-resistant GNB (Peirano and Pitout, 2010; Kumarasamy et al., 2010; Johnson et al., 2010). Clinical settings are important reservoirs and sources of drug-resistant GNB pathogens and drug-resistance genes. Antimicrobial agents used in hospitals may facilitate the transfer of mobile drug-resistance genes across different lineages of the same bacterial species or across different bacterial species by horizontal gene transfer. Notably, the emergence and spread of MDR *E. coli* and *K. pneumoniae* are associated with significant morbidity and mortality (Sievert et al., 2013; Carbonne et al., 2013). In recent years, a significant increase in the number of MDR isolates of *E. coli* and *K. pneumoniae* has been reported from the clinical microbiology laboratory in the Third People's Hospital of Yunnan Province (Tan et al., 2014), a tertiary-care hospital in Kunming, the capital city of Yunnan Province, China. To better understand the phenotype and molecular characterization of the drug resistance determinants of local *E. coli* and *K. pneumoniae* isolates and to monitor the emergence of novel antimicrobial resistance isolates, a joint investigation was conducted between the National Institute for Communicable Disease Control and Prevention, China CDC, People's Republic of China and the Third People's Hospital of Yunnan Province, People's Republic of China, from June to September 2013.

MATERIALS AND METHODS

Ethics statement

All protocols in the study were approved by the institutional ethics committee of the Third People's Hospital of Yunnan Province. Written consent was given by the patients for the use of pathogens isolated from them and for the evaluation of information in their medical records for research purposes. All samples and information were made anonymous.

Study design, specimen collection and patient demographics

This study was a prospective investigation of *E. coli* and *K. pneumoniae* isolates recovered from patients in the microbiology laboratories of the Third People's Hospital of Yunnan Province (a 1,000-bed tertiary-care hospital serving approximately 110,000 inpatients per year) between June and September 2013. Among these patients who had positive *E. coli* cultures, four were from the department of nephrology, three were from the intensive care unit (ICU), three were from the department of urinary surgery, three were from the department of geriatrics, two were from the department of traditional chinese medicine, one was from the department of physiatry, one was from the department of endocrinology, one was from the department of gastroenterology, one was from the department of general surgery, one was from the department of respiration, one was from the department of internal neurology, one was from the department of neurosurgery and one was from the department of chest surgery. Of the patients who had positive *K. pneumoniae* cultures, three were from the ICU, two were from the department of respiration, one was from the department of physiatry, one was from the department of neurosurgery, one was from the department of nephrology and one was from the department of urinary surgery.

All patient demographics were recorded, including age, sex, hospital ward, and the types of specimens. The first isolate was chosen in the case of duplicate patient samples.

Identification and antimicrobial susceptibility testing of isolates

Bacteria were isolated from patients according to the standard protocol from the Manual of Clinical Microbiology Laboratory (Zhou et al., 2010). Identification and antimicrobial susceptibility testing of isolates were conducted with standard biochemical tests using the bioMérieux VITEK-2 system following the manufacturer's instructions. Identification of isolates to the species level was further confirmed by amplifying and sequencing the 16S rRNA gene with universal prokaryotic bacterial 16S rRNA primers (Weisburg et al., 1991). A total of 21 drugs were included in the antimicrobial susceptibility testing: ampicillin (AMP), piperacillin/tazobactam (TZP), ampicillin/sulbactam (SAM), cefuroxime (CXM), cefazolin (CFZ), ceftriaxone (CRO), ceftazidime (CAZ), cefoperazone (SCF), cefepime (FEP), cefotetan (CTT), ertapenem (ETP), meropenem (MEM), imipenem (IMP), aztreonam (ATM), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GM), tobramycin (TM), kanamycin (AN), trimethoprim-sulfamethoxazole (SXT) and furadantin (FD). The *E. coli* strains: ATCC 25922 and ATCC 35218 and the *K. pneumoniae* strain ATCC 700603 were used as quality control strains. According to the standardized international definitions of multidrug-resistant (MDR) (Magiorakos et al., 2012), MDR was defined as resistant to at least one agent in three or more agent categories, extensively drug-resistant (XDR) was defined as resistant to at least one agent in all but two or fewer antimicrobial categories, and pandrug-resistant (PDR) was defined as resistant to all agents in all antimicrobial categories.

Amplification and sequencing of the *bla*gene and gene-capturing elements

Genomic DNA was extracted using a DNeasy® Blood and Tissue Kit (QIAGEN, Hilden, German, Cat No. 69506) and plasmid DNA was extracted using a high-purification plasmid mini-preparation kit (BioTeke Corporation, Beijing, China, Cat#DP1002). PCR was performed using a SensoQuest LabCycler standard plus (SensoQuest GmbH, Goettingen, Germany) with *Taq* DNA polymerase (SBS Genetech Co., Ltd, China, Lot#042512). All bacteria were subjected to PCR for the detection of the *bla* genes with the primers listed in Table 1 using genomic and plasmid DNA of the bacteria as templates. The PCR conditions were based on the reference sources listed in Table 1. The PCR products were sequenced in both directions by two separate commercial sequencing companies in China (Beijing Tsingke BioTech Co., Ltd.

*Corresponding author. E-mail: zhanglijuan@icdc.cn. Tel/Fax: 0086-10-58900780.

#Contributed equally to this work.

Table 1. Primers used for PCR amplification of the *bla* gene and gene-capturing elements

Target	Primer name	Primer sequence (5'–3')	Product size (bp)	Reference
***bla* gene**				
TEM	TEM-F	TCCGCTCATGAGACAATAACC	931	Sturenburg et al., 2004
	TEM-R	TTGGTCTGACAGTTACCAATGC		
SHV	SHV-F	TGGTTATGCGTTATATTCGCC	868	Pai et al.,1999
	SHV-R	GGTTAGCGTTGCCAGTGCT		
CTX-M	CTX-F	TCTTCCAGAATAAGGAATCCC	909	Sturenburg et al., 2004
	CTX-R	CCGTTTCCGCTATTACAAAC		
VEB	VEB-F1	GATAGGAGTACAGACATATG	914	Pastera´n et al., 2006
	VEB-R1	TTTATTCAAATAGTAATTCCACG		
OXA-2 group	OXA-2-F	AAGAAACGCTACTCGCCTGC	478	Yan et al., 2006
	OXA-2-R	CCACTCAACCCATCCTACCC		
OXA-10 group	OXA-10-F	GTCTTTCGAGTACGGCATTA	720	Bert et al., 2002
	OXA-10-R	ATTTTCTTAGCGGCAACTTAC		
PER	PER-F	ATGAATGTCATCACAAAATG	927	Celenza et al., 2006
	PER-R	TCAATCCGGACTCACT		
GES	GES-F	ATGCGCTTCATTCACGCAC	864	Vourli et al., 2004
	GES-R	CTATTTGTCCGTGCTCAGG		
Gene-capturing elements				
int1	*int 1* F	CCTCCCGCACGATGATC	280	Chen et al., 2013
	int 1 R	TCCACGCATCGTCAGGC		
IS*CR1*	*CR1* F	ATGTCTCTGGCAAGGAACGC	1450	Chen et al., 2013
	CR1 R	AGACGACTCTGTGATGGATC		
IS*Ecp1*	IS-F	GTGCCCAAGGGGAGTGTATG	615	Kiratisin et al., 2007
	IS-R	ACYTTACTGGTRCTGCACAT		

and Shanghai Sangon BioTech Co., Ltd.). Sequencing was performed with an ABI 3100 genetic analyzer (Applied Biosystems) using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The sequences were analyzed using the nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov/).

IS*Ecp1*, which plays a key role in gene transfer (Kiiru et al., 2013; Dhanji et al., 2011; Tian et al., 2011), is often located in the upstream region preceding bla_{CTX-M}, an emerging and highly prevalent CTX-M genotype among *E. coli* and *K. pneumoniae* strains worldwide. The IS*Ecp1* distribution among the isolates was assayed by PCR using primers targeting IS*Ecp1* (Table 1).

In addition, the presence of the key gene-capturing elements *int1* and IS*CR1* was determined by PCR using primers targeting *int1* and IS*CR1* (Table 1) and genomic DNA (gDNA) from the bacteria because the expression of *int1* and IS*CR1* is closely associated with the dissemination of MDR bacteria among clinical isolates.

MLST and PFGE analysis

To study the clonal relationships of the isolates tested, MLST and PFGE were conducted. According to the MLST protocol described online (www.pasteur.fr/mlst), 7 genes, including *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB*, were used for genotyping the *K. pneumoniae* isolates, and 8 genes, including *dinB*, *icdA*, *pabB*, *polB, putP*, *trpA*, *trpB* and *uidA*, were used for genotyping the *E. coli* isolates. The PCR products from MLST were sequenced as described in the aforementioned methods. Sequence types (STs) were assigned using the MLST database (www.pasteur.fr/mlst).

PFGE analysis of *E. coli* was performed using a CHEF Mapper XA apparatus (Bio-Rad Laboratories, Hercules, CA) according to the standard protocol of the International Molecular Subtyping Network for Foodborne Disease Surveillance (http://www.pulsenetinternational.org/protocols/), and the PFGE protocol for *K. pneumoniae* was performed as previously described (Han et al., 2013). The PFGE banding patterns were analyzed using Fingerprinting II Software, version 3.0 (Bio-Rad Laboratories, Hercules, CA). A PFGE group was defined as having more than 80% similarity.

RESULTS

Specimen collection and patient demographics

During the study period, a total of 23 non-duplicate *E. coli* isolates and nine *K. pneumoniae* isolates were obtained from hospitalized patients. The proportions of males and females were 61.0% (n= 19) and 41.2% (n= 13), respectively, and the median age of the patients was 61 years (range 3.0–88.0 years). 23 *E. coli* isolates were recovered from 12 urine specimens, four blood cultures, three sputum specimens, two secretion specimens, one pus specimen and one sterile cavity fluid specimen, while nine *K. pneumoniae* isolates were isolated from five sputum specimens, two urine specimens, one blood culture and one throat swab specimen.

Table 2. Drug-resistance rates of *E. coli* and *K. pneumonia*

Antimicrobial category	Agent[a]	*E. coli* % (No. positive/No. tested)			*K. pneumoniae* % (No. positive/No. tested)			p
		S[b]	I[b]	R[b]	S	I	R	
Ampicillin	AMP	0	0	100(23/23)			100(9/9)	
Antipseudomonal penicillin + beta-lactamase inhibitors	TZP	87.0(20/23)	4.3(1/23)	8.7(2/23)	33.3(3/9)	55.6(5/9)	11.1(1/9)	<1.0
penicillin + beta-lactamase inhibitors	SAM	4.3(1/23)	13.0(3/23)	82.6(19/23)		11.1(1/9)	88.9(8/9)	<1.0
1st and 2nd generation cephalosporin	CXM	0	0	100(23/23)	0	0	100(9/9)	
	CFZ	0	0	100(23/23)	0	0	100%(9/9)	
3rd and 4th generation cephalosporin	CRO	0	0	100(23/23)	0	0	100(9/9)	
	CAZ	47.8(11/23)	0	52.2(12/23)	11.1(1/9)	11.1(1/9)	77.8(7/9)	0.2
	SCF	73.9(17/23)	17.4(4/23)	2/23	44.4(4/9)	33.3(3/9)	22.2(2/9)	0.6
	FEP	52.2(12/23)	4.3(1/23)	43.5(10/23)	33.3(3/9)	11.1(1/9)	55.6(5/9)	0.7
Cephamycin	CTT	91.3(21/23)	4.3(1/23)	4.3(1/23)	77.8(7/9)	11.1(1/9)	11.1(1/9)	0.5
Carbapenem	ETP	100(23/23)	0	0	88.9(8/9)	0	11.1(1/9)	
	MEM	100(23/23)	0	0	88.9(7/9)	0	11.1(1/9)	
	IMP	100(23/23)	0	0	88.9(8/9)	0	11.1(1/9)	
Monobactam	ATM	13.0(3/23)	0	87.0(20/23)	11.1(1/9)	0	88.9(8/9)	<1.0
Fluoroquinolone	CIP	8.7(2/23)	0	91.3(21/23)	44.4(4/9)	0	55.6(5/9)	0.0006
	LEV	17.4(4/23)		82.6(19/23)	66.7(6/9)	0	33.3(3/9)	0.01
Aminoglycoside	GM	34.8(8/23)	0	65.2(15/23)	55.6(5/9)	0	44.4(4/9)	0.4
	TM	39.1(9/23)	21.7(5/23)	39.1(9/23)	33.3(3/9)	11.1(1/9)	55.6(5/9)	0.5
	AN	95.6(22/23)	4.3(1/23)	0	55.6(5/9)	0	44.4(4/9)	0.004
Folate pathway inhibitors	SXT	39.1(9/23)	0	60.9(14/23)	44.4(4/9)	0	55.6(5/9)	<1.0
Nitrofurantoin	FD	65.2(15/23)	30.4(7/23)	4.3 (1/23)	22.2(2/9)	11.1%(1/9)	66.7%(6/9)	0.0006
Total		57.0(223/391)	12.5(23/184)	63.8(235/368)	49.3(71/144)	19.4(14/72)	54.5(103/189)	<0.0001

[a]Abbreviations of drugs: AMP, Ampicillin; TZP, Piperacillin/Tazobactam; SAM, Ampicillin/Sulbactam; CXM, Cefuroxime; CFZ, Cefazolin; CRO, Ceftriaxone; CAZ, Ceftazidime; SCF, Cefoperazone; FEP, Cefepime; CTT, Cefotetan; ETP, Ertapenem; MEM, Meropenem; IMP, Imipenem; ATM, Aztreonam; CIP, Ciprofloxacin; LEV, Levofloxacin; GM, Gentamycin; TM, Tobramycin; AN, Kanamycin, SXT, Trimethoprim-Sulfamethoxazole; FD, Furadantin. [b]S, susceptible; I, intermediate; R, resistant.

Antimicrobial susceptibility

Detailed information on the resistance rates to all tested drugs is listed in Table 2. All of the *E. coli* and *K. pneumoniae* isolates exhibited resistance to AMP, CXM and CFZ and even to CRO (3rd generation cephalosporin). In addition, most *K. pneumoniae* and *E. coli* isolates were resistant to ATM (88.9% for *K. pneumoniae* and 87.0% for *E. coli*), SAM (88.9% for *K. pneumoniae* and 82.6% for *E. coli*) and CAZ (77.8% for *K. pneumoniae* and 52.2% for *E. coli*). Notably, higher resistance rates to CIP, LEV, GM, TM, AN, SXT and FD were detected in 55.6%, 33.3%, 44.4%, 55.6%, 44.4%, 55.6% and 66.7% of *K. pneumoniae*.

Table 3. Molecular characterization of *bla*genes among *E. coli* and *K. pneumoniae* isolates

Organism	Genotype of *bla* gene	No. of isolates
E. coli	TEM-1	1
	TEM-1, CTX-M-55	5
	TEM-1, CTX-M-15	2
	TEM-1, CTX-M-3	1
	CTX-M-15	5
	CTX-M-55	3
	TEM-1, CTX-M-55, SHV-11	1
	TEM-1, CTX-M-15, SHV-1/148	1
	TEM-1, CTX-M-55, SHV-2	1
K. pneumoniae	TEM-1, CTX-M-15,SHV-1b-b	1
	CTX-M-55, SHV-11	3
	CTX-M-15, SHV-108	1
	CTX-M-15, SHV-11	1

Table 4. Sequence analysis of the IS*Ecp1*-carrying bla_{CTX-M}.

Organism	Type of bla_{CTX-M}	IS element	No. of isolates
E. coli	CTX-M-3	IS26	1
	CTX-M-55	IS*Ecp1*	6
	CTX-M-15	IS*Ecp1*	4
	CTX-M-15	IS1 interrupting IS*Ecp1*	2
	CTX-M-15	IS1	1
K. pneumoniae	CTX-M-55	IS1 interrupting IS*Ecp1*	4
	CTX-M-15	IS*Ecp1*	3

The isolates, and higher resistance to CIP, LEV, GM, TM and SXT was seen in 91.3%, 82.6%, 65.2%, 39.1%, and 60.9% of E. coli isolates, respectively. The lowest resistance rates were observed for carbapenems, and all 23 *E. coli* isolates and eight of nine *K. pneumoniae* isolates, except for one XDR isolate, were sensitive to ETP, MEM and IMP (Table 2). In this study, 100% (23/23) of *E. coli* and 88.9% (8/9) of *K. pneumoniae* isolates (except one isolate defined as XDR) were confirmed as being MDR according to the standardized international definitions for drug resistance.

Amplification and sequencing of the *bla* genes and gene-capturing elements

The bla_{TEM} and bla_{CTX} groups were detected in 39.1% (9/23) and 69.6% (16/23) of *E. coli* and 44.4% (4/9) and 100% (9/9) of *K. pneumoniae*, respectively. All of the bla_{TEM}-positive isolates, including the 9 *E. coli* isolates and the 4 *K. pneumoniae* isolates, encoded bla_{TEM-1}. For the CTX-M-producing isolates, bla_{CTX-M}-encoding CTX-M-3, CTX-M-15, and CTX-M-55 was found in 6.2, 43.8 and 50.0% of *E. coli* isolates and 0, 44.4 and 55.6% of *K. pneumoniae* isolates, respectively (Table 3). All *K. pneumoniae* isolates, but none of the *E. coli* isolates, included bla_{SHV} genes, and bla_{SHV} genes encoding SHV-2, SHV-11, SHV-1b-b, SHV-108, and SHV-148/-1 was identified in 11.1, 55.5, 11.1, 11.1 and 11.1% of *K. pneumoniae*, respectively (Table 3). Other *bla* genes, including bla_{OXA-2} group, bla_{OXA-10} group, bla_{VEB}, bla_{PER} and bla_{GES}, were not detected in any of the isolates in the study. IS*Ecp1* was identified in the upstream region of bla_{CTX-M} in 14 bla_{CTX-M}-positive *E. coli* isolates (87.5%) and 7 bla_{CTX-M}-positive *K. pneumoniae* isolates (77.8%). Sequence analysis of the IS*Ecp1*-carrying bla_{CTX-M} is shown in Table 4. IS1 was found to interrupt the IS*Ecp1* gene in 2 *E. coli* isolates and 4 *K. pneumoniae* isolates within the sequences upstream of bla_{CTX-M}.

Amplification and sequencing of *int1* and the IS*CR1* gene-capturing elements indicated that 91.3% (21/23) of *E. coli* isolates and 77.8% (7/9) of *K. pneumoniae* isolates carried the *int1* gene, and 44.4% of *K. pneumoniae* and 0% of *E. coli* contained the IS*CR1* gene.

MLST and PFGE typing

The analysis of MLST sequence types (STs) showed that

Table 5. MLST profiles of *E. coli* and *K. pneumoniae* based on bla_{CTX-M} type

Organism	bla_{CTX-M} type	MLST type (No. of type)
E. coli	CTX-M-3	ST7 (n=1)
	CTX-M-15	ST7 (n=7)
	CTX-M-55	ST7 (n=3)
		ST6 (n=3)
		ST5 (n=1)
		ST2 (n=1)
	Non-CTX-M	ST7 (n=6)
		ST2 (n=1)
K. pneumoniae	CTX-M-15	ST45S (n=1); ST11 (n=1); ST29 (n=1); ST7 (n=1)
	CTX-M-55	ST395 (n=3); ST65 (n=1); ST629 (n=1)

ST7 was the dominant MLST type among the *E. coli* isolates, which accounted for 68.8% (11/16) of the bla_{CTX-M}-positive *E. coli* isolates and 85.7% (6/7) of the non-bla_{CTX-M}-positive *E. coli* isolates. In addition, ST6, ST5 and ST2 were detected in 18.8, 6.3 and 6.3%, respectively, of the 16 bla_{CTX-M}-positive *E. coli* isolates. Further, ST6, ST5 and ST2 types uniquely co-existed with CTX-M-55 (Table 5). In contrast, a large diversity of MLST types was identified among the nine bla_{CTX-M}-positive *K. pneumoniae* isolates, and a total of seven types were found. Four CTX-M-15 *K. pneumoniae* isolates shared different MLST types (Table 5). The five CTX-M-55 *K. pneumoniae* isolates were divided into three MLST types, in which ST395, ST629 and ST65 accounted for 60% (3/5), 20% (1/5) and 20% (1/5), respectively.

No banding patterns were obtained by PFGE analysis in three *E. coli* and two *K. pneumoniae* isolates because their DNA samples were consistently auto-digested. In contrast to the MLST typing, a large genetic diversity of *E. coli* isolates was demonstrated by PFGE typing, and 13 clusters were obtained based on the definition of more than 80% similarity (Figure 1A). However, there was little relatedness among the PFGE types, ST types and CTX-M types (Figure 1A). Similarly, the PFGE banding patterns of the seven *K. pneumoniae* isolates were very different (Figure 1B).

DISCUSSION

The emergence and rapid spread of multidrug-resistant *Enterobacteriaceae* is a substantial challenge to public health. We first reported the high frequency and molecular characterization of MDR *E. coli* and *K. pneumoniae* in Yunnan Province, China. Although, extended-spectrum beta-lactamases have been recognized among *Enterobacteriaceae* worldwide (Rossolini et al., 2008; Bonnet, 2004) and are known for their rapid spread in the US, Europe and Asia (Wang et al., 2013; Sidjabat et al., 2009; Johnson et al., 2010), it was remarkable that in this study 100% (9/9) of *K. pneumoniae* and 69.6% (16/23) of *E. coli* carried the bla_{CTX-M} genes. In contrast, less than 50 cases of CTX-M-producing *K. pneumoniae* isolates had been described in the United States before 2013 (Wang et al., 2013). Because the isolates recovered from patients were from different hospital departments and displayed high genetic diversity, the high rates of MDR *E. coli* and *K. pneumoniae* isolates in the hospital may be a considerable challenge for antimicrobial drug-use and infection control. Unlike the distributions of the dominant CTX-M genotypes in Beijing (CTX-M-10, 35.9%) (Li et al., 2012), Hunan Province (CTX-M-14), Jiangxi Province (CTX-M-14), Fujian Province (CTX-M-14) and Guangdong Province (CTX-M-14) (Wang et al., 2012), the CTX-M-55 and CTX-M-15 genotypes were the leading CTX-M genotypes in Yunnan Province in this study and they were identified in 50 and 43.8% of *E. coli* isolates, 55.6% and 44.4% of *K. pneumoniae* isolates, respectively. It is noteworthy that CTX-M-15 is the most prevalent genotype throughout the world (Carbonne et al., 2013; Kiratisin et al., 2007; Wang et al., 2013; Li et al., 2012; Paterson et al., 2003; Parveen et al., 2012), and it has frequently been reported in several parts of China (Li et al., 2012; Wang et al., 2012), however, CTX-M-55 has only been described a few times in China. Only one *E. coli* isolate was identified as CTX-M-3 in the study. Strikingly, all of the CTX-M genotypes identified in the study, including CTX-M-15, CTX-M-55 and CTX-M-3, have traditionally been classified in the CTX-M-1 subgroup, and increasing evidence indicates that the CTX-M-1 subgroup of bacteria exhibits higher levels of resistance to several antibiotics than other CTX-M subgroups (Wang et al., 2013; Paterson et al., 2003; Parveen et al., 2012). Here, we propose that the high frequency of CTX-M-1 subgroup isolates may have contributed to the high level of MDR agents identified in the study. A survey conducted from 1998 to 1999 in Thailand, a country neighboring the Yunnan Province, reported the first detection of bla_{CTX-M} at a hospital. The prevalence of bla_{CTX-M} was 52% (CTX-M-9 only) and

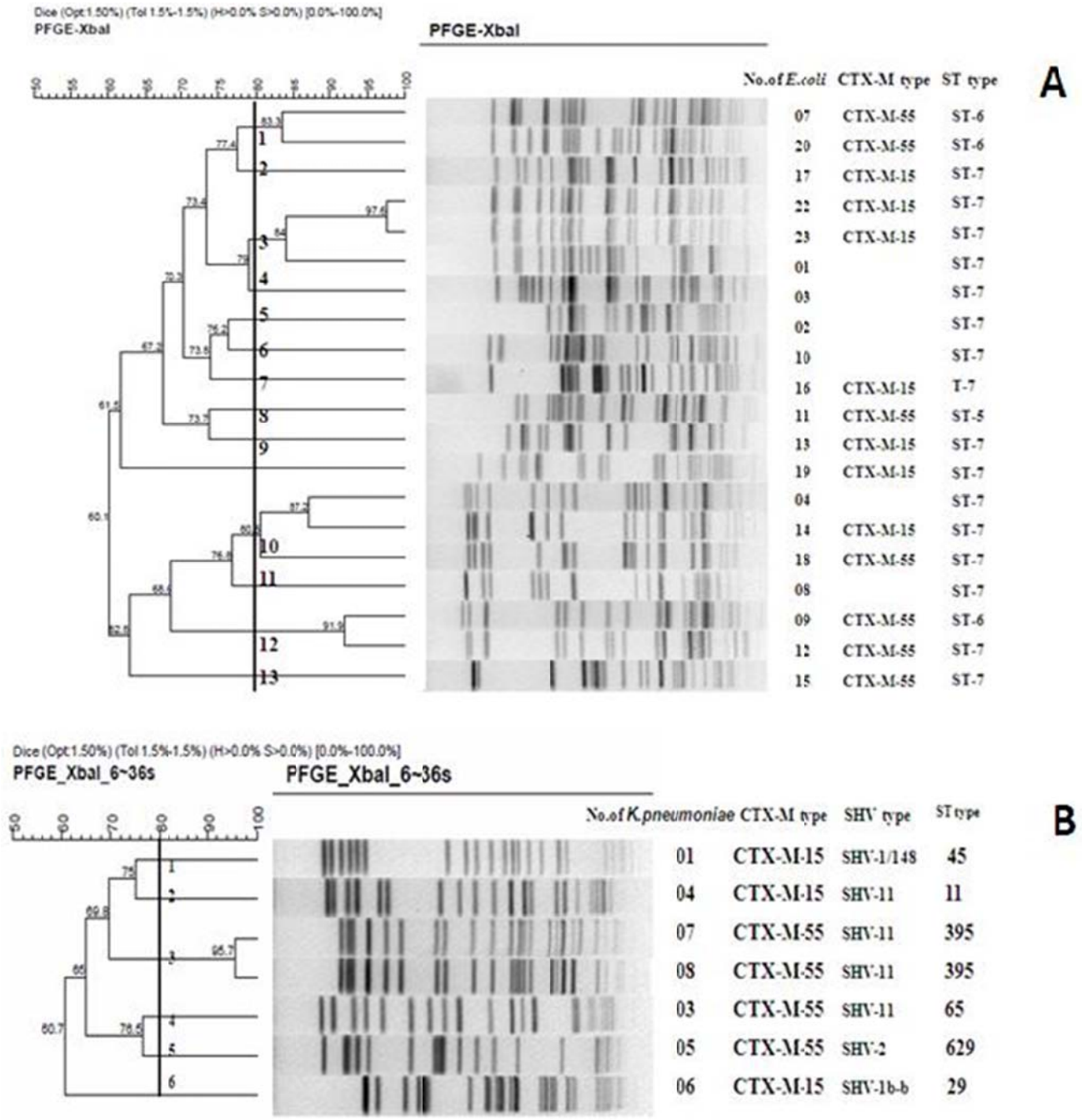

Figure 1. Dendrogram of pulsed-field gel electrophoresis (PFGE) patterns of *E. coli* (A) and *K. pneumoniae* (B) isolates and their genetic relatedness to the CTX-M genotype and sequence type (ST), if available.

subsequently increased to 65% in 2003 (CTX-M-15, 44%; CTX-M-14, 11% and CTX-M-9, 10%) (Chanawong et al., 2007). More recently, the frequency has increased to 99.6% of bla_{CTX-M}-producing *E. coli* (CTX-M-14, 43.6%; CTX-M-15, 37.2%; CTX-M-27, 1.3%; CTX-M-40, 1.3% and CTX-M-55, 17.5%) and 99.2% of bla_{CTX-M}-producing *K. pneumoniae* (CTX-M-3, 3.2%; CTX-M-14, 52.4%; CTX-M-15, 38.9%; CTX-M-27, 0.8% and CTX-M-55, 4.7%) in 2004-2005. These results indicate that CTX-M-55 is also an emerging CTX-M genotype in Thailand.

Significantly, an XDR *K. pneumoniae* isolated from the blood culture sample of a 66-year-old male patient exhibited resistance to all the carbapenem drugs tested in the study. This XDR *K. pneumoniae* exhibited the CTX-M-15, SHV-1b-b and TEM-1 genotypes, and it also carried the IS*Ecp1* and IS*CR*1 gene-capturing elements.

In this study, TEM-types were less numerous among the *E. coli* and *K. pneumoniae* isolates than CTX-M-types (39.1 vs 69.6% for *E. coli* and 44.4 vs 100% for *K. pneumoniae*). This result is not surprising because cefotaxime and ceftriaxone are used nationwide. The bla_{SHV} genes were not present in the *E. coli* isolates, but all of the *K. pneumoniae* isolates contained the bla_{SHV} genes. Moreover, these bla_{SHV} *K. pneumoniae* isolates all coexisted with CTX-M genes. The dominant SHV genotype was SHV-11, and it was identified in 55.6% of *K. pneumoniae* isolates, which was significantly higher than that identified in the Beijing area (Li et al., 2012). The other SHV genotypes found in the study includes SHV-2, SHV-1b-b, SHV-108, and SHV-148/-1 was unique, and each accounted for 11.1% of the isolates.

A high frequency of IS*Ecp1*, which is regarded as having a key role both in drug-resistance gene transfer and as a promoter for bla_{CTX-M}, was detected in bla_{CTX-M}-carrying *E. coli* isolates and *K. pneumoniae* isolates in this study. Two other gene-capturing elements, *int1* and IS*CR1*, which were previously noted elsewhere (Li et al., 2012) and are involved in multidrug resistance among clinical isolates, were also demonstrated in *E. coli* and *K. pneumoniae* isolates, respectively, further supporting our proposal that these gene-capturing elements might contribute to the high detection rates of MDR *E. coli* isolates and *K. pneumoniae* isolates in the Yunnan area. In addition, the genetic heterogeneity of *E. coli* and *K. pneumoniae* was noticed among the isolates (Figure 1A and 1B) analyzed in the study, suggesting that the emergence and polyclonal spread of multidrug-resistant *E. coli* and *K. pneumoniae* occurred among clinical isolates with diverse genetic backgrounds.

To summarize, we report for the first time the molecular characterization and phenotypes of *E. coli* and *K. pneumoniae* isolates in Yunnan Province, China. Our data revealed a high frequency of MDR *E. coli* and *K. pneumoniae* isolates among polyclonal strains in local areas. The CTX-M-1 subgroups which are involved in the development of higher levels of drug resistance were highly endemic among hospitalized patients. Active monitoring of novel antibiotic resistance is critical to avoid the rapid spread or outbreaks of these multidrug-resistant isolates in local health care facilities.

REFERENCES

Adams-Sapper S, Sergeevna-Selezneva J, Tartof S, Raphael E, Diep BA, Perdreau-Remington F, Riley LW (2012). Globally dispersed mobile drug-resistance genes in gram-negative bacterial isolates from patients with bloodstream infections in a US urban general hospital. J. Med. Microbiol. 61:968-74.

Bert F, Branger C, Lambert-Zechovsky N (2002). Identification of PSE and OXA beta-lactamase genes in *Pseudomonas aeruginosa* using PCR restriction fragment length polymorphism. J. Antimicrob. Chemother.50:11–18.

Bonnet R (2004). Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob. Agents. Chemother. 48:1–14.

Carbonne A, Arnaud I, Maugat S, Marty N, Dumartin C, Bertrand X, Bajolet O, Savey A, Fosse T, Eveillard M, Sénéchal H, Coignard B, Astagneau P, Jarlier V;MDRB Surveillance National Steering Group (BMR-Raisin) (2013). National multidrug-resistant acteria (MDRB) surveillance in France through the RAISIN network: a 9 year experience. J. Antimicrob. Chemother. 68:954-59.

Celenza C, Pellegrini G, Caccamo M, Segatore B, Amicosante G, Perilli M (2006). Spread of *bla*(CTX-M-type) and *bla*(PER-2) beta-lactamase genes in clinical isolates from Bolivian hospitals. J. Antimicrob. Chemother.57:975–978.

Chanawong A, Lulitanond A, Kaewkes W, Lulitanond VL, Srigulbutr S, Homchampa P (2007).CTX-M extended-spectrum beta-lactamases among clinical isolates of *Enterobacteriaceae* in a Thai university hospital. Southeast. Asian. J. Trop. Med. Public. Health. 38:493–500.

Chen X, Yuan M, Li GX, Chen Y, Yu HL, Li J (2013). Dissemination of *int*1 gene and IS*CR1* and their relations with multi-drug resistance in clinical isolates. China J. Znoonoses. 29: 646-652.

Dhanji H, Doumith M, Hope R, Livermore DM, Woodford N (2011). IS*Ecp1*-mediated transposition of linked *bla*CTX-M-3 and *bla*TEM-1b from the IncI1 plasmid pEK204 found in clinical isolates of *Escherichia coli* from Belfast, UK. J. Antimicrob. Chemother. 66:2263-5.

Drees M, Pineles L, Harris AD, Morgan DJ (2014). Variation in definitions and isolation procedures for multidrug-resistant Gram-negative bacteria: a survey of the society for healthcare epidemiology of America research network. Infect Control Hosp. Epidemiol. 35:362-6.

Han H, Zhou HJ, Li HS, Gao Y, Lu Zhi, Hu KX, Xu BL (2013). Optimization of pulse-field gel electrophoresis for subtyping of *Klebsiella pneumoniae.* Int. J. Environ. Res. Public Health 10:2720-31

Johnson JR, Johnston B, Clabots C, Kuskowski M, Castanheira M (2010). *Escherichia coli* sequence type ST131 as the major cause of serious multidrug-resistant *E. coli* infections in the United States. Clin. Infect. Dis. 51:286–94.

Kiiru J, Butaye P, Goddeeris BM, Kariuki S (2013). Analysis for prevalence and physical linkages amongst integrons, IS*Ecp1*, IS*CR1*, Tn21 and Tn7 encountered in *Escherichia coli* strains from hospitalized and non-hospitalized patients in Kenya during a 19-year period (1992-2011). BMC. Microbiol.13:109.

Kiratisin P, Apisarnthanarak A, Saifon P, Laesripa C, Kitphati R, Mundy LM (2007). The emergence of a novel ceftazidime-resistant CTX-M extended-spectrum beta-lactamase, CTX-M-55, in both community-onset and hospital-acquired infections in Thailand. Diagn. Microbiol. Infect. Dis. 58:349–55.

Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet. Infect. Dis.10:597-602.

Li B, Yi Y, Wang Q, Woo PCY, Tan L, Jing H, Gao GF, Liu CH (2012). Analysis of drug resistance determinants in *Klebsiella pneumoniae* isolates from a tertiary-care hospital in Beijing,China. Plos.ONE. 7: e42280.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB,Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18: 268–81.

Pai H, Lyu S, Lee JH, Kim J, Kwon Y, Kim JW, Choe KW (1999). Survey of extended-spectrum beta-lactamases in clinical isolates of Escherichia coli and *Klebsiella pneumoniae*: prevalence of TEM-52 in Korea. J. Clin. Microbiol. 37:1758–63.

Pastera´n F, Rapoport M, Petroni A, Faccone D, Corso A, Galas M, Vázquez M, Procopio A, Tokumoto M, Cagnoni V (2006). Emergence of PER-2 and VEB-1a in *Acinetobacter baumannii* strains

in the Americas. Antimicrob. Agents. Chemother. 50:3222–24.
Parveen MR, Manivannan S, Harish BN, Parija SC (2012). Study of CTX-M type of extended spectrum b-lactamase among nosocomial isolates of *Escherichia coli* and *Klebsiella pneumoniae* in south India. Indian J. Microbiol. 52:35–40.
Paterson DL, Hujer KM, Hujer AM, Yeiser N, Bonomo MV, Rice LB, Bonomo RA; International Klebsiella Study Group (2003). International *Klebsiella* Study Group: Extended-spectrum-lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV-and CTX-M-Type-Lactamases. Antimicrob. Agents Chemother. 47:3554–60.
Peirano G, Pitout JD (2010).Molecular epidemiology of *Escherichia coli* producing CTX-Mβ-Lactamase:the worldwide emergence of clone ST131 O25:H4. Int. Antimicrob. Agents. 35:316-21.
Rossolini GM, Andrea DMM, Mugnaioli C (2008). The spread of CTXM-type extended-spectrum beta-lactamases. Clin. Microbiol. Infect.14 (Suppl1):33–41.
Sidjabat HE, Paterson DL, Adams-Haduch JM, Ewan L, Pasculle AW, Muto CA, Tian GB, Doi Y (2009).Molecular epidemiology of CTX-M-producing *Escherichia coli* isolates at a tertiary medical center in western Pennsylvania. Antimicrob. Agents. Chemother. 53:4733–9.
Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, Kallen A, Limbago B, FridkinS, National Healthcare Safety Network (NHSN) Team andParticipating N HSN Facilities (2013). Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. Infect. Control. Hosp. Epidemiol. 34:1-14.
Smith SP, Manges AR, Riley LW (2008).Temporal changes in the prevalence of community-acquired antimicrobial-resistant urinary tract infection affected by *Escherichia coli* clonal group composition. Clin. Infect. Dis. 46:689-95.
Sturenburg E, Kuhn A, Mack D, Laufs R (2004). A novel extended spectrum beta-lactamase CTX-M-23 with a P167T substitution in the active site omega loop associated with ceftazidime resistance. J. Antimicrob. Chemother. 54:406–409. Tacconelli E, Cataldo MA, Dancer SJ, De Angelis G, Falcone M, Frank U, Kahlmeter G, Pan A, Petrosillo N, Rodríguez-Baño J, Singh N, Venditti M, Yokoe DS, Cookson B; European Society of Clinical Microbiology (2014). ESCMID guidelines for the management of the infection control measures to reduce transmission of multidrug-resistant Gram-negative bacteria in hospitalized patients. Clin. Microbiol. Infect. 20:1-55.
Tan HL, Wang Y, Cheng XQ, Huang YM, Zhang LJ (2014). Molecular epidemiological analysis of multi-drug resistant *E.coli* and *K.pneumoniae* isolates in Yunnan, China. Chin.Trop.Med.14:777-81.(In Chinese)
Tian SF, Chu YZ, Chen BY, Nian H, Shang H (2011). IS*Ecp1* element in association with *bla*(CTX-M) genes of *E. coli* that produce extended-spectrum β-lactamase among the elderly in community settings. Enferm. Infecc. Microbiol. Clin. 29:731-4.
Vourli S, Giakkoupi P, Miriagou V, Tzelepi E, Vatopoulos AC, Tzouvelekis LS (2004). Novel GES/IBC extended-spectrum beta-lactamase variants with carbapenemase activity in clinical enterobacteria. FEMS. Microbiol. Lett. 234:209–213
Wang GQ, Huang T, Surendraiah KPM, Wang K, Komal R, Zhuge J, Chern CR, Kryszuk AA, King C, Wormser GP (2013). CTX-M β-Lactamase–producing *Klebsiella pneumoniae* in Suburban New York, New York, USA. Emerg. Infect. Dis.19:1803-10.
Wang XR, Chen JC, Kang Y, Jiang N, An SC, Gao ZC (2012). Prevalence and haracterization of plasmid-mediated *bla*ESBL with their genetic environment in Escherichia coli and *Klebsiella pneumoniae* in patients with pneumonia. Chin. Med. J.125: 894–900.
Wei ZQ, Chen YG, Yu YS, Lu WX, Li LJ (2005). Nosocomial spread of multi-resistant *Klebsiella pneumoniae* containing a plasmid encoding multiple beta-lactamases. J. Med. Microbiol. 54:885-8.
Weisburg WG, Barns SM, Peleetier DA, Lane DJ (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol.173:697–703.
Yan JJ, Tsai SH, Chuang CL, Wu JJ (2006). OXA-type betalactamases among tended-spectrum cephalosporin-resistant *Pseudomonas aeruginosa* isolates in a university hospital in southern Taiwan. J. Microbiol. Immunol. Infect. 9:130–134.
Zhou TY, Ni YX (2010). “Protocol of bacterial isolation of clinical specimen: Manual of Clinical Microbiology Laboratory”. pp. 325-412. (In Chinese).