

Identification of extended-spectrum beta-lactamase (ESBL) and carbapenemase genes in carbapenem-resistant Enterobacteriaceae (CRE) clinical isolates from hospitals in Nan province

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Objective To identify extended-spectrum beta-lactamase (ESBL) and carbapenemase genes in *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates from hospitals in Nan province and to characterize the antibiotic susceptibility of these isolates

Methods The antibiotic susceptibility of *E. coli* and *K. pneumoniae* against imipenem, meropenem, and ertapenem in specimens collected at Pua Crown Prince Hospital and Nan Hospital between May and September 2016 was determined using Kirby–Bauer disk diffusion and minimum inhibitory concentration (MIC) and the agar dilution method based on Clinical and Laboratory Standards Institute (CLSI) guidelines 2016. Polymerase chain reactions (PCR) were performed to identify the ESBL genes, *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M-1 group}, *bla*_{CTX-M-9 group} and *bla*_{VEB} and the carbapenemase genes, *bla*_{KPC}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM} and *bla*_{OXA-48-like}.

Results Antibiotic susceptibility tests using disk diffusion found that 9 isolates of *E. coli* and 37 isolates of *K. pneumoniae* were carbapenem-resistant Enterobacteriaceae (CRE). PCR assays for ESBL gene identification revealed that the most prevalent genes found in *E. coli* were *bla*_{TEM} (88.9%) followed by *bla*_{CTX-M-1 group} (66.7%), whereas *K. pneumoniae* mainly carried *bla*_{SHV} (91.9%) and/or *bla*_{TEM} (89.2%). Carbapenemase-encoding genes detected in both *E. coli* and *K. pneumoniae* were *bla*_{NDM} and *bla*_{OXA-48-like}. The *bla*_{NDM} was the most common carbapenemase gene and was always co-harbored with ESBL genes. All isolates that carried *bla*_{NDM} were resistant to all tested carbapenems (meropenem, imipenem, and ertapenem).

Conclusion All CRE isolates from Pua Crown Prince Hospital and Nan Hospital, Northern Thailand harbored ESBL and carbapenemase genes. *bla*_{TEM}, *bla*_{SHV}, and *bla*_{NDM} are the most common beta-lactamase. Some isolates were observed co-carrying more than 3 different types of beta-lactamase genes in each single cell. The finding that ESBL genes were co-harbored with carbapenemase genes in all but 3 CRE isolates suggests that these isolates may have previously contained ESBL genes and developed carbapenem resistance by later obtaining carbapenemase genes. The data from this study provides important information on the epidemiology and the distribution of beta-lactamase genes among Enterobacteriaceae in hospitals in Northern Thailand. **Chiang Mai Medical Journal 2018;57(4):183-94.**

Keywords: carbapenem-resistant enterobacteriaceae (CRE), carbapenemase genes, ESBL genes

Introduction

Cephalosporins are commonly used for treatment of several bacterial infections ranging from mild to severe, eg, uncomplicated cellulitis, urinary tract infection, pyelonephritis, bacteremia, and septic shock (1). Presently, a gradual increase in the use of cephalosporins, especially the third and fourth generation cephalosporins, has led to cephalosporin resistance in Enterobacteriaceae (2). Widespread antibiotic usage acts as a selective pressure and a driving force accelerating adaptability of bacterial resistance to antibiotics (3). Bacterial evolutions based on natural mutations and acquired horizontal gene transfers are known major mechanisms in their survival when they are exposed to harmful factors including antibiotics (4). Dissemination of genes involved in drug resistance has an extreme effect on increasing the number of antibiotic resistant bacteria and limiting the choices of therapeutic drugs.

Extended-spectrum β -lactamases (ESBLs) are powerful enzymes that can hydrolyze not only penicillin, ampicillin, and amoxicillin but also hydrolyze extended-spectrum cephalosporins with an oxyimino side chain (3rd generation cephalosporin) (5, 6). Therefore, ESBLs are involved with a cephalosporin resistance mechanism in Enterobacteriaceae, especially *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*). ESBLs can hydrolyze 3rd generation cephalosporins such as cefotaxime, cefotaxime, ceftriaxone, and aztreonam, but do not hydrolyze cephamycins or carbapenems and are inhibited by the serine beta-lactamase inhibitors sulbactam, tazobactam, and clavulanic acid (7,8). ESBLs are classified as class A beta-lactamases, eg, SHV (sulfhydryl-variable), TEM (temoniera), CTX-M (cefotaximase), VEB (Vietnam extended-spectrum beta-lactamase), and as class D beta-lactamases; eg, OXA (oxacillinase). CTX-M enzymes are very diverse. More than 50 different types have been identified. They can be grouped based on amino acid sequence similarity into five clusters: CTX-M-1 group, CTX-M-2 group, CTX-M-9 group, CTX-M-8 group, and CTX-M-25 group (9, 10). Reports by Kiratisin

et al. in 2008 and Netikul et al. in 2015 showed that CTX-M-15 enzyme in CTX-M-1 group and CTX-M-14 in CTX-M-9 group were predominant among the ESBL-producing *E. coli* and *K. pneumoniae* clinical strains from Siriraj Hospital and Thammasat Hospital, Bangkok, Thailand (11,12). Most ESBL genes can be commonly horizontally transferred among Enterobacteriaceae via plasmid-mediated rather than chromosomally mediated transfer. That process leads to the dissemination of cephalosporin-resistant strains (13, 14).

Carbapenems are used as the drug of choice for treatment of cephalosporin-resistant Enterobacteriaceae infection (15). However, it was not long after the widespread use of carbapenems that the carbapenem-resistant strains were found. One of the major mechanisms of carbapenem resistance is based on the activity of the carbapenemase produced by these strains (16). Carbapenemase-producing Enterobacteriaceae are often resistant to most beta-lactam antibiotics including 3rd generation cephalosporins and carbapenems as well as many other non-beta-lactam drugs, eg, aminoglycosides and fluoroquinolones. Furthermore, carbapenemases are not inhibited by beta-lactamase inhibitors (7). The emergence and spread of carbapenem-resistant Enterobacteriaceae (CRE) is a major global health concern; the available treatment options for infections with these strains are limited to tigecycline and colistin (polymyxin E) (17-19). Carbapenemases, eg, KPC (*K. pneumoniae* carbapenemase), are classified as beta-lactamases class A; IMP (imipenem), VIM (Verona integron-encoded metallo beta-lactamase), and NDM (New Delhi metallo-beta-lactamase) as class B, and OXA (oxacillinase) as class D (20, 21). Beside the production of carbapenemase, other carbapenem resistance mechanisms have been identified including porin down-regulation and mutation, efflux pump hyperexpression, and drug target alteration (22, 23).

In Thailand, a high prevalence of ESBL-producing Enterobacteriaceae and CRE has been reported in several studies, but the identification of ESBL and carbapenemase genes in the same types of clinical isolates in North-

ern Thailand has not yet been reported (11, 12, 24-26). The screening of beta-lactamase genes by molecular PCR used as information for the emergence and spread of ESBL-producing Enterobacteriaceae and CRE.

Objectives

The aims of this study were to characterize the presence of the ESBL and carbapenemase genes in CRE collected from Pua Crown Prince Hospital and Nan Hospital and to characterize the antibiotic susceptibility of these isolates.

Methods

Bacterial strains and antimicrobial susceptibility tests

The clinical isolates of *E. coli* and *K. pneumoniae* were collected at Pua Crown Prince Hospital and Nan Hospital between May and September 2016. The bacterial strains were isolated from clinical specimens including urine, pus, sputum, and rectal swabs. Antimicrobial susceptibility to imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), and ertapenem (ETP, 10 µg) was evaluated by Kirby–Bauer disk diffusion. The MIC of meropenem was determined using the agar dilution method in accordance with the criteria recommended in the CLSI guidelines 2016. *E. coli* ATCC 25922 was used as the reference strain for antimicrobial susceptibility testing (27).

Molecular identification of beta-lactamase encoding genes by polymerase chain reaction

Whole genomic DNA was extracted from fresh bacterial colonies using heat lysis procedures. Bacterial colonies were suspended in sterile deionized water and boiled at 95°C for 10 min. The suspensions were cooled on ice and centrifuged at 13,000 rpm for 2 min at room temperature. After removing cell debris by centrifugation, bacterial genomic DNA in the supernatant was collected. DNA concentration was adjusted to 100 ng/µL and used as a DNA template for PCR analysis. All clinical strains were screened using PCR for the presence of the ESBL genes, *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M-1 group}, *bla*_{CTX-M-9 group} and *bla*_{VEB} as well as the carbapenemase genes, *bla*_{NDM}, *bla*_{OXA-48-like}, *bla*_{IMP}, *bla*_{KPC}, and *bla*_{VIM}. The reference strains contained each ESBL gene and carbapenemase gene used as a positive control for PCR. All control strains were generously provided by Assoc. Prof. Dr. Aroonwadee Chanawong and Dr. Anusak Kerdsin. Nucleotide sequences of oli-

gonucleotide primers and PCR cycling conditions are shown in Table 1. All of the PCR amplicons were subjected to 1% agarose gel electrophoresis and visualized using a ultraviolet transilluminator system.

Results and discussion

Disk diffusion patterns against imipenem, meropenem, and ertapenem identified 9 isolates of *E. coli* and 37 isolates of *K. pneumoniae* as CRE based on the definition by the Centers for Disease Control and Prevention (CDC) 2015 (28). Most *E. coli* isolates used in this study were obtained from urine (66.7%), while most *K. pneumoniae* isolates were from rectal swabs (81.1%). Antimicrobial susceptibility results showed that all tested isolates were resistant to ertapenem, indicating that ertapenem was an inefficient antibiotic for treating CRE infections in Pua Crown Prince Hospital and Nan Hospital (Figure 3). Interestingly, 44.4% (4/9) of *E. coli* and 70.3% (26/37) of *K. pneumoniae* resisted all tested carbapenem disks (meropenem, imipenem, and ertapenem) and exhibited a high level of resistance to meropenem with an MIC of ≥32 µg/mL (Tables 2 and 3). Among these isolates, one isolate (CRKP10) of *K. pneumoniae* showed meropenem MIC of 32 µg/mL, but was susceptible to imipenem in the disk diffusion study.

Detection of five ESBL genes, *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M-1 group}, *bla*_{CTX-M-9 group}, and *bla*_{VEB}, revealed that all the *E. coli* isolates except CREC03 harbored at least one ESBL gene (Table 2 and Figure 1). The highest prevalence of ESBL genes found in *E. coli* was *bla*_{TEM} (88.9%, 8 isolates), followed by *bla*_{CTX-M-1 group} (66.7%, 6 isolates), and *bla*_{CTX-M-9 group} (11.1%, 1 isolate). Interestingly, *bla*_{SHV} and *bla*_{VEB} were absent in all *E. coli* isolates in this study. However, reports of characterization of ESBL genes in *E. coli* isolates in northeastern Thailand have stated that during 1998 and 1999, all 3 isolates harbored *bla*_{TEM-1} and *bla*_{VEB}, while in 2003, 18 isolates out of 19 isolates contained *bla*_{TEM-1} (25). In central Thailand, most ESBL-producing *E. coli* isolated during 2004 and 2005 possessed *bla*_{CTX-M} (99.6%), followed by *bla*_{TEM-1} (77.0%), *bla*_{VEB-1} (8.5%), *bla*_{OXA-10} (8.1%), and *bla*_{SHV} (3.8%) (11). These data support that the

Table 1. Primers for characterization of beta-lactamase genes in Enterobacteriaceae by PCR

Gene target	Primer name	Primer Sequence (5'to 3')	Amplicon size (bp)	Amplification condition
<i>bla</i> _{TEM}	TEM (F)	CATTTCCGTGTCGCCCTTATTC	800	Initial denaturation: 94°C, 3 min 30 cycles of denaturation: 94°C, 40 s annealing: 60°C, 30 s elongation: 72°C, 90 s Final elongation: 72°C, 7 min
	TEM (R)	CGTTCATCCATAGTTGCCTGAC		
<i>bla</i> _{SHV}	SHV (F)	AGCCGCTTGAGCAAATAAAC	713	
	SHV (R)	ATCCCGCAGATAAATCACCAC		
<i>bla</i> _{CTX-M-1 group}	CTXM-G1 (F)	TTAGGAARTGTGCCGCTGYA	688	
	CTXM-G1 (R)	CGATATCGTTGGTGGTRCCAT		
<i>bla</i> _{CTX-M-9 group}	CTXM-G9 (F)	TCAAGCCTGCCGATCTGGT	561	
	CTXM-G9 (R)	TGATTCTCGCCGCTGAAG		
<i>bla</i> _{VEB}	VEB (F)	CGACTTCCATTTCCCAGATGC	642	
	VEB (R)	GGACTCTGCAACAAATACGC		
<i>bla</i> _{NDM}	NDM (F)	GGTTTGGCGATCTGGTTTTTC	621	
	NDM (R)	CGGAATGGCTCATCACGATC		
<i>bla</i> _{OXA-48-like}	OXA-48 (F)	GCGTGGTTAAGGATGAACAC	438	
	OXA-48 (R)	CATCAAGTTCAACCAACCG		
<i>bla</i> _{IMP}	IMP (F)	GGAATAGAGTGGCTTAATTCTC	232	
	IMP (R)	GGTTTAACAAAACAACCACC		
<i>bla</i> _{VIM}	VIM (F)	GATGGTGTGTTGGTCGCATA	390	
	VIM (R)	CGAATGCGCAGCACCAG		
<i>bla</i> _{KPC}	KPC (F)	TGTCACTGTATCGCCGTC	881	
	KPC (R)	CTTGTCATCCTTGTTAGGCG		

Table 2. Phenotypic and genotypic characterization of *E. coli* clinical isolates

Bacterial ID.	Specimen type	Antibiotic resistance profile	MEM MIC (μg/mL)	Genotype of <i>bla</i> gene	
				ESBL gene	Carbapenemase gene
CREC01	urine	ETP	≤1	<i>bla</i> _{TEM} , <i>bla</i> _{CTX-M-1 group}	-
CREC02	urine	MEM, IPM, ETP	8	<i>bla</i> _{TEM}	-
CREC03	rectal swab	MEM, ETP	16	-	<i>bla</i> _{OXA-48-like}
CREC04	urine	MEM, IPM, ETP	32	<i>bla</i> _{TEM}	<i>bla</i> _{NDM} , <i>bla</i> _{OXA-48-like}
CREC05	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{CTX-M-1 group}	<i>bla</i> _{NDM}
CREC06	urine	MEM, IPM, ETP	16	<i>bla</i> _{TEM} , <i>bla</i> _{CTX-M-1 group}	-
CREC07	urine	MEM, IPM, ETP	8	<i>bla</i> _{TEM} , <i>bla</i> _{CTX-M-1 group} , <i>bla</i> _{CTX-M-9 group}	<i>bla</i> _{NDM}
CREC08	urine	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{CTX-M-1 group}	<i>bla</i> _{NDM}
CREC09	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{CTX-M-1 group}	<i>bla</i> _{NDM}

high prevalence of *bla*_{TEM} and *bla*_{CTX-M-1} group genes in northern Thailand is similar to that of northeastern and central Thailand. During

2000 and 2001 in a neighboring country, Vietnam, the most prevalent ESBL genes in *E. coli* were *bla*_{TEM} (84.3%), *bla*_{SHV} (44%), and *bla*_{CTX-M}

Table 3. Phenotypic and genotypic characterization of *K. pneumoniae* clinical isolates

Bacterial ID	Specimen type	Antibiotic resistance profile	MEM MIC (µg/mL)	Genotype of <i>bla</i> gene	
				ESBL gene	Carbapenemase gene
CRKP01	sputum	MEM, IPM, ETP	32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP02	rectal swab	MEM, IPM, ETP	32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-9} group, <i>bla</i> _{VEB}	<i>bla</i> _{NDM}
CRKP03	rectal swab	MEM, IPM, ETP	32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-9} group, <i>bla</i> _{VEB}	<i>bla</i> _{NDM}
CRKP04	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-9} group, <i>bla</i> _{VEB}	<i>bla</i> _{NDM}
CRKP05	rectal swab	MEM, IPM, ETP	32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{VEB}	<i>bla</i> _{NDM}
CRKP06	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP07	rectal swab	MEM, IPM, ETP	32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{VEB}	<i>bla</i> _{NDM}
CRKP08	rectal swab	MEM, IPM, ETP	16	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{VEB}	<i>bla</i> _{NDM}
CRKP09	rectal swab	MEM, ETP	16	-	<i>bla</i> _{OXA-48-like}
CRKP10	rectal swab	MEM, ETP	32	<i>bla</i> _{SHV}	-
CRKP11	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-9} group, <i>bla</i> _{VEB}	<i>bla</i> _{NDM}
CRKP12	rectal swab	MEM, IPM, ETP	32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-9} group, <i>bla</i> _{VEB}	<i>bla</i> _{NDM}
CRKP13	rectal swab	MEM, IPM, ETP	16	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP14	urine	ETP	16	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	-
CRKP15	urine	ETP	4	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	-
CRKP16	rectal swab	MEM, IPM, ETP	32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-9} group, <i>bla</i> _{VEB}	<i>bla</i> _{NDM}
CRKP17	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-9} group, <i>bla</i> _{VEB}	<i>bla</i> _{NDM}
CRKP18	rectal swab	MEM, IPM, ETP	16	<i>bla</i> _{SHV}	-
CRKP19	pus	MEM, IPM, ETP	8	-	-
CRKP20	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP21	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP22	rectal swab	MEM, IPM, ETP	16	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	-
CRKP23	rectal swab	MEM, ETP	16	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	-
CRKP24	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	-
CRKP25	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP26	sputum	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	-
CRKP27	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-1} group	<i>bla</i> _{NDM}
CRKP28	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP29	rectal swab	MEM, IPM, ETP	32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	-
CRKP30	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP31	sputum	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{CTX-M-1} group	<i>bla</i> _{NDM}
CRKP32	urine	MEM, ETP	16	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	-
CRKP33	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP34	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP35	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV}	<i>bla</i> _{NDM}
CRKP36	rectal swab	MEM, IPM, ETP	32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-1} group	-
CRKP37	rectal swab	MEM, IPM, ETP	>32	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-1} group	<i>bla</i> _{NDM}

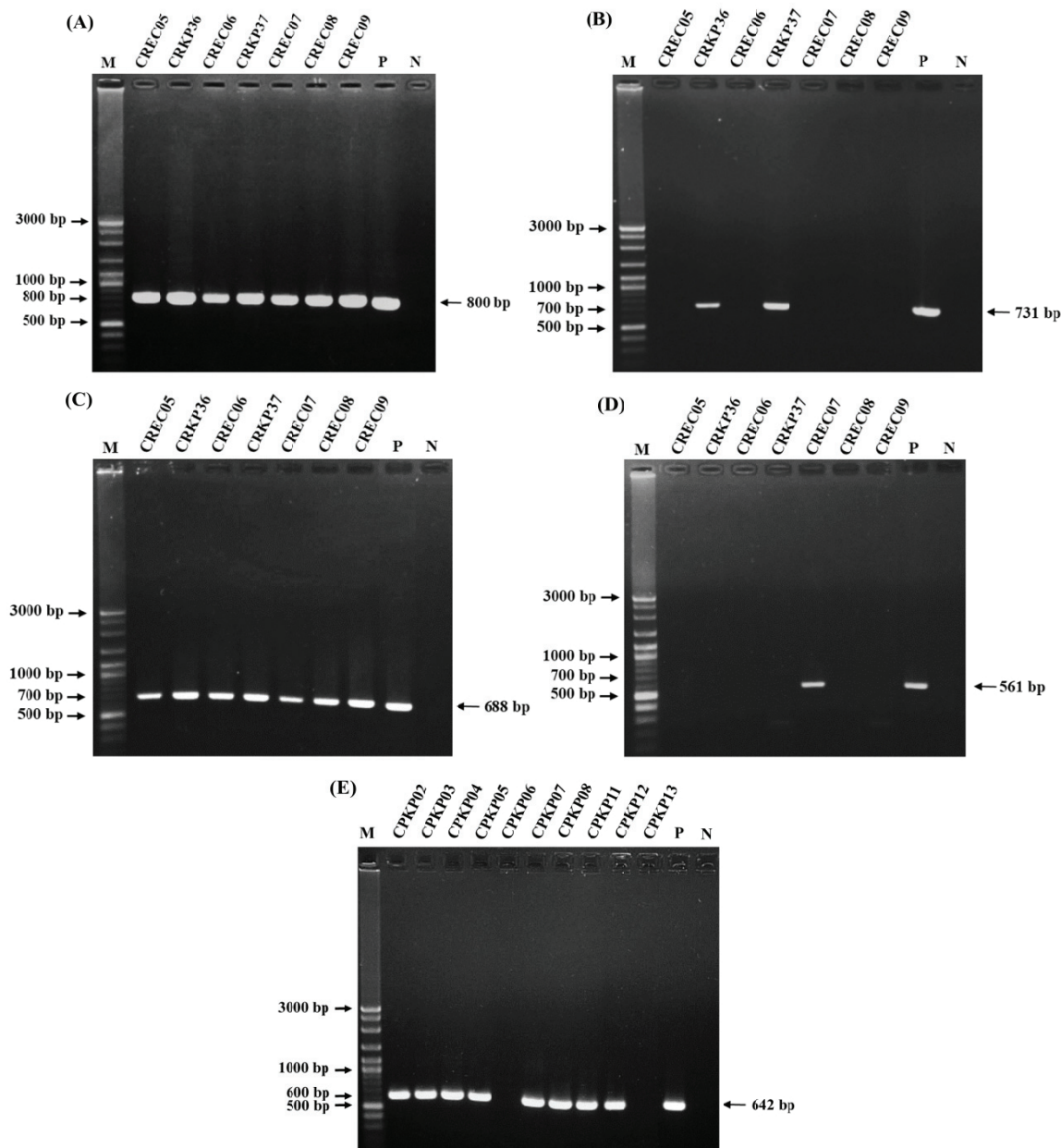


Figure 1. ESBL genes detected by PCR. The amplicons of *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M-1 group}, *bla*_{CTX-M-9 group} and *bla*_{VEB} genes are shown in figures (A) to (E), respectively. Lane M, 100 bp standard DNA marker; P, positive control; N, negative control.

and *bla*_{VEB-1} (18.7%) (29). Based on this information, *bla*_{TEM} is the most prevalent ESBL gene found in at least two Southeast Asian countries, Thailand and Vietnam.

In contrast to *E. coli*, all five ESBL genes, *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M-1 group}, *bla*_{CTX-M-9 group}, and *bla*_{VEB}, were identified in most *K. pneumo-*

niae isolates with the exception of two isolates (CRKP09, CRKP19) as shown in Table 3. *bla*_{SHV} and *bla*_{TEM} were the most common ESBL genes found in *K. pneumoniae* with a prevalence of 91.9% and 89.2%, respectively. *bla*_{VEB} (27.0%), *bla*_{CTX-M-9 group} (18.9%), and *bla*_{CTX-M-1 group} (10.8%) were found with a lower

Table 4. Summary of *bla* gene combinations in *E. coli* clinical isolates

<i>bla</i> gene	No. of isolates
TEM, CTX-M-1 group, CTX-M-9 group, NDM.....	1
TEM, CTX-M-1 group, NDM.....	3
TEM, CTX-M-1 group	2
TEM, NDM, OXA-48-like	1
TEM.....	1
OXA-48-like.....	1

Table 5. Summary of *bla* gene combinations in *K. pneumoniae* clinical isolates

<i>bla</i> gene	No. of isolates
TEM, SHV, CTX-M-9 group, VEB, NDM.....	7
TEM, SHV, CTX-M-1 group, NDM	2
TEM, SHV, CTX-M-1 group.....	1
TEM, SHV, VEB, NDM	3
TEM, SHV, NDM	11
TEM, SHV	8
TEM, CTX-M-1 group, NDM.....	1
SHV.....	2
OXA-48-like	1
Not found	1

prevalence. In contrast to the data from this study, *bla*_{CTX-M} was found to be highly endemic in central Thailand (126/127 isolates) and in Vietnam (8/13 isolates) in 2000 to 2001 (11, 29). In northeastern Thailand, *K. pneumoniae* isolates collected in 2003 were *bla*_{SHV} (74%), *bla*_{TEM-1} (74%), *bla*_{CTX-M-15} (48%), *bla*_{VEB} (17%), *bla*_{CTX-M-9} (9%), and *bla*_{CTX-M-14} (9%) (25). These data indicate that the high prevalence of *bla*_{SHV} and *bla*_{TEM} genes are similar in northern and northeastern Thailand.

The carbapenemase gene, *bla*_{NDM}, was found in 5 isolates (55.6%) of *E. coli* (Table 4) and 24 isolates (64.9%) of *K. pneumoniae* (Table 5 and Figure 2). Interestingly, *bla*_{NDM} was always co-harbored with ESBL genes. In contrast, *bla*_{OXA-48-like} was a less common carbapenemase gene in both organisms compared with *bla*_{NDM}, and could be present alone without the ESBL gene in CREC03 and CRKP09.

Carbapenemase genes were not found in some isolates, but these bacteria showed resistance to at least one carbapenem agent. It is possible that other mechanisms, eg, overexpression of plasmid-mediated AmpC enzymes in combination with loss of outer membrane porins or increased expression of efflux pump could be involved in carbapenem resistance in these isolates (18,30). In this study, *bla*_{IMP}, *bla*_{KPC}, and *bla*_{VIM} were absent in both *E. coli* and *K. pneumoniae*, whereas two isolates of *K. pneumoniae*-produced IMP-14a were identified in northeastern Thailand during 2010 and 2011 (26).

The intensive usage of third-generation cephalosporins leads to the detection of ESBL-producing strains. Carbapenem has been used for treatment of infections of ESBL-producing strains resulting in infection caused by CRE to increase greatly. Our study found that

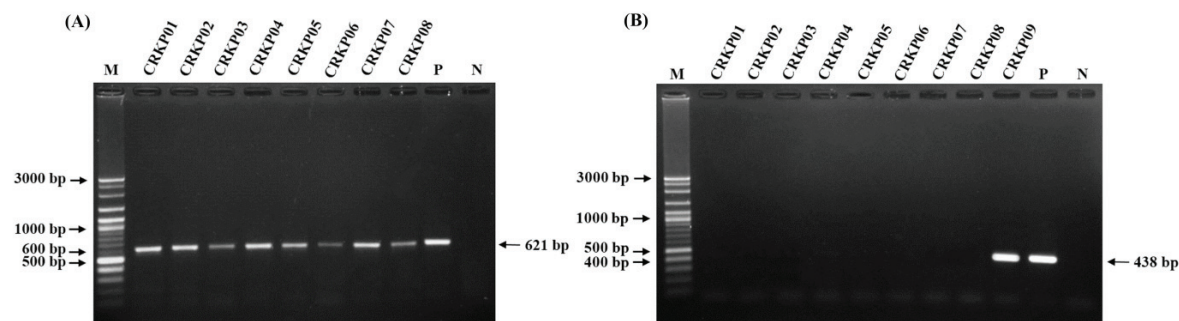


Figure 2. Carbapenemase genes detected by PCR. The amplicons of *bla*_{NDM} and *bla*_{OXA-48-like} genes are shown in figures (A) and (B), respectively. Lane M, 100 bp standard DNA marker; P, positive control; N, negative control.

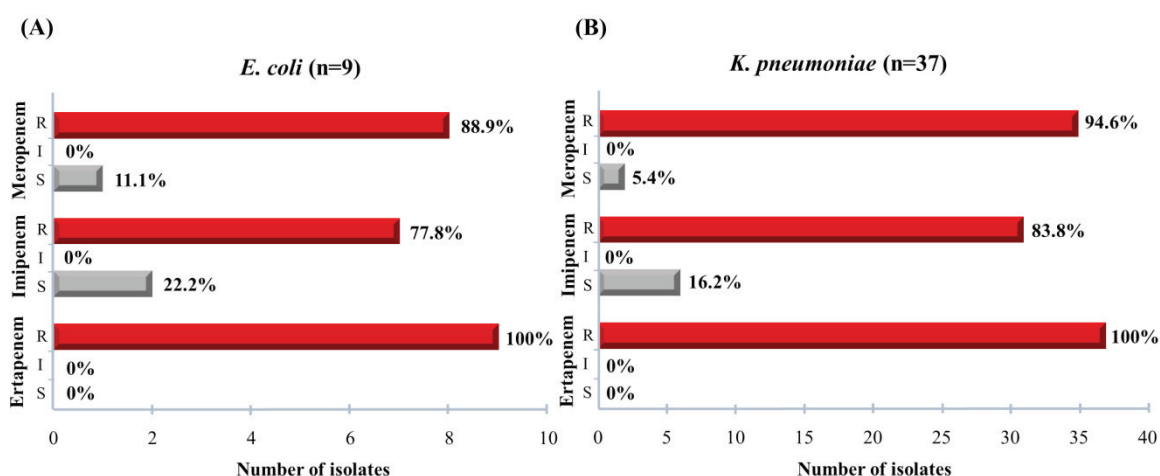


Figure 3. Summary of antimicrobial susceptibility for *E. coli* (A) and *K. pneumoniae* (B) from Pua Crown Prince Hospital and Nan Hospital.

all tested CRE except CRKP19 harbored at least one carbapenemase gene as well as an ESBL gene. This indicates that the pressure of third-generation cephalosporin usage leads to the spread of ESBL genes in the bacterial strains. The response of bacteria to carbapenem treatment leads to the distribution of carbapenemase genes among bacterial strains that already harbor ESBL genes. Hence, the detection of ESBL genes and carbapenemase genes in the same bacterial isolate, such as those found in this study, represents the response of bacteria to the antibiotic treatment in the form of spreading antibiotic resistance genes to bacterial populations. The most frequent beta-lactamase gene profile was the co-carrying of *bla*_{TEM}, *bla*_{CTX-M-1 group} and *bla*_{NDM} (33.3%) in *E. coli*, while the co-harboring of

*bla*_{TEM}, *bla*_{SHV} and *bla*_{NDM} (29.8%) was found in *K. pneumoniae* isolates. Four beta-lactamase genes, *bla*_{TEM}, *bla*_{CTX-M-1 group}, *bla*_{CTX-M-9 group}, and *bla*_{NDM}, were found together in a single *E. coli* isolate (CREC07). Seven isolates of *K. pneumoniae* contained all 5 genes, *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M-9 group}, *bla*_{VEB}, and *bla*_{NDM}, in each single cell. Castanheira et al (2013) observed that *K. pneumoniae* frequently carried multiple beta-lactamases, indicating that this bacterium is able to accumulate multidrug- or resistant determinants which is similar to what we found in this study (31). In the present study, both *E. coli* and *K. pneumoniae* isolates that co-carried more than 3 different types of ESBL and carbapenemase genes showed a high level of meropenem MIC (≥ 32 μ g/mL) and conferred resistance to all tested carbapenems (mero-

penem, imipenem, and ertapenem). It should be noted that bacterial isolates that were resistant to all 3 carbapenems in disk diffusion susceptibility tests and which showed a high level of meropenem MIC ($>32 \mu\text{g/mL}$ in *E. coli* and *K. pneumoniae*) always carried the *bla*_{NDM} gene.

The results from this study provide the information on the presence of ESBL and carbapenemase genes related to the changes in carbapenem resistance in CRE strains over the period of time. Therefore, this data may extend the understanding of molecular epidemiology of resistance genes in Northern Thailand.

Conclusions

ESBL and carbapenemase genes were identified in all carbapenem-resistant *E. coli* and *K. pneumoniae* isolates in Pua Crown Prince Hospital and Nan Hospital, Northern Thailand. Therefore, the major mechanism of carbapenem resistance used by these isolates is most likely to be carbapenemase production. Most isolates were observed to be carrying multiple beta-lactamase genes, particularly as ESBLs/carbapenemase combinations. Both *E. coli* and *K. pneumoniae* isolates that co-carried four or five ESBL and carbapenemase genes showed high levels of meropenem MIC and conferred resistance to all tested carbapenems. In this study, among *E. coli* and *K. pneumoniae*, *bla*_{TEM} was the predominant ESBL gene, while *bla*_{NDM} was the most common carbapenemase gene. *bla*_{NDM} was always found in combination with ESBL genes. The finding that ESBL genes co-harbor with carbapenemase genes in all but 3 of the CRE isolates indicates that these isolates may have previously contained ESBL genes then later developed carbapenem resistance by obtaining carbapenemase genes. The most prevalent ESBL genes seem to vary in different parts of Thailand. However, it should be noted that differences in the most common types of ESBL genes may due to the number of bacterial isolates as well as to the collection time. Molecular screening for the presence of ESBL and carbapenemase genes may provide im-

portant information for controlling the spread of this gene among Enterobacteriaceae in hospitals.

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Conflicts of interest

The authors certify that there are no conflicts of interests related to the publication of this paper.

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การตรวจหายีน extended-spectrum beta-lactamase (ESBL) และยีน carbapenemase ในเชื้อ carbapenem-resistant Enterobacteriaceae (CRE) สายพันธุ์ที่คัดแยกได้จากผู้ป่วยในโรงพยาบาลในจังหวัดน่าน

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วัตถุประสงค์ เพื่อตรวจหายีน extended-spectrum beta-lactamase (ESBL) และยีน carbapenemase ในเชื้อ *Escherichia coli* และ *Klebsiella pneumoniae* ที่คัดแยกได้จากผู้ป่วยในโรงพยาบาลในจังหวัดน่าน และศึกษารูปแบบความไวต่อยาต้านจุลชีพของเชื้อดังกล่าว

วิธีการศึกษา เก็บตัวอย่างเชื้อ *E. coli* และ *K. pneumoniae* จากโรงพยาบาลปัว และโรงพยาบาลจังหวัดน่าน ระหว่างเดือนพฤษภาคม ถึงเดือนกันยายน พ.ศ. 2559 และนำเชื้อมาทดสอบความไวต่อยาต้านจุลชีพ ได้แก่ imipenem, meropenem และ ertapenem โดยวิธี Kirby-Bauer disk diffusion พร้อมทั้งหาค่า minimum inhibitory concentration (MIC) โดยวิธี agar dilution ตามวิธีมาตรฐานของ Clinical Laboratory Standard Institute (CLSI) ปี ค.ศ. 2016 และทำการตรวจสอบหายีน ESBL ได้แก่ bla_{SHV} , bla_{TEM} , $bla_{CTX-M-1-group}$, $bla_{CTX-M-9-group}$ และ bla_{VEB} และยีน carbapenemase ได้แก่ bla_{KPC} , bla_{IMP} , bla_{VIM} , bla_{NDM} และ $bla_{OXA-48-like}$ โดยเทคนิค Polymerase chain reactions (PCR)

ผลการศึกษา ผลการทดสอบความไวต่อยาต้านจุลชีพ โดยวิธี disk diffusion พบเชื้อ carbapenem-resistant Enterobacteriaceae (CRE) *E. coli* จำนวน 9 ตัวอย่าง และ *K. pneumoniae* จำนวน 37 ตัวอย่าง เมื่อทำการตรวจสอบหายีน ESBL พบว่า เชื้อ *E. coli* ส่วนใหญ่พบยีน bla_{TEM} (ร้อยละ 88.9) และยีน $bla_{CTX-M-1-group}$ (ร้อยละ 66.7) ส่วนเชื้อ *K. pneumoniae* ส่วนใหญ่พบยีน bla_{SHV} (ร้อยละ 91.9) และ bla_{TEM} (ร้อยละ 89.2) และผลการตรวจหายีน carbapenemase พบว่า ทั้งเชื้อ *E. coli* และ *K. pneumoniae* พบยีน bla_{NDM} และ $bla_{OXA-48-like}$ โดยพบยีน bla_{NDM} มากที่สุดในเชื้อกลุ่ม Enterobacteriaceae และยีน bla_{NDM} มักจะพบอยู่ร่วมกับยีน ESBL ทั้งนี้เชื้อที่พบยีน bla_{NDM} จะคือต่อยากลุ่ม carbapenem ทั้ง 3 ชนิดที่นำมาทดสอบ (meropenem, imipenem และ ertapenem)

สรุป เชื้อกลุ่ม Enterobacteriaceae ที่คือต่อยากลุ่ม carbapenem จากโรงพยาบาลปัว และโรงพยาบาลจังหวัดน่าน พบยีนคือยา ESBL และยีน carbapenemase ในทุกตัวอย่าง โดยส่วนใหญ่พบยีนคือยาชนิด bla_{TEM} , bla_{SHV} และ bla_{NDM} และพบยีนคือยา beta-lactamase จำนวนมากกว่า 3 ยีน ภายในเซลล์แบคทีเรียเดียวกัน การที่เชื้อ CRE เกือบทั้งหมด (ยกเว้น 3 ตัวอย่าง) พบยีน ESBL ร่วมกับยีน carbapenemase มีความเป็นไปได้ว่าเชื้อ CRE ได้รับยีน ESBL มาก่อน และค่อยรับยีน carbapenemase เข้ามาภายหลัง แล้วสามารถพัฒนาตนเองให้เกิดการคือต่อยากลุ่ม carbapenem การศึกษานี้จะทำให้ทราบข้อมูลทางระบาดวิทยา และการแพร่กระจายของยีน beta-lactamase ในเชื้อ Enterobacteriaceae ภายในโรงพยาบาลในจังหวัดน่าน เชียงใหม่เวชสาร 2561;57(4):183-94.

คำสำคัญ: carbapenem-resistant enterobacteriaceae (CRE), ยีน carbapenemase, ยีน ESBL