คุณลักษณะของยีนที่สร้างเอนไซม์ carbapenemases ในเชื้อกลุ่ม Enterobacteriaceae ที่แยกได้
จากผู้ป่วยไทย

นางสาวศศิเพ็ญ แซ่จู

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CHARACTERIZATION OF CARBAPENEMASE-
ENCODING GENES IN ENTEROBACTERIACEAE ISOLATES FROM THAI PATIENTS

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ศศิเพ็ญ แซ่จู : คุณลักษณะของยีนที่สร้างเอนไซม์ carbapenemases ในเชื้อกลุ่ม Enterobacteriaceae ที่แยกได้จากผู้ป่วยไทย (CHARACTERIZATION OF CARBAPENEMASE-ENCODING GENES IN ENTEROBACTERIACEAE ISOLATES FROM THAI PATIENTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ธนิษฐา ฉัตรสุวรรณ, 134 หน้า.

การดื้อยากลุ่ม carbapenems ในเชื้อกลุ่ม Enterobacteriaceae มีรายงานเพิ่มสูงขึ้นในหลายประเทศทั่วโลก โดยการสร้างเอนไซม์ carbapenemases เป็นกลไกหลักที่เชื้อใช้ในการดื้อยากลุ่ม carbapenems ในการศึกษาครั้งนี้ทำการตรวจหาคุณลักษณะของยีนที่สร้างเอนไซม์ carbapenemases ในเชื้อกลุ่ม Enterobacteriaceae ที่แยกได้จากผู้ป่วยไทย โดยเชื้อจำนวน 3,854 สายพันธุ์ ที่แยกได้จากผู้ป่วยในโรงพยาบาลจุฬาลงกรณ์ ตั้งแต่เดือนสิงหาคม พ.ศ. 2556 ถึงเดือนมกราคม พ.ศ. 2557 ถูกนำมาตรวจคัดกรองเพื่อหาเชื้อที่สร้างเอนไซม์ carbapenemases โดยใช้ MacConkey agar ที่มี ertapenem, cloxacillin และ ZnSO$_4$ ผลการศึกษาพบเชื้อที่ให้ผลบวกจำนวน 104 สายพันธุ์ (2.7%) นำมาทดสอบหาค่า MICs (minimum inhibitory concentrations) โดยวิธี agar dilution พบว่าเชื้อดื้อต่อยา ertapenem จำนวน 100 สายพันธุ์ (96.2%), imipenem จำนวน 39 สายพันธุ์ (37.5%), meropenem จำนวน 48 สายพันธุ์ (46.2%), cefotaxime จำนวน 102 สายพันธุ์ (98%), ceftazidime จำนวน 102 สายพันธุ์ (98%), ceftriaxone จำนวน 102 สายพันธุ์ (98%) และ cefoxitin จำนวน 99 สายพันธุ์ (95.2%) พบความชุกของการดื้อยากลุ่ม carbapenems เป็น 2.7% ทำการตรวจหายีนที่สร้างเอนไซม์ carbapenemases ได้แก่ ยีน *bla* ชนิด IMP, VIM, GIM, SIM, SPM, AIM, DIM, BIC, KPC, OXA-48, NDM ด้วยวิธี multiplex PCR และตรวจหาการทำงานของเอนไซม์ carbapenemases ด้วยวิธี modified Hodge test, boronic acid-based inhibition test และ EDTA-meropenem combined-disk test พบเชื้อที่มียีนที่สร้างเอนไซม์ carbapenemases และมี carbapenemase activity จำนวน 19 สายพันธุ์ พบยีน *bla*$_{IMP}$ จากเชื้อ *E. cloacae* จำนวน 1 สายพันธุ์ (5.3%), พบยีน *bla*$_{OXA-48}$ จากเชื้อ *E. cloacae* จำนวน 1 สายพันธุ์ (5.3%), พบยีน *bla*$_{NDM}$ จากเชื้อ *E. cloacae* จำนวน 2 สายพันธุ์ (10.5%), *C. freundii* จำนวน 2 สายพันธุ์ (10.5%), *E. coli* จำนวน 3 สายพันธุ์ (15.8%) และ *K. pneumoniae* จำนวน 10 สายพันธุ์ (52.6%) พบเชื้อที่สร้างเอนไซม์ carbapenemases ร่วมกับ เอนไซม์ ESBLs ชนิด CTX-M-15 มากที่สุดคิดเป็น 63.1 % แต่ไม่พบเชื้อที่สร้างเอนไซม์ carbapenemases ร่วมกับเอนไซม์ AmpC beta-lactamases ผลการวิเคราะห์ลำดับเบสด้วยวิธี DNA sequencing พบยีน IMP-14a, OXA-48, NDM-1 และ NDM-4 จากนั้นทำการศึกษาการถ่ายทอดยีนดื้อยาด้วยวิธี conjugation พบว่าเชื้อจำนวน 15 สายพันธุ์ที่มียีน NDM-1 และ OXA-48 สามารถส่งผ่านยีนได้ด้วยวิธีนี้ แบบแผนทางพันธุกรรมโดยวิธี PFGE แสดงให้เห็นว่ามีการแพร่กระจายยีน NDM-1 และCTX-M-15 ในเชื้อ *E. coli* และ *K. pneumoniae* ในโรงพยาบาลจุฬาลงกรณ์ ซึ่งมีความสัมพันธ์กับclonal spread ผลจาก Southern blot hybridization พบว่า พลาสมิดที่มียีน OXA-48 มีขนาด 51 kb และพลาสมิดที่มียีน NDM-1 มีขนาด 24 kb และ 159 kb จากผลการศึกษานี้พบเอนไซม์ carbapenemases ชนิด NDM-1 มากที่สุดในเชื้อกลุ่ม Enterobacteriaceae และรายงานการพบเอนไซม์ชนิด NDM-4 ในเชื้อ *E. coli* เป็นครั้งแรกในประเทศไทย

สาขาวิชา   จุลชีววิทยาทางการแพทย์   ลายมือชื่อนิสิต ....

ปีการศึกษา 2557   ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

SASIPEN SAE-JOO: CHARACTERIZATION OF CARBAPENEMASE-ENCODING GENES IN ENTEROBACTERIACEAE ISOLATES FROM THAI PATIENTS. ADVISOR: TANITTHA CHATSUWAN, Ph.D., 134 pp.

Carbapenem resistance in Enterobacteriaceae has been increasingly reported worldwide. Carbapenemase production is the main mechanism of carbapenem resistance. In this study, carbapenemase-encoding genes in Enterobacteriaceae isolates from Thai patients were characterized. A total of 3,854 Enterobacteriaceae isolates from King Chulalongkorn Memorial Hospital during August 2013 to January 2014 were screened for carbapenemase producers by using MacConkey agar containing ertapenem, cloxacillin and $ZnSO_4$. A total of 104 isolates were positive on screening plates. MICs (minimum inhibitory concentrations) were performed by agar dilution method. Of the 104 isolates, 100 (96.2%) were resistant to ertapenem, 39 (37.5%) to imipenem, 48 (46.2%) to meropenem, 102 (98%) to cefotaxime, 102 (98%) to ceftazidime, 102 (98%) to ceftriaxone and 99 (95.2%) to cefoxitin. The prevalence of carbapenem resistace in Enterobacteriaceae was 2.7%. Carbapenemase genes, including bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{BIC}, bla_{KPC}, bla_{OXA-48}, bla_{NDM}, were determined by multiplex PCR. Carbapenemase activity was determined by ertapenem modified Hodge test (MHT), boronic acid-based inhibition test and EDTA-meropenem combined-disk test. The detection of ESBL and AmpC phenotypes was performed by combination disk test and cefoxitin-MHT, respectively. There were 19 isolates carrying carbapenemase genes and had carbapenemase activity. The bla_{IMP} was found in one isolate (5.3%) of *E. cloacae*. The bla_{OXA-48} was present in one isolate (5.3%) of *E. cloacae. The* bla_{NDM} was found in two isolates (10.5%) of *E. cloacae,* two isolates of *C. freundii* (10.5%), three isolates (15.8%) of *E. coli* and ten isolates (52.6%) of *K. pneumoniae*. The majority of carbapenemase producers coproduced CTX-M-15 ESBL (63.1 %). None of carbapenemase producers coproduced AmpC beta-lactamases. DNA sequence analysis revealed that carbapenemase genes were bla_{NDM-1}, bla_{NDM-4}, $bla_{IMP-14a}$, and bla_{OXA-48}. The transfers of resistance genes by conjugation showed that the bla_{NDM-1} and bla_{OXA-48} genes from 15 Enterobactericeae isolates were successfully transferred to *E. coli* recipient strain. The PFGE patterns demonstrated that *E. coli* and *K. pneumoniae* isolates carrying bla_{NDM} and $bla_{CTX-M-15}$ genes were associated with clonal spread. Southern blot hybridization showed that the plasmid carrying the bla_{OXA-48} gene was approximately 51 kb in size and the plasmids carrying the bla_{NDM-1} gene were approximately 24 kb and 159 kb in size. The results showed that NDM-1 was the most common carbapenemase among Enterobacteriaceae isolates. This is the first report of NDM-4 in *E. coli* from Thailand.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µg	microgram
bp	base pair
CAZ	ceftazidime
CLA	clavulanic acid
CLSI	Clinical and Laboratory Standards Institute
CRO	ceftriaxone
CTX	cefotaxime
DNA	deoxynucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenediamine tetraacetic acid
ESBLs	Extended-spectrum beta-lactamases
FOX	cefoxitin
hr	hour
I	intermediate resistant
IPM	imipenem
M	molar
MHT	Modified Hodge Test
MIC	minimum inhibitory concentration

ml	milliliter
mM	milimolar
NDM-1	New Delhi metallo beta-lactamase-1
NDM-4	New Delhi metallo beta-lactamase-4
NSS	Normal Saline Solution
$^{\circ}$C	degree Celcius
OD	optical density
PCR	polymerase chain reaction
PFGE	Pulse Field Gel Electropheresis
R	resistant
rpm	revolutions per minute
S	susceptible
TBE	Tris-borate ethylenediamine tetraacetic acid
TE	Tris-EDTA
TSB	Trypticase soy broth
U	unit
UV	ultraviolet
V	voltage

CHAPTER I

INTRODUCTION

The family Enterobacteriaceae is rod-shaped, facultative anaerobic Gram-negative bacteria that commonly inhabit the intestinal tract system in human and animals. This family includes the most important human pathogens, such as; *Klebsiella pneumoniae, Escherichia coli, Enterobacter* spp. and *Proteus mirabilis*. Theses bacteria cause infections that range from cystitis to pyelonephritis, bacteremia, pneumonia, peritonitis, meningitis, and device-associated infections [1]. They can spread easily between humans by hand carriage as well as contaminated food, water and cause both hospital- and community-acquired infections. The acquisition of resistance genes is usually derived from horizontal gene transfer, mediated mostly by plasmids and transposons.

Since 1950s, beta-lactam antibiotics, including penicillins and cephalosporins, have been used to treat Enterobacteriaceae infections [2]. They bind to penicillin-binding proteins (PBPs), which are responsible for peptidoglycan synthesis in cell wall, and ultimately cause cell death. However, resistance to these antibiotics in Enterobacteriaceae has emerged. The main mechanism is due to the production of beta-lactamases, enzymes that target the beta-lactam ring of penicillins, cephalosporins, monobactams, and carbapenems.

Carbapenems are the last resort antimicrobial agents for treatment of infection caused by multi-drug resistant Enterobacteriaceae. The emergence of carbapenem-resistant Enterobacteriaceae has rapidly spread worldwide. The most common mechanism of carbapenem resistance is the acquisition of carbapenemase genes that encode for enzymes capable of degrading carbapenems. Other mechanisms include penicillin binding proteins (PBPs), overexpression of efflux pump, overproduction of AmpC beta-lactamases, porin loss and the decrease of outer membrane permeability [3]. Various beta-lactamases including ESBLs and AmpC beta-lactamases and carbapenemases have been reported in

Enterobacteriaceae. ESBL and AmpC production have been the major mechanisms of 3rd generation cephalosporins resistance.

Carbapenemases belong to class A, B and D beta-lactamases and can separate into two groups, including serine carbapenemases (class A, D) and metallo beta-lactamases (class B) [4]. The enzymes which have serine at active site of them, are serine carbapenemases. Metallo beta-lactamases required zinc ion as a co-factor at the active site for enzyme activity.

Class A beta-lactamases can hydrolyze penicillins, cephalosporins, aztreonam and carbapenems. They are inhibited by beta-lactamase inhibitors such as clavulanic acid, boronic acid and tazobactam [5, 6]. The class A enzymes include *Klebsiella pneumoniae* carbapenemase (KPC), Bicetre carbapenemase (BIC), Guiana extended spectrum (GES), *Serratia marcescens* enzyme (SME), imipenem-hydrolyzing-beta-lactamase (IMI) and non-metallo-enzyme carbapenemase (NMC-A). In 1996, the first of KPC was detected in the US [7]. The KPC enzyme were identified in *K. pneumoniae, E. coli* and *Enterobacter* spp. [5, 8-10]. Global spread of KPC was reported in Israel, Greece, Korea, China, Italy and Taiwan [6, 11-15].

The class B beta-lactamases can hydrolyze all beta-lactams except aztreonam and are inhibited by EDTA and dipicolinic acid [16]. These enzymes include Imipenemase (IMP), Verona integron-encoded metallo-beta-lactamase (VIM), New Delhi metallo-beta-lactamase (NDM), German imipenemase (GIM), Seoul imipenemase (SIM) and Sao Paulo metallo-beta-lactamase (SPM). The spread of IMP and VIM have been reported worldwide and mostly identified in enterobacterial species and *Pseudomonas* spp. [17] In 1991, the first report of IMP was identified in *Serratia marcescens* from Japan. Also it has report in other countries such as America, Singapore, China and Australia [18-21]. In 1997, the first report of VIM enzyme was detected in *Pseudomonas aeruginosa* from Italy [4]. Later, these were reported in Greece, South Africa, German, and South Korea [22-26]. Recently, the NDM-1 was first reported in Enterobacteriaceae isolates from an Indian decent patient in Sweden [27]. The NDM-1 carbapenemases were disseminated in India and

Pakistan and now have been reported worldwide [1]. They were commonly found in *K. pneumoniae, E. cloacae*, *P. mirabilis*, *E. coli* and *Providencia rettgeri* [28-31].

Oxacillinases (OXA) carbapenemases are the class D beta-lactamases, which can hydrolyze almost all beta-lactams, including broad-spectrum cephalosporins, cephamycins, monobactam and carbapenems. These enzymes are inhibited by NaCl [32]. The first report of class D beta-lactamases in Enterobacteriaceae was OXA-48 identified in *K. pneumoniae* from Turkey and has been spread worldwide [33]. Other enzymes in this class are OXA-181, OXA-204 and OXA-232. These enzymes have been reported in *K. pneumoniae, E. coli, E. cloacae* and *Citrobacter freundii* [33, 34].

The Centers for Disease Control and Prevention (CDC), reported that carbapenem-resistant Enterobacteriaceae (CRE) has increased from 1% to 4% and CRE infections have been reported in medical facilities across the 42 states of USA during the last 10 years. In 2009, the mortality rate of CRE infections patients was 21.9% in Israel [35]. In China, the prevalence of carbapenemase enzymes in *E. cloacae* was KPC-2 (8.6%), IMP-8 (14.3%), IMP-26 (2.9%) and NDM-1 (2.9%) [36]. The prevalence of KPC-2 in *K. pneumoniae* was 1.67% in Taiwan [37]. In 2012, IMP-14a and NDM-1 were found in *E. coli, K. pneumoniae and C. freundii* in Thailand. In 2009, screening of stool specimens is recommended by CDC to identify carriers of carbapenem-resistant Gram-negative rods and initiate appropriate infection control measures. The detection of carbapenem-resistant Enreobacteriaceae by modified hodge test (MHT) with ertapenem, as a screening test, was recommended by the Clinical and Laboratory Standards Institute (CLSI 2013).

In Thailand, the emergence of carbapenem-resistant Enterobacteriaceae has become global concern. The report of carbapenemase-producing Enterobacteriaceae was rare and there were few studies on resistance mechanism. The aims of this study were to characterize carbapenemase-encoding gene in Enterobacteriaceae isolates and to determine the prevalence of carbapenem-resistant Enterobacteriaceae. The genetic relationships among carbapenemase-producing Enterobacteriaceae were also investigated.

CHAPTER II
OBJECTIVES

1. To characterize carbapenemase-encoding genes in Enterobacteriaceae isolates from Thai patients.
2. To detect the prevalence of carbapenem-resistant Enterobacteriaceae.
3. To investigate genetic relationships among carbapenemase-producing Enterobacteriaceae.

CHAPTER III

LITERATURE REVIEW

Enterobacteriaceae

Enterobacteriaceae are a family of gram-negative, facultative anaerobic, rod-shaped bacteria that do not form endospores. They are usually found in human intestinal flora and are major causes of both hospital-acquired and community-acquired infections [1]. Organisms in this family are *Citrobacter* spp., *Enterobacter* spp., *E. coli, Klebsiella* spp*., Morganella* spp., *Proteus* spp., *Providencia* spp., *Salmonella* spp., *Serratia* spp., *Shigella* spp*.* and *Yersinia* spp.

The enterobacteria are the important causes of urinary tract infections (UTIs), bloodstream infections, hospital and healthcare-associated pneumonias, also various intra-abdominal infections [38]. They can spread easily between humans by contaminated food and water, or hand carriage and have the propensity to acquire multiple genetic elements conferring resistance through horizontal transmission via plasmids and transposons [39].

E. coli is the most commonly isolated organism in the clinical laboratory and a major of enteric pathogens. *E. coli* is the most common cause of urinary tract infections, prostatitis and pyelonephritis. *Proteus* spp*., Klebsiella* spp*.,* and *Enterobacter* spp. are also common urinary tract pathogens. *K. pneumoniae* causes a severe pneumonia [40]. *Shigella* causes shigellosis with watery diarrhea, dehydration, vomiting, bloody dysentery, mucoid stools, and abdominal pain (cramps and tenesmus) and *Salmonella* spp. causes gastroenteritis, septicemia, and enteric fevers (typhoid fever) [41]. Enteric fever is caused by *Salmonella typhi*.

Beta-lactam antibiotic

In 1940s, the first beta-lactam antibiotic, penicillin discovered by Alexander Fleming from *Penicillium notatum* leads to the introduction of beta-lactam antibiotics into medical practice [42]. Since then, beta-lactam are important antimicrobial agents used in the treatment of a variety of Gram-negative and Gram-positive infections [43].

Beta-lactams are a broad class of antibiotics and contain a beta-lactam ring in their molecular structures. The beta-lactam ring displays a characteristic structural similarity with the back bone of the D-alanyl-D-alanine, the substrate of penicillin-binding proteins (PBP) (figure 1). PBPS are transpeptidases, which are essential for the cell wall biosynthesis. They bind to the active site of PBP and cause inhibition of bacterial cell wall biosynthesis, consequently leading to cell death [44].
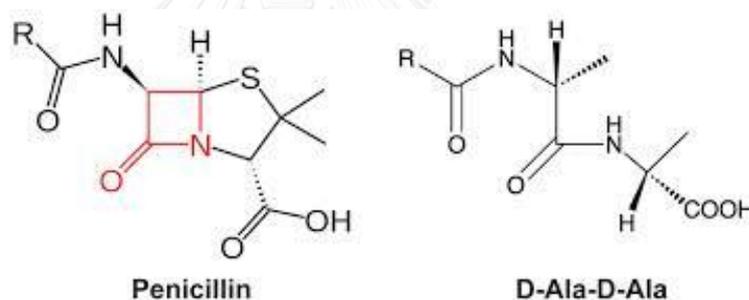
Figure 1 The mimicry of beta-lactam antibiotics to D-alanyl-D-alanine(D-Ala-D-Ala). The four-member lactam ring in penicillin was highlighted in red [44].

Later, variety of beta-lactam antibiotics with different antimicrobial profiles have been discovered or synthesized such as penicillin derivatives (penams) production by *Penicillium chrysogenum*, cephalosporins (cephems) formation by *Cephalosporium acremonium*, cephamycin, clavam and carbapenem production by actinomycetes and monocyclic beta-lactam production by actinomycetes and unicellular bacteria [45]. However, all these groups of beta-lactam antibiotics have a common core containing four-member beta-lactam ring. Beta-lactam antibiotics can be subdivided into 6 different structural subtypes [46]. These include penem (e.g.

benzylpenicillin, ampicillin), cephems which include 2nd generation cephalosporins (e.g. cefuroxime, cefotiam), and 3rd generation cephalosporins (e.g. ceftriaxone, ceftazidime, cefotaxime), cephamycins as 7-alpha-methoxy cephalosporins (e.g. cefoxitin), monobactams as monocyclic molecules (e.g. aztreonam), penems with a 2,3-double bond in the fused thiazoline ring (e.g. faropenem), Carbapenems (e.g. imipenem, meropenem, doripenem and ertapenem) with an unsaturated fused 5-membered ring differing from penem structure by possession of a carbon atom at position 1 (figure 2).
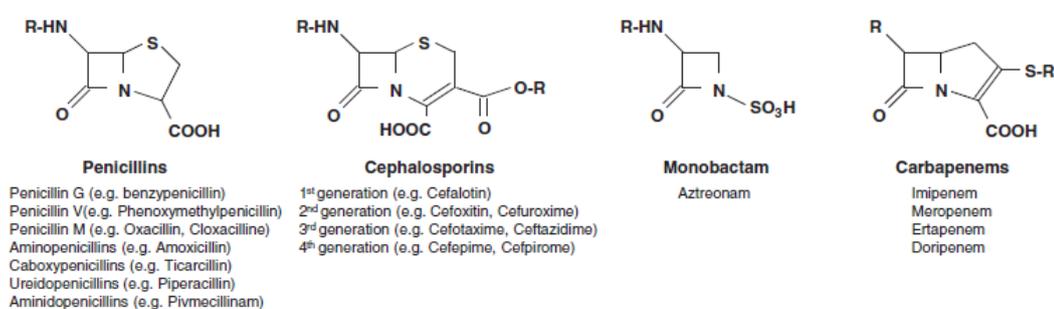
Figure 2 The structure of beta-lactams. [39]

Beta-lactam antibiotics have been frequently used to treat bacterial infections caused by Enterobacteriaceae, However, the emergence of highly resistant bacteria is rapidly changing the way of treatment and control in bacterial infections. Gram-negative bacteria have adapted to broad spectrum beta-lactam antibiotics by modifying the substrate spectrum of common plasmid-mediated beta-lactamases and mobilizing resistance-promoting chromosomal beta-lactamase genes into plasmids, allowing their spread to new hosts [47]. In the early 1950s, Enterobacteriaceae had been reported resistant to the first penicillins [46]. Then, Enterobacteriaceae isolates are increasingly resistant to several antibiotics, including cephalosporins and fluoroquinolones which were commonly used to treat urinary tract and bacteremia [48].

Mechanisms of beta-lactam resistance

The bacterial resistance mechanisms against the beta-lactam antibiotics have been based on 3 basic principles. These include the production of beta-lactamases, the possession of an altered or acquired penicillin binding protein (PBP) with low affinity for beta-lactams, porin loss and efflux pumps [46] (figure 3).
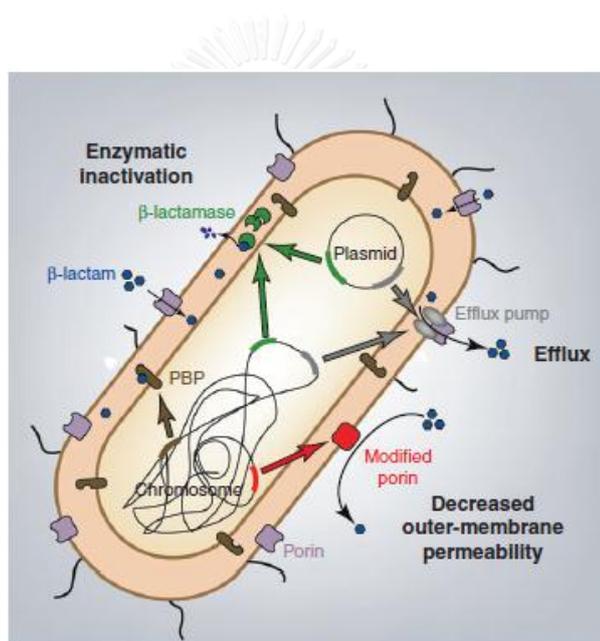
Figure 3 Mechanisms of beta-lactam resistance in Enterobacteriaceae [39].

Beta-lactamases

Beta-lactamases, enzymes that target the beta-lactam ring, were the most frequently found among the Enterobacteriaceae. They cause chromosomally or plasmid mediated resistance, and can be induced or constitutively expressed.

The classification schemes of beta-lactamases, which were introduced first time in 1980 and divided into two groups, based on analogies of the peptide sequences including Class A enzymes (serine-beta-lactamases), Class B enzymes (metallo-beta-lactamases) by Ambler [49]. Moreover, in 1981, Jaurin *et al.* reported AmpC cephalosporinase (class C enzymes) [50] and Ouellette *et al.* reported a OXA-1 enzyme (class D enzymes) in 1987 [51].

However, Bush *et al.* classified another group of classification schemes for beta-lactamases [52] (table 1), are classified into four molecular classes, A, B, C, and D, based on conserved and distinguishing amino acid motif [53]. The Classes A, C, and D hydrolyze their substrates by forming an acyl enzyme through an active site serine. Class B beta-lactamases, metalloenzymes, differ from other beta-lactamases in that they require zinc for the active site instead of the amino acid serine to facilitate beta-lactam hydrolysis [49, 53]. These enzymes cleave the amide bond of the beta-lactam ring thus inactivating the antibiotic activity [54].

Table 1 The classification scheme of beta-lactamases [53]

Molecular classes	Beta-lactamases	Examples
A	Broad-spectrum beta-lactamases	TEM-1, TEM-2, SHV-1
	ESBLs	TEM-3, SHV-12, CTX-M-15
	carbapenemases	KPC, GES, SME
B	Metallo-beta-lactamases	VIM, IMP
C	AmpC cephamycinases (chromosomal-encoded)	
D	AmpC cephamycinases (plasmid-encoded)	CMY, DHA, MOX, FOX
	Broad-spectrum beta-lactamases	OXA-1, OXA-9
	ESBL OXA-type	OXA-2, OXA-10
	carbapenemases	OXA-48, -23, -24, -58
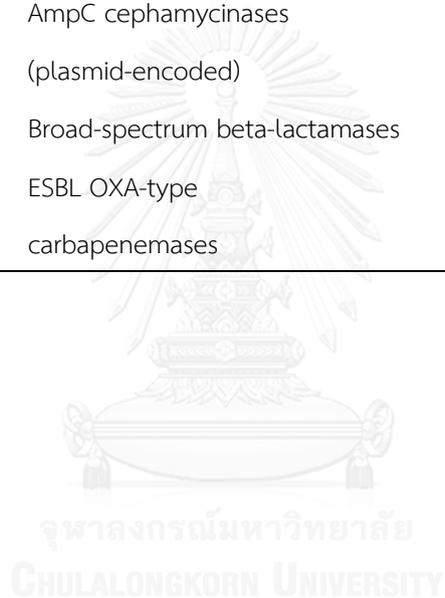
ESBLs (Extended-spectrum beta-lactamases)

Extended-spectrum beta-lactamases (ESBLs) are a group of enzymes that are able to hydrolyze and confer resistance to penicillins 2nd, 3rd generation cephalosporins, monobactams, except carbapenems [53, 55]. However, ESBLs can be inhibited by the classical beta-lactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam. The major cause of 3rd generation cephalosporin resistance in Enterobacteriaceae are ESBL enzymes of Ambler Class A [46]. ESBL-producing organisms are also a common cause of nosocomial pneumonia, central venous in-related bacteremia urinary tract infections, peritonitis, cholangitis, intra-abdominal abscesses, and meningitis [56].

The gene responsible for ESBLs is usually found in large plasmids. Also those plasmids with ESBLs frequently carry resistance genes to other drug classes (aminoglycosides, chloramphenicol, sulfonamide, trimethoprim, and tetracycline) [57]. The most common broad-spectrum beta-lactamases are TEM-1, TEM-2 and SHV-1. These enzymes hydrolyze penicillins and early cephalosporins such as cephaloridine and cephalothin. They are not active against third generation cephalosporins and can be inhibited by clavulanic acid and tazobactam.

ESBLs enzymes are the derivatives of TEM-1, TEM-2 and SHV-1 by amino acid substitutions. The most common ESBLs are TEM, SHV type ESBLs and CTX-M enzymes. In 1960, TEM-1 (Temoneira) enzyme was recovered in Greece from *E. coli* [58]. These TEM-type ESBLs were found in *K. pneumoniae, E. coli, P. mirabilis, Salmonella* spp. and *M. morganiii* [59, 60]. SHV-2 was found in *Klebsiella ozaenae* from Germany in 1983 [57]. The SHV-type ESBLs was found in *K. pneumoniae, E. coli* and *Citrobacter* spp. [61]. Other type of ESBLs of Ambler class A enzymes, which are naturally able to hydrolyze third generation cephalosporins and/or several carbapenems include CTX-M, VEB, GES and IBC, PER, TLA, BES, and SFO [57]. The most widespread ESBLs are CTX-M enzyme.

In 1989, CTX-M-1 in *E. coli* has been reported from Germany. CTX-M type ESBL preferentially hydrolyze cefotaxime rather than ceftazidime [62]. Tazobactam was found to inhibit CTX-M at least an order of magnitude better than by clavulanic acid

[63]. These enzymes are divided into 5 subgroups, based on amino acid similarity. CTX-M-1 group includes CTX-M-1, -3, -10, -12, -15, -22, -23, -28, -29, -30, -32, -33, -36, -54 and FEC-1. CTX-M-2 group includes CTX-M-2, -4, -4L, -5, -6, -7, -20, and Toho-1. CTX-M-8 group includes CTX-M-8 and CTX-M-40. CTX-M-9 group includes CTX-M-9, -13, -14, -16, -17, -19, -21, -24, -27, -45, -46, -47, -48, -49, -50 and Toho-2. CTX-M-25 group includes CTX-M-25, -26, -39 and CTX-M-41 [64].

A natural ancestor of each subgroup had representation by a chromosomal gene of the different environmental *Kluyvera* species as *K. ascorbata* and *K. georgiana* [65]. The CTX-M enzymes were often reported in *E. coli* and *K. pneumoniae* [61]. The surrounding of *bla*$_{CTX-M}$ associated with IS*Ecp1* and IS*CR1* are in complex class 1 integrons. However, the first clinical strain producing CTX-M was detected in Japan in 1993 with the characterization of the Toho-1 enzyme from an *E. coli* isolate [66]. The CTX-M enzymes were reported in several countries, including China, Japan, Vietnam, Korea, Taiwan and India [67-71]. The OXA-type ESBLs are able to hydrolyze benzylpenicillins, aminopenicillins, carboxypenicillins, oxacillin and cloxacillin, and can be inhibited by NaCl [32]. The members of OXA-type ESBLs are OXA-18, derivatives of OXA-2 and OXA-10 [43]. The most common of this type are OXA-1, OXA-2 and OXA-10 subgroup [32]. These enzymes were reported in *E. coli*, *K. pneumoniae, S. flexneri* and *Salmonella* spp. [72-74].

AmpC beta-lactamases

AmpC beta-lactamase is belong to class C cephalosporinase [53]. These enzymes hydrolyze penicillins and cephalosporins, oxyimino-cephalosporins (ceftazidime, cefpodoxime and cefotaxime), cephamycins (e.g. cefotetan, cefoxitin) and monobactams (aztreonam). They are usually retaining susceptibility to carbapenems and, in some instances, fourth-generation cephalosporins (e.g. cefepime, cefpirome). They can be chromosomal or plasmid encoded [43]. These enzymes are not affected by available beta-lactamase inhibitors (clavulanate, sulbactam and tazobactam) [75]. The fourth-generation cephalosporin, cefepime, is active against organisms producing AmpC beta-lactamases [76].

Cefoxitin induces ampC expression by binding to transpeptidases (penicillin-binding proteins) [77]. Expression of chromosomal *ampC* genes are typically influenced by the AmpR regulator (negatively in the absence of inducer, positively in the presence of inducer) and the ampD-encoded cytosolic amidase, whose activity (in the absence of inducer) negatively impacts on ampC expression [78]. The mutations of *ampC* gene , *ampD* gene and *amp*R can cause high- level ampC expression [46]. Chromosomal AmpC beta-lactamases are recovered in several Enterobacteriaceae such as *C. freundii, E. coli, Enterobacter* spp., *P. stuartii, M. morganii, S. marcescens* [43].

The plasmid-encoded AmpC beta-lactamases are divided into five families, based on amino acid sequence homologies. C-1 includes CMY-2, -3, -4, -5, -6, -7, -12 -13, LAT-1 and CFE-1. C-2 includes CMY-1, -8, -9, -10, -11, MOX-1, -2 and FOX-1, -2, -3, -4, -5 and FOX-6. C-3 includes the ACT-1 and MIR-1. C-4 includes DHA, C-5 includes ACC-1 [43].

The first report of AmpC enzymes was CMY-1 (cephamycins) which was detected in *K. pneumoniae* from South Korea, in 1989 [79]. Plasmid-encoded AmpC beta-lactamases were found in *K. pneumoniae, K. oxytoca, Salmonella* spp*., P. mirabilis, E. coli, C. freundii* and *E. aerogenes* [80]. The CMY and DHA beta-lactamases were frequently found in *E. coli* and *K. pneumoniae* [46]. Plasmid-encoded AmpC were reported worldwide, such as in Europe, Africa, the Middle East, Asia, North,

Central and South America [75]. Many strains with plasmid-mediated AmpC enzymes were reported to co-produce TEM-1, TEM-2, or an ESBL, such as SHV-5 [81-83]. The plasmids carrying *ampC* genes frequently harboured multiple resistance genes which cause resistance to chloramphenicol, aminoglycosides, tetracycline, trimethoprim, sulfonamide, or mercuric ion [75]. However, the AmpC enzymes together with loss of outer membrane porins can cause resistance to carbapenem.

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Carbapenem-resistant Enterobacteriaceae (CRE)

The past several decades, the spread of Enterobacteriaceae which were resistant to broad-spectrum antimicrobials has been increasingly reported. The carbapenems are mostly used to treat infections caused by these resistant organisms [84] and are recommended as first-line therapy for severe infections caused by ESBL-producing Enterobacteriaceae [56]. Then, carbapenem-resistant Enterobacteriaceae (CRE) was discovered in clinical isolates which were commonly isolated from the urinary tract infection [85]. The identification of carbapenem-resistant isolates is an emerging phenomenon as a consequence of the dissemination of community-acquired CTX-M-producing isolates worldwide [55]. Antibiotics which are currently used to treat infections caused by carbapenem-resistant Enterobacteriaceae include polymyxins, aminoglycosides, fosfomycin, temocillin and tigecycline. These are the most commonly used " drugs of last resort" [85].

CRE infection is important for health-care settings and community. There are 4 reasons as follows. First, invasive infection with CRE is associated with high mortality and morbidity. Second, pan-resistant CRE have been reported. Third, CRE can spread rapidly in health-care settings. Fourth, CRE could spread into the community among otherwise healthy persons [84]. CRE have been reported worldwide as a consequence of acquisition of carbapenemase genes [4].

Carbapenems

The carbapenems are a group of beta-lactam antibiotics with the widest spectrum of antibacterial activity against the Gram-positive and Gram-negative bacteria. Thienamycin was the first discovered carbapenem, and later become the model compound for all other carbapenems [86]. The basic mechanism of this is to inhibit the cell wall synthesis. Carbapenems enter to the cell through outer membrane proteins called porins. Then, they irreversibly bind to the PBP, and block the formation of peptide cross-linking in cell wall. Ultimately, cells are autolysis due to the peptidoglycan weakens making the cell bursts and dead.

Carbapenems include imipenem, meropenem, ertapenem, doripenem, panipenem and biapenem (figure 4). They are potent against Gram-positive and Gram-negative bacteria. Carbapenems can be combine with other antibiotics for treatment. Imipenem in combination with tigecycline was used to treat ESBL-producing *K. pneumoniae* and *E. coli* [87].
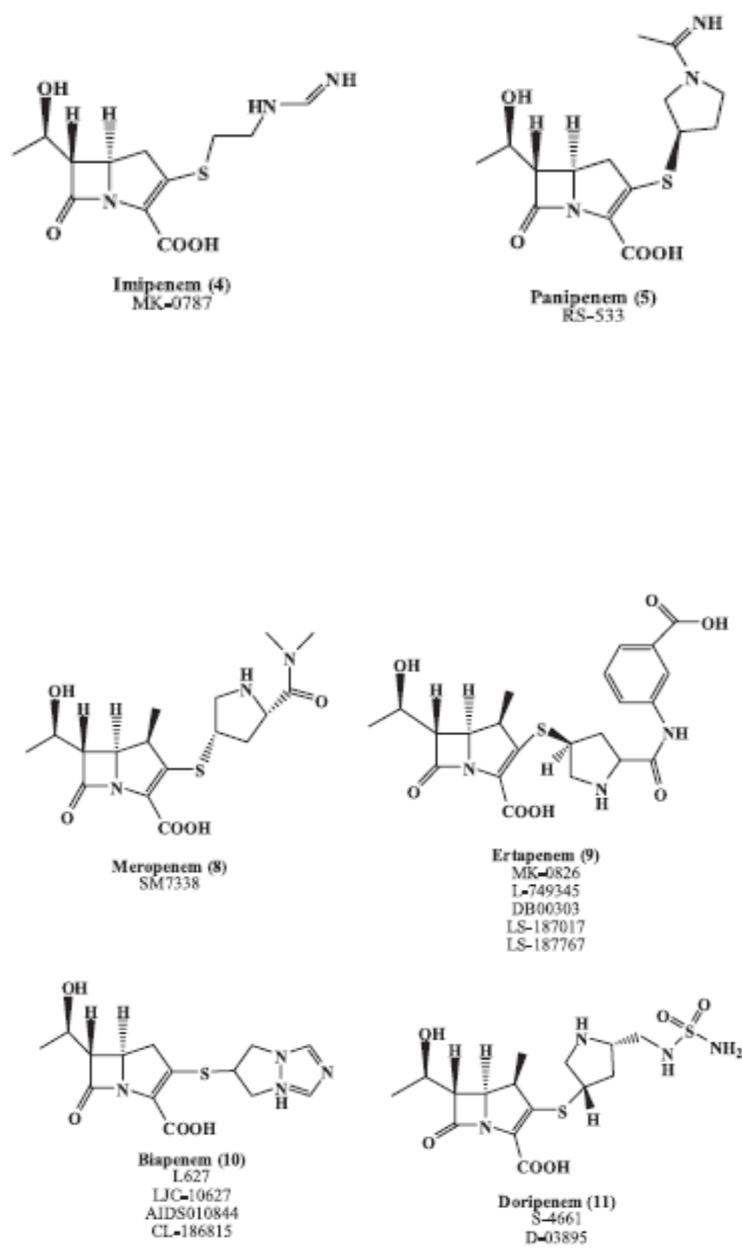
Figure 4 Chemical structures of clinically available carbapenems [86]

Mechanism of carbapenem resistance

The family Enterobacteriaceae has two main mechanisms of carbapenem resistance, including the production of carbapenemases and a decrease in uptake of antibiotics due to qualitative and/or quantitative deficiency of porin expression in association with overexpression of beta-lactamases that possess very weak affinity for carbapenems [39].

Non-carbapenemase mediated carbapenem resistance in Enterobacteriaceae

The outer membrane of Gram-negative bacteria contains specific proteins, called porins. They form hydrophilic channels and allow with selective uptake essential nutrients and other compounds, including antibiotics. There are four types of porins, including genera/nonspecific porins, substrate-specific porins, gated porins, and efflux porins [88]. However, the main types of carbapenem resistance are due to genera/nonspecific porins, substrate-specific porins and efflux porins [86]. The OmpF or OmpC families are the primary porins found in Enterobacteriaceae [89]. The changes in the number or activity of porins may effect on antibiotic resistance [39].

The substitutions, or decreased expression, or non-efflux porins decrease the level of carbapenem in periplasm and mostly found in *K. pneumonia* [90], *E. aerogenes* [91], *E. coli* [92], *S. marcescens* [93], *P. mirabilis* [94], *C. freundii* [95], *Shigella dysenteriae* [96] and *Salmonella enterica* [97]. Carbapenem-resistant Enterobacteriaceae isolates with overexpression of efflux pump were mostly found in *E. aerogenes* [98]. The overexpression of AcrA, an efflux pump component, was responsible for imipenem resistance in *E. aerogenes* [99]. Moreover, mutations in the PBPs or decreases in PBP transcription also result in carbapenem resistance phenotype [86]. The decrease of PBPs was found in carbapenem-resistant *P. mirabilis* [100]. Acquisition of a novel PBP or amino acid substitutions in PBPs active site caused carbapenem resistance in *P. mirabilis* [100], and *E. coli* [101].

The plasmid-mediated AmpC beta-lactamases were found to be associated with decreased cell membrane permeability, due to modifications in OmpF for *S* typhimurium, OmpF and OmpC for *E. coli* and OmpK35/36 for *K. pneumoniae* [102-

104]. The production of ESBLs in combination with low outer membrane permeability increased carbapenem resistance in Enterobacteriaceae. This mechanism was reported in enterobacterial species, including *K. pneumoniae, E. coli, Salmonella* spp. and *Enterobacter* spp. [39].

Carbapenemases in carbapenem-resistant Enterobacteriaceae

Class of carbapenemases

Carbapenemases mainly belong to 3 main groups of beta-lactamases A, B and D. The class A and D enzymes have serine in their active site, namely serine carbapenemases. Class B enzymes require zinc ions at active site for hydrolysis, namely metallo-beta-lactamases. The genes encoding for carbapenemases are on plasmid or chromosome with association mobile genetic elements such as integron and transposon [4]. They were often co-produced with ESBL enzymes or AmpC beta-lactamases [6, 105].

Class A (Serine carbapenemases)

Class A enzymes are resistant to all beta-lactams including penicillins, cephalosporins, carbapenems and aztreonam by a hydrolytic mechanism, involving an active site at position 70 [106]. The hydrolytic activity is inhibited (*in vitro*) by clavulanic acid, tazobactam and boronic acid [5, 39]. Enzymes in class A, include *Serratia marcescens* enzyme (SME), non-metallocarbapenemase-A (NMC-A), Imipenem-hydrolyzing beta-lactamase (IMI), Guiana extended spectrum (GES), Biceˆtre carbapenemase (BIC), and the most common *K. pneumoniae* carbapenemase (KPC) [107].

SME (*Serratia marcescens* enzyme)

The SME enzymes have been only identified in *S. marcescens*, with chromosomally encoded gene [108]. Basically, this family of enzymes include 5 variants (www.lahey.org/Studies). This enzyme was identified from two *S. marcescens* isolates in London in 1982 [109]. These strains were resistant to aminopenicillins, ureidopenicillins and carboxypenicillins, early-generation cepharosporins, imipenem and aztreonam [110]. However, these enzymes also were found in USA [111, 112]. An enzyme activity is regulated by a LysR-type regulator [113].

IMI (imipenem-hydrolyzing beta-lactamase)

In 1984, IMI-1 carbapenemase was identified from two *E. cloacae* strains, isolated at Southern California and encoded on chromosome [114], with a very similar hydrolysis profile. It was inducible, due to an upstream-located LysR-type regulatory gene [110]. Recently, 8 variants of this enzyme have been reported (www.lahey.org/Studies).

NMC-A (non-metallocarbapenemase-A)

The first carbapenemase producer in NMC-A was identified in 1993 from *E. cloacae* [115]. This chromosomally-encoded enzyme can hydrolyze aminocarboxypenicillins, cephalothin, imipenem, and aztreonam. The activity of NMC-A is partially inhibited by clavulanic acid, tazobactam and sulbactam. The bla_{NMC-A} gene was regulated by a LysR-type regulatory gene, similar to those found upstream of the naturally encoded cephalosporinase (AmpC-type) genes [110].

KPC (Klebsiella pneumoniae carbapenemase)

In 1996, KPC-1 was first identified in North Carolina from *K. pneumoniae* [3]. However, In 2005 KPC were identified as a outbreak in USA [116]. These KPC producers were found in several countries around the world such as France [117], Israel [118, 119], China [9], Taiwan [15], Brazil [120], Argentina [121]. The main endemic focus in Europe is located in Greece [105, 122], Italy [123], Scotland [124], Germany [125], Belgium [126], Finland [127], Scandinavian [128] and Switzerland [129] (figure 5). However, 11 KPC variants have been identified (http://www.lahey.org). The KPC enzymes have been frequently reported from *K. pneumoniae* isolates, followed by *E. coli* and other enterobacterial species [130]. The KPC enzymes were predominantly found in *Enterobacter* spp. and *Salmonella* spp. [131, 132]. KPC-2 was first identified in 2003 from Baltimore in *S. enterica* [132]. KPC-3 was first reported in *K. pneumoniae* outbreak in New York [133] and was also detected in *Enterobacter* spp. [131].

Outbreak of KPC-producing *E. coli* was reported in many countries, including Greece, the United States and Israel [134-136]. Among those, the endemic area for the KPC producers are USA, Greece and Israel [130]. The bla_{KPC-1} was found to be located on a 50-kb transferable plasmid of *K. pneumoniae* [110]. The bla_{KPC} genes

are always associated with *Tn4401* transposon. The specific *K. pneumoniae* clone (ST258), expressing *bla*$_{KPC-2}$, has been extensively identified worldwide [137]. Attributed mortality to infections due to KPC-producing strains was reported to be high (50% or more) [35].
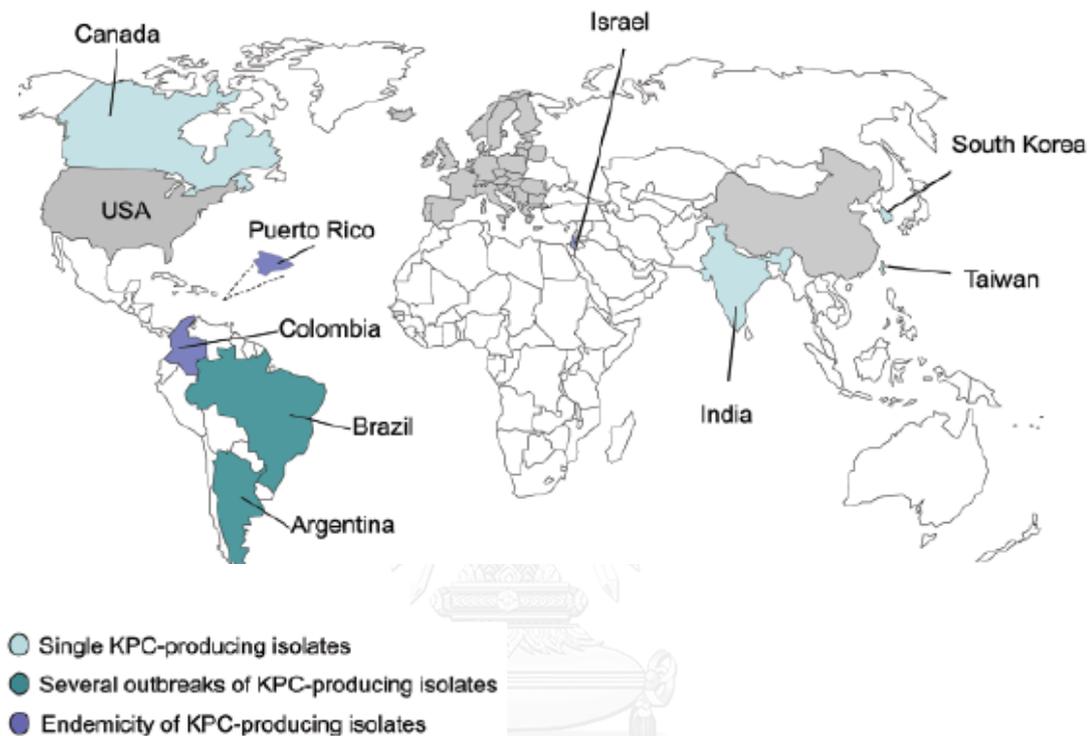
Figure 5 The distribution of *Klebsiella pneumoniae* carbapenemase (KPC) producer
[1].

GES (Guiana extended spectrum)

All the GES variants can hydrolyze broad-spectrum cephalosporins, but some variants possess amino acid substitutions within their active sites (position 104 and 170, causing broad spectrum activity against carbapenems [138]. Ges-2, the frequently reported class A enzyme with carbapenemase activity, is the point-mutant derivative of GES-1 [139]. GES-2 was identified in South Africa from *P. aeruginosa* [140]. GES-2 differs from GES-1 by a substitution of Gly-to-Asp in Ambler position 170, which was located inside the omega loop [110]. The GES gene is located on transposons and this family includes 25 variants (http://www.lahey.org).

Class B (Metallo-beta-lactamases)

Class B enzymes have serine at active site of enzyme and required one or two zinc ions for their activity. These are classified into subclasses B1, B2 and B3 [54]. Three subclasses base on a combination of structural features, zinc affinities for the two binding sites, and hydrolysis characteristic [4]. Subclasses B1 and B3 are divided by amino acid homology. They bind two zinc atoms for optimal hydrolysis. The subclass B2 enzymes are inhibited when the second zinc is bound. The subclass B2 also differs in hydrolysis spectrum, since it preferentially hydrolyzes carbapenems. Incontrast to B1 and B3, they have broad hydrolysis spectrum [141]. Subclasses B1 includes VIM, IMP, GIM, SIM, SPM, AIM, DIM and NDM gene [142]. These enzymes can hydrolyze all penicillins, cephalosporins, and carbapenems with the exception of aztreonam. They are not inhibited by beta-lactamase inhibitors, including clavulanic acid, sulbactam and tazobactam [143]. EDTA which is a chelator of divalent cations [39], dipicolinic acid, and 1,10-*o*-phe-nanthroline [53] are the inhibitors of these enzyme. The first MBLs (metallo-beta-lactamases) were detected from environment and opportunistic bacteria such as *Bacillus cereus, Aeromonas* spp. and *Stenotrophomonas maltophilia* [144-147]. The most common types of MBLs identified in Enterobacteriaceae include the IMP, VIM and NDM group. The KHM-1 is rare [17]. The SPM-1, GIM-1, SIM-1, DIM-1 and AIM-1 enzyme have not been identified in Enterobacteriaceae [148]. The attributed mortality was associated with MBL production, is ranging from 18% to 67% [149].

IMP (Imipinemase)

In Japan, IMP-1 was first reported from a *S. marcescens* isolate [150]. IMP-type carbapenemase producers have spread worldwide which has been reported in Greece, Taiwan and Japan [4, 17]. IMP-positive enterobacteria was reported to be Japan [151]. Dissemination of IMP-producing Enterobacteriaceae was identified at Turkey, Lebanon, Brazil, Japan, Korea and Taiwan [152-154]. The IMP-1 and IMP-2 enzyme were reported at the US, China, Australia and Singapore [9, 18-21]. IMP-3 was identified in *Shigella flexneri* from Japan [155]. IMP-4 was recovered in *Citrobacter youngae* from Guangzhou [156] and in *K. pneumoniae* from Wuhan [157]. IMP-6 was found in *S. marcescens* from Japan [158]. In Thailand, IMP-14a was reported from *K.*

pneumoniae isolate [159]. IMP-14 differs from IMP-14a at position 179 (from adenine to cytosine).

IMP enzymes can divided into three groups based on amino acid identity. Group 1 includes IMP-1, -3, -4, -5, -6 and IMP-7, Group 2 includes IMP-2 and IMP-8, and Group 3 includes IMP-9 [110] (figure 6). The mainly IMP-producing Enterobacteriaceae isolates were *S. marcescens*, *E. coli* and *E. cloacae* [142]. These *bla*$_{IMP}$ genes were often located on plasmids which are transferable to other bacteria. The genetic environment of this enzyme is most often associated with class 1 integron, which often carries other resistance gene such as *aacA4, aadA1 and aadB*, class D beta-lactamases (*bla*$_{OXA}$) and *catB* [160]. The 48 IMP variants have been assigned (http://www.lahey.org).

```
                40               60               80              100
                |                |                |                |
IMP-1  MSKLSVFFIFLFCSIATAAESLPDLKIEKLDEGVYVHTSFEEVNGMGVVPKHGLVVLVNAZAYLIDTPFTAKDTEKLVTWFVERG
IMP-3  ------------------------------------------------------------------------------------
IMP-4  -------------------P-----------------------------------D-----------------------------
IMP-5  ----F---M---------------------------------------------T-----------------------------
IMP-6  ------------------------------------------------------------------------------------
IMP-7  -K------M-------ASG-A---------------------------------TD-----------------------------
IMP-2  -K--F-LCVCFL---TA-GAR---------E---------------S-------TD------------T-----N-----------
IMP-8  -K--F-LCVCFL---TA-GAA---------E---------------S-------TD------------T-----N-----------
IMP-9  ----F----MCFL---TA-G--------------------------I-------TD---------------N--N----------


             118 120          140              180              200
              |   |            |                |                |
IMP-1  YKIRGSISSHFHSDSTGGIEWLNSRSIPTYASELTNELLKKDGKVQATNSFSGVNYWLVKNKIEVFYPGPGHTPDNVVVWLPERK
IMP-3  -----------------------G------------------------------------------------------------
IMP-4  ----------H-H---------Q-----------------------------------------------L------------
IMP-5  ----------H-H---------Q---------------------K-----AS-----K----------------------NR
IMP-6  ----------H-H--------------------------------------------------------------------
IMP-7  ----------H-H---------Q---------------------K-----AS-----K---I-----------------HR
IMP-2  -----T----H-H--------Q----------------------K------S--------------Q---------K--
IMP-8  -----T----H-H--------Q----------------------K------S--------------Q---------K-
IMP-9  -R--------H-H--------Q----------------------KY-----S-----K--------A-------------NR


           220          240          260    280          300
            |            |            |      |            |
IMP-1  ILPGGCFIKPYGLGNLGDANIEAWPKSAKLLESKYGKAKLVVPSHSEVGDASLLKLTLEQAVKGLNESKKPSKPSN
IMP-3  ------C-------------------------G------------------------------------------
IMP-4  ------C------------L---------I----------A-------------------------L--
IMP-5  V----C-V---------Y--------M----------------R-------------------------
IMP-6  ------C-----------------------------G------------------------------
IMP-7  V----C-V-------L---------V----------R--------------------L-----
IMP-2  ------C-V-D-------L-------I-M--V------S----I-------R-N-----------------Q---
IMP-8  ------C-V-D-------L-------I-M---------S----I-------R-N-----------------Q---
IMP-9  V-----C-V-----------L--------M---S--------DI--S------N--T--F------------
```

Figure 6 The alignment of amino acid sequences for IMP variants enzymes.

The arrow indicates the putative position of the leader peptide cleavage site [110].

VIM (Verona integron-encoded metallo-beta-lactamase)

In 1997, first VIM-1 was identified in Italy from *P. aeruginosa* [161]. It has been reported in Greece, Taiwan and Japan [4, 17]. The VIM-2 variant was reported in France from *P. aeruginosa* [162]. VIM-2 is commonly reported worldwide, it was endemic in Southeast Asia (Taiwan, South Korea) and Southern Europe (Greece, Italy, Spain) [163] (figure 7). VIM-producing *K. pneumoniae* was first observed around 2001 to 2003, in Southern Europe. It was later spread to the United States and Northern Europe [164]. This enzyme was frequently reported in Mediterranean countries [165, 166]. The most endemic area of VIM producers are Greece [1, 167]. The VIM genes are located class 1 integrons. The VIM producers were often associated with ESBLs such as SHV. Recently, 42 VIM variants have been identified (http://www.lahey.org)
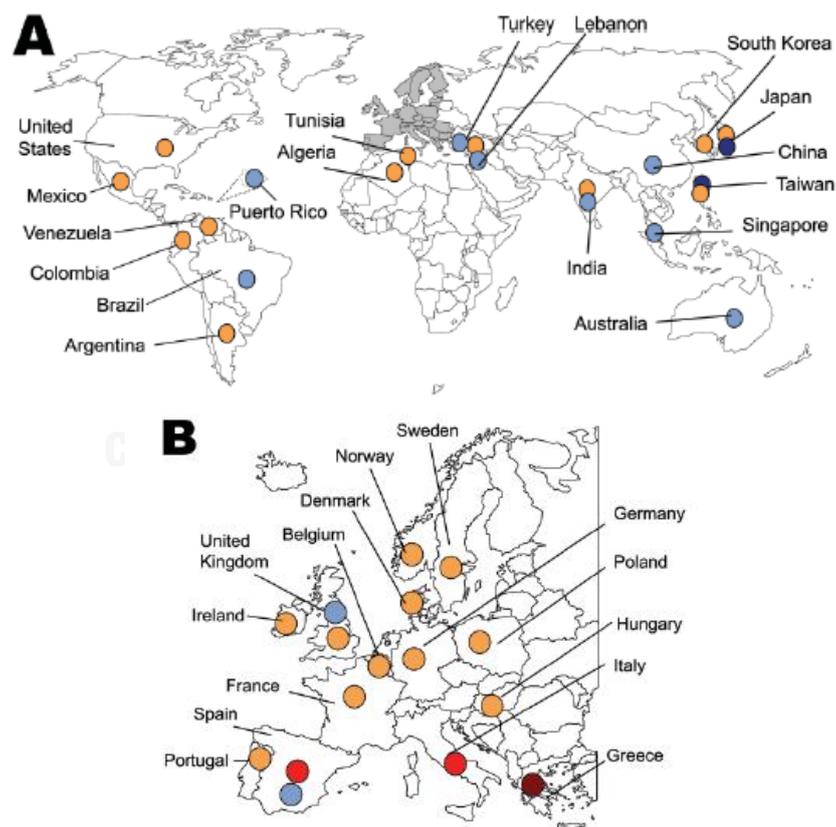
Figure 7 The distribution of Verona integron–encoded metallo-beta-lactamase (VIM) and IMP enterobacterial producers.

Worldwide (A) and European (B) geographic distribution of Verona integron–encoded metallo-beta-lactamase (VIM) and IMP enterobacterial producers [1].

SPM (Sao Paulo metallo-beta-lactamase), GIM (German imipenemase) and SIM (Seoul imipenemase)

SPM, GIM and SIM metallo beta-lactamases have not spread beyond their countries of origin [4]. The SPM-1 (Sao Paulo metallo-beta-lactamase) was first isolated in *P. aeruginosa* at Sao Paolo, Brazil in 1997 [168]. Later, GIM-1 (German imipenemase) was isolated at Germany in 2002 from *P. aeruginosa* [169]. SIM-1 (Seoul imipenemase) was discovered at Korea from *A. baumannii* [170].

KHM (Kyorin Health Science MBL)

KHM-1 was identified in Japan from a *C. freundii* clinical isolate in 2008 [171]. This enzyme was identified from a patient with catheter-associated urinary tract infection in Japan. The KHM enzyme was susceptible to monobactams (carumonam and aztreonam) and resistant to all beta-lactams and inhibited by EDTA. The similarity of KHM-1 to other MBLs is shown in figure 8.
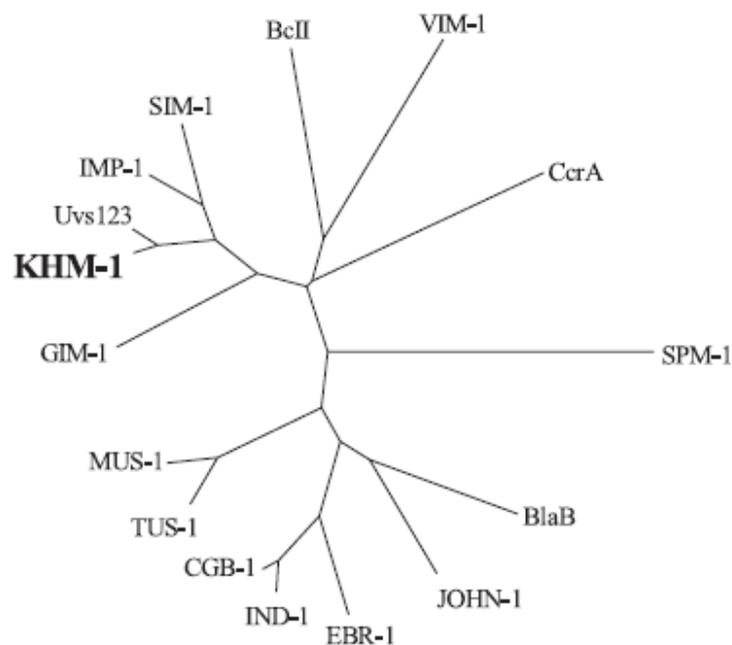
Figure 8 Dendrogram showing the similarity of KHM-1 to other MBLs
. KHM-1 and MBLs from a variety of organisms [171].

NDM (New Delhi metallo-beta-lactamases)

First NDM-1 was recovered in 2009 from *K. pneumoniae* isolated from patient of Indian origin in Sweden [27]. The NDM-producing isolates were usually resistant to almost all antibiotics, except to tigecycline and colistin [107]. NDM enzymes were often found in India [1], Pakistan [172], Bangladesh [173] and Sri Lanka [174]. NDM producers are *K. pneumoniae, E. coli, P. mirabilis, P. rettgeri, Enterobacter* spp*., Serratia* spp. and *Citrobacter* spp. [28-31, 107]. NDM producers were also reported in Great Britain, Canada, the USA, South Africa, Kenya, Saudi Arabia, Malaysia, Australia, France, Morocco, Vietnam and Thailand [30, 175-179].

The bla_{NDM-1} gene was not spread by specific clones, plasmids or by genetic structure [180]. The NDM gene was carried on different plasmid types, such as IncA/C, IncF, IncL/M, or untypeable [1, 172, 181]. The genetic environment of bla_{NDM-1} revealed a strong association between bla_{NDM-1} and bla_{MBL} , which encode a functional bleomycin resistance protein [182]. The bla_{NDM-1} gene was always associated at its 5'-end with a remnant of insertion sequence IS*Aba125,* an IS previously found in *A. baumannii* [183].

The NDM-1-producing strains were reported to be co-harboured many other resistance genes, such as those that encode OXA-48 and VIM carbapenemases, AmpC beta-lactamase, ESBLs and also resistant to aminoglycosides (16S RNA methylase), macrolides (esterases), rifampicin (rifampicin-modifying enzymes), and sulfamethoxazole [39]. The NDM-1-producing Enterobacteriaceae isolates carried ESBL genes, mostly the $bla_{CTX-M-15}$ gene [184]. The bla_{TEM-1}, bla_{OXA-1}, and bla_{OXA-10} genes were also frequently detected [180]. NDM-1 has been identified in *E. coli* ST-type 131, as a source of community-acquired infection [185]. The ST type has been reported to be strongly linked with CTX-M-15 [184]. NDM-1 producers were reported to be susceptible only to tigecycline, colistin and to a lesser extent to fosfomycin [186].

The first NDM-2-producing *A. baumannii* isolates were reported in Israel from patients with no previous travel or hospitalization in India. However, this NDM-2 differs from NDM-1 by a single amino acid substitution (Pro28Ala) [187]. NDM-3 was identified in a multidrug-resistant *E. coli* isolate, obtained from the feces of a patient

in Japan [188]. Recently, NDM-4 was found in *E. coli* isolated from India, Cameroon, Vietnam and Italy [189-191], and also in *E. cloacae* from Sri Lanka [192]. The NDM-4 differs from NDM-1 by a single substitution of amino acid at position 154 (Met154Leu). NDM-4 had increased hydrolytic activity to carbapenems and several cephalosporins compared to that of NDM-1 [190].

NDM-5 was identified in *E. coli* from India, Japan, Algeria and the United Kingdom [193-196]. NDM-5 differs from NDM-1, due to substitutions at positions 88 (Val88Leu) and 154 (Met154Leu) [195]. NDM-6 was recovered in *E. coli* from India and New Zealand [196, 197]. NDM-7 was reported in *E. coli* from India, Germany and France [196, 198, 199]. Alignment of the amino acid sequences of the seven NDM variants are shown in figure 9. The endemic area of NDM producers are in Indian subcontinent and possibly the Balkans [130]. Currently, 12 NDM variants have been identified (http://www.lahey.org and recently, NDM-positive *E. coli* was reported in the US from companion animals [178].
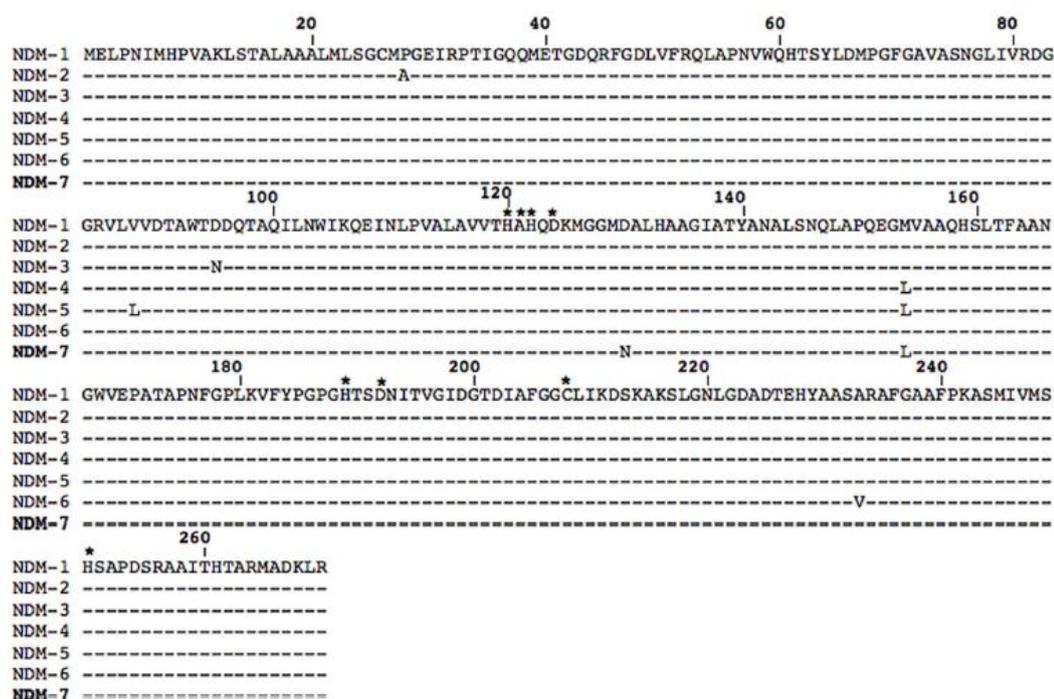
Figure 9 Alignments of the amino acid sequences of seven NDM variants [199].

Class D (Oxacillinases)

Class D enzymes are hydrolyze penicillins, 1st generation cephalosporins, and carbapenems. However, they are not inhibited by either clavulanic acid or by EDTA, tazobactam and sulbactam. They are inhibited by NaCl and weakly active against 2nd and 3rd generation cephalosporins such as cefotaxime, cetriaxone or ceftazidime, and hydrolyze only partly carbapenems [33, 39]. The first acquired class D beta-lactamase with carbapenemase activity was OXA-23. This enzyme was identified in an *A. baumannii* isolate from Scotland [200]. This enzyme was found in *P. mirabilis* in 2002 from France [201]. The first OXA-48 producers were recovered in Turkey from *K. pneumoniae* in 2001 [33]. OXA-48-positive *K. pneumoniae* was co-expressed several beta-lactamases, including SHV-2a, TEM-1 and OXA-47, and had defects in several outer membrane proteins [202]. The OXA-48 gene was mostly found in *K. pneumoniae* and *E. coli* [1]. OXA-48-positive *K. pneumoniae* isolates were reported in Middle Eastern and North African countries, India, Senegal and Argentina [203-207] (figure 10).

The endemic area of OXA-48 producers was in North Africa and Turkey [1]. OXA-48 gene was reported to be often located on a single plasmid, which has spread in many different Enterobacteriaceae strains and species [208]. The major source of the bla_{OXA-48} gene dissemination was the plasmid with approximately 62 kb in size [209]. It was a conjugative plasmid with an IncL/M type backbone and no other antibiotic resistance gene [208]. The bla_{OXA-48} gene was found to be bracketed by two IS*1999* elements to form a functional composite transposon [39]. Recently, Several $bla_{OXA-48-like}$ variants have been identified, including $bla_{OXA-162}$, $bla_{OXA-163}$, $bla_{OXA-181}$, $bla_{OXA-204}$, and $bla_{OXA-232}$ [210].
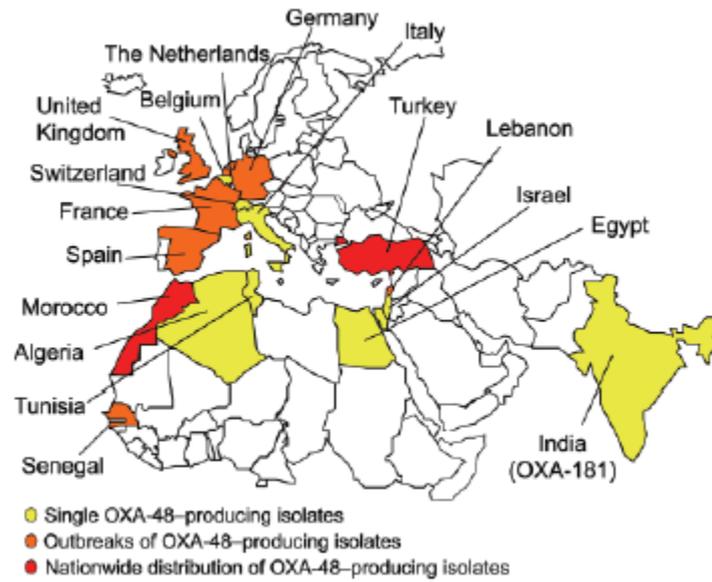
Figure 10 Geographic distribution of OXA-48 producers [1].

CHAPTER IV

MATERIAL AND METHOD

Methodology Scheme

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                    ┌─────────────────────────────────────┐
                    │   Enterobacteriaceae isolates         │
                    │ (screening for carbapenemase producers)│
                    └─────────────────────────────────────┘
                                      │
                                      ▼
                    ┌─────────────────────────────────────┐
                    │   Antimicrobial susceptibility test   │
                    └─────────────────────────────────────┘
                                      │
                                      ▼
        ┌──────────────────────────────────┐     ┌──────────────────────────┐
        │      Modified Hodge test          │────▶│  PCR and sequencing for   │
        │ Boronic acid-based inhibition test│     │   carbapenemase genes     │
        │      Combined disk test           │     └──────────────────────────┘
        └──────────────────────────────────┘                  │
                                                               ▼
             ┌──────────────────────┐     ┌──────────────────────────┐
             │  Combination disk     │     │   Modified Hodge test     │
             │  For ESBLs phenotypes  │     │    for AmpC phenotypes    │
             └──────────────────────┘     └──────────────────────────┘
                                                       │
    ┌──────────┐                          ┌──────────────────────────┐
    │   PFGE    │                          │   PCR and sequencing for  │
    └──────────┘                          │    ESBLs and AmpC genes    │
                                           └──────────────────────────┘
    ┌──────────────────┐                             │
    │ Conjugation assay │                   ┌──────────────────┐
    └──────────────────┘                   │  Sounthern blot   │
                                            └──────────────────┘
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Figure 11 Methodology Scheme

PART I Bacterial strains

1. **Enterobacteriaceae isolates**

A total of 3,854 Enterobacteriaceae were isolated from patients in King Chulalongkorn Memorial Hospital during August 2012 to January 2013. They were obtained from urine (48%), sputum (15%), pus and wound (10%), body fluids (9%), blood (9%) and other specimen (9%). These isolates were plated onto MacConkey agar (Oxoid, Basingstoke, Hampshire, England), containing ertapenem (0.25 µg/ml) (Laboratories Merck Sharp & Dohme-Chibret, France), cloxacillin (250 µg/ml) (M&H Manufacturing, Thailand) and $ZnSO_4$ (70 µg/ml) (Sigma-Aldrich, St.Louis, MO, USA), as modified from previous report [211]. Of the 3,854 Enterobacteriaceae, 104 isolates grew on this media. All isolates were identified by conventional methods. The biochemical tests included oxidase, urease, indole, citrate, motile and methyl-red/voges-proskauer (MR-VP). All isolates were kept in tripicase soy broth (BBL, Becton Dickinson and Company, Coskeysville, MD) containing 20% glycerol and stored at -80 $^{\circ}$C.

2. **Quality control strains for MIC determination**

Escherichia coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains for susceptibility test.

3. **Recipient strains for conjugation assay**

The recipient strain used for conjugation assay was the sodium azide-resistant *E. coli* (UB1637 Az^R).

PART II Antimicrobial susceptibility test and minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentrations (MICs) of imipenem (Merck Sharp & Dohme Corp., USA), meropenem (AstraZeneca UK Limited, United Kingdom), ertapenem (Laboratories Merck Sharp & Dohme-Chibret), cefotaxime (Sigma-Aldrich,co., St.Louis, MO, USA), ceftriaxone (Siam Bheasach Co.,Ltd., Thailand) and ceftazidime (Sigma-Aldrich, St.Louis, MO, USA) were determined by agar dilution method. Antimicrobial agents were adjusted to two-fold dilution with sterile DDW and mixed well in Mueller-Hinton agar (BBL, Becton Dickinson and Company, Coskeysville, MD). The concentration of antibiotics were 0.015 to 256 µg/ml, as shown in table 2. The clinical isolates were subcultured on MacConkey agar and incubated at 35°C for 18-24 hours. Each isolate was cultured in 1 ml. of trypticase soy broth at 35°C for 3-4 hours. The suspension was adjusted to 0.5 McFarland and diluted 1:10 with 0.9% sodium chloride solution (normal saline, NSS). The isolates were applied on the plate by the multi-inoculator. The final concentration was approximately 10^{4} CFU/spot. Finally, these plates were incubated at 35°C for 16-20 hours. The MIC is defined as the lowest concentration of antimicrobial agent at which there is no visible growth. MIC interpretation used breakpoint criteria recommended by CLSI (2013) guidelines, shown in table 3. The quality control strains were *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213 (table 4).

Table 2 Scheme for preparing dilutions of antimicrobial agents to be used in agar
dilution susceptibility tests

Antimicrobial solution

Step	Conc. (μg/ml)	Source	Volume (ml)	Diluent (ml)	Intermediate concentration (μg/ml)	Final conc. at 1:10 dilution in agar (μg/ml)
	5,120	stock	-	-	5,120	512
1	5,120	stock	1.05	1.05	2,560	256
2	5,120	stock	0.6	1.8	1,280	128
3	5,120	stock	0.6	4.2	640	64
4	640	Step 3	1.05	1.05	320	32
5	640	Step 3	0.6	1.8	160	16
6	640	Step 3	0.6	4.2	80	8
7	80	Step 6	1.05	1.05	40	4
8	80	Step 6	0.6	1.8	20	2
9	80	Step 6	0.6	4.2	10	1
10	10	Step 9	1.05	1.05	5	0.5
11	10	Step 9	0.6	1.8	2.5	0.25
12	10	Step 9	0.6	4.2	1.25	0.125
13	1.25	Step 12	1.05	1.05	0.625	0.0625
14	1.25	Step 12	0.6	1.8	0.3125	0.03125
15	1.25	Step 12	0.6	4.2	0.15625	0.015625

Note: This table is modified from Ericsson HM. Sherris JC. Antibiotic sensitivity testing.
Report of an international collaborative study. (Acta Pathol Microbiol Scand. 1971;
217 (suppl B) : 1-98).

Table 3 Standard MICs for interpretation of Enterobacteriaceae isolates

Antimicrobial agents	MICs for interpretation (μg/ml)		
	Susceptible	Intermediate	Resistant
Ertapenem	≤ 0.5	1	≥ 2
Imipenem	≤ 1	2	≥ 4
Meropenem	≤ 1	2	≥ 4
Cefotaxime	≤ 1	2	≥ 4
Ceftazidime	≤ 4	8	≥ 16
Cetriaxone	≤ 1	2	≥ 4
Cefoxitin	≤ 8	16	≥ 32

Table 4 Acceptable limits for quality control strains used to monitor accuracy of MICs

Antimicrobial agents	MIC (μg/ml)			
	E. faecalis ATCC 29212	E. coli ATCC 25922	P. aeruginosa ATCC 27853	S. aureus ATCC 29213
Ertapenem	4 - 16	0.004 - 0.015	2 - 8	0.06 - 0.25
Imipenem	0.5 – 2	0.06 - 0.25	1 - 4	0.015 - 0.06
Meropenem	2 - 8	0.008 - 0.06	0.25 - 1	0.03 - 0.12
Cefotaxime	-	0.03 - 0.12	8 - 32	1 - 4
Ceftazidime	-	0.06 - 0.5	1 - 4	4 - 16
Cetriaxone	-	0.03 - 0.12	8 - 64	1 - 8
Cefoxitin	-	2 - 8	-	1 - 4

PART III Detection of carbapenemase and cephalosporin producers

1. **Detection of carbapenemase phenotypes by modified Hodge test (MHT) with ertapenem**

The MHT using ertapenem disk (10 μg) (Oxoid) was used to screen for carbapenemase production (CLSI 2013). Enterobacteriaceae isolates were cultured on MacConkey agar at 35 $^\circ$C for 18-24 hours. *E. coli* ATCC 25922 was adjusted to 0.5 McFarland standard with NSS and spread onto Mueller-Hinton agar plate. An ertapenem disk was placed in the center of area. Then, the test isolates were streaked from the edge of the disk to the edge of the plate. Finally, the plate was incubated at 35 $^\circ$C for 18-24 hours. The presence of a clover leaf-like indentation of the *E. coli* ATCC 25922 growing along the test organism growth streak within the disk diffusion zone was interpreted as a positive result for the MHT. Positive control strains were OXA-48-producing *K. pneumoniae*, NDM-producing *E. coli* and IMP-producing *K. pneumoniae*.

2. **Detection of class A carbapenemase phenotypes by the boronic acid-based inhibition test**

The class A carbapenemases were detected by the boronic acid-based inhibition test using meropenem disk (10 μg MEM) (Oxoid) and meropenem disk, containing 400 μg of phenylboronic acid (PBA) (Sigma-Aldrich, St.Louis, MO, USA). The test isolate was adjusted to 0.5 McFarland standard with NSS and spread onto Mueller-Hinton agar plate. The MEM disk and MEM-PBA disk, containing 400 μg of PBA were put onto Mueller-Hinton agar plate. The plate was incubated at 35 $^\circ$C for 18-24 hours. The increase of inhibition zones of MEM-PBA and MEM ≥ 5 mm was considered positive [212]. Negative control strain and positive control strain were *E. coli* ATCC 25922 and KPC-producing *K. pneumoniae*, respectively.

3. **Detection of class B carbapenemase phenotypes by the EDTA-meropenem combined-disk test**

The class B carbapenemases were detected by the EDTA-meropenem combined-disk test using meropenem disk (10 µg) and meropenem disk, containing 292 µg of EDTA (Affymetrix, Netherlands). The protocol and the interpretation of this test was similar to boronic acid-based inhibition test [212]. Negative control strain and positive control strain were *E. coli* ATCC 25922 and NDM producing *K. pneumoniae*, respectively.

4. **Detection of ESBL phenotypes by combination disk test**

All carbapenemase-producing Enterobacteriaceae isolates were investigated for ESBL phenotypes by combination disk test. The test isolate was adjusted to 0.5 McFarland standard with NSS and spread onto Mueller-Hinton agar plate. The antibiotic disks, including 30 µg cefotaxime , 30 µg cefotaxime containing 10 µg clavulanic acid, 30 µg ceftazidime and 30 µg ceftazidime containing 10 µg clavulanic acid (BBL, Becton Dickinson and company, Coskeysville, MD), were placed onto the plates and incubated at 35 $^{\circ}$C for 18-24 hours. The ≥ 5 mm increase in inhibition zone of either cephalosporin with clavulanic acid versus cephalosporin alone was considered positive for ESBL production.

5. **Detection of AmpC beta-lactamase phenotype by MHT with cefoxitin**

All carbapenemase-producing Enterobacteriaceae isolates were investigated for AmpC beta-lactamase phenotypes by MHT with cefoxitin. *E. coli* ATCC 25922 was adjusted to 0.5 McFarland with NSS and spread onto Mueller-Hinton agar plate. A 10 µg cefoxitin disk was placed in the center of the plate. Then, the isolates were streaked from edge of cefoxitin disk to edge of the plate at 35 $^{\circ}$C for 18-24 hours. The positive result showed the growth of test isolates into inhibition zone of cefoxitin. Negative control strain and positive control strain were *E. coli* ATCC 25922 and CMY-2-producing *E. coli*, respectively.

PART IV DETECTION OF CARBAPENEMASE AND CEPHALOSPORINASE GENES

1. **Detection of carbapenemase genes**

A total of 44 Enterobacteriaceae isolates positive for MHT were determined for class A, class B and class D carbapenemase genes.

1.1 Plasmid DNA extraction

Plasmid DNA extraction was performed by HiYield^{TM}Plasmid Mini Kit (RBC Bioscience's product, Taiwan). The 1.5 ml of bacterial cell suspensions were put into microcentrifuge tube and centrifuged at 13,000 rpm for 1 minute. Then, the supernatant was discarded. The 200 μl of resuspension buffer were added and mixed by vortex. The 200 μl of lysis buffer were added and mixed by inverting. The mixture tube was stored at room temperature for 2 min. Then, the 300 μl of neutralization buffer were added, mixed by inverting and centrifuged at 13,000 rpm for 3 minutes. The clear supernate was applied to a plasmid mini column and centrifuged at 13,000 rpm for 30 seconds. The filtrate was discarded. The first 400 μl of wash buffer were added into the mini column and centrifuged at 13,000 rpm for 30 seconds. Then, the 600 μl of second wash buffer were added into the mini column and centrifuged at 13,000 rpm for 30 seconds. The wash solution was removed. The mini column matrix was dried by centrifugation at 13,000 rpm for 2 minutes. The mini column with plasmid was transferred to a microcentrifuge tube and 32 μl of elution buffer were added into the mini column and placed at room temperature for 2 minutes. The mini column was centrifuged at 13,000 rpm for 2 min. Finally, the eluted plasmid DNA samples were kept at -20°C.

1.2. Primers

Class A carbapenemase genes, including bla_{KPC} and bla_{BIC}, Class B carbapenemase genes, including bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, bla_{SPM}, bla_{NDM-1}, bla_{AIM} and bla_{DIM}, and bla_{OXA-48} Class D carbapenemase gene were determined by multiplex PCRs. The specific primers for screening carbapenemase genes are listed in table 5.

Table 5 Specific primers for amplification of carbapenemase genes

Carbapenemase genes	Primers	Sequence (5'-3')	Product size (bp)	Reference
KPC	KPC-F	5'-CGTCTAGTTCTGCTGTCTTG-3'	798	[213]
	KPC-R	5'-CTTGTCATCCTTGTTAGGCG-3'		
BIC	BIC-F	5'-TATGCAGCTCCTTTAAGGGC-3'	537	[213]
	BIC-R	5'-TCATTGGCGGTGCCGTACAC-3'		
AIM	AIM-F	5'-CTGAAGGTGTACGGAAACAC-3'	322	[213]
	AIM-R	5'-GTTCGGCCACCTCGAATTG-3'		
DIM	DIM-F	5'-GCTTGTCTTCGCTTGCTAACG-3'	699	[213]
	DIM-R	5'-CGTTCGGCTGGATTGATTTG-3'		
IMP	IMP-F	5'-GGAATAGAGTGGCTTAAYTCT-3'	188	[214]
	IMP-R	5'-CCAAACYACTASGTTATCT-3'		
VIM	VIM-F	5'-GATGGTGTTTGGTCG CATA-3'	390	[214]
	VIM-R	5'-CGAATGCGCAGCACCAG-3'		
GIM	GIM-F	5'-TCGACACACCTTGGTCTGAA-3'	477	[214]
	GIM-R	5'-AACTTCCAACTTTGCCATGC-3'		
SIM	SIM-F	5'-TACAAGGGATTCGGCATCG-3'	304	[215]
	SIM-R	5'-CCAACCAAAAGCTCTCTTTATC-3'		
SPM	SPM-F	5'-AAAATCTGGGTACGCAAACG-3'	271	[214]
	SPM-R	5'-ACATTATCCGCTGGAACAGG-3'		
NDM	NDM-F	5'-GGTTTGGCGATCTGGTTTTC-3'	621	[213]
	NDM-R	5'-CGGAATGGCTCATCACGATC-3'		
OXA-48	OXA-48-F	5'-GCGTGGTTAAGGATGAACAC-3'	438	[213]
	OXA-48-R	5'-CATCAAGTTCAACCCAACCG-3'		

1.3 Amplification of bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM} and bla_{SPM} by multiplex PCR

The specific primers for bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM} and bla_{SPM} were previously described by Ellington *et al.* [216] and Samarnthai *et al.* [217]. The PCR was performed in final volume of 25 μl containing 1X buffer, 2 mM of $MgCl_2$, 0.2mM of each deoxynucleotide triphosphates (dNTPs), 0.4 mM for IMP-F, IMP-R, VIM-F and VIM-R primers, 0.08mM for GIM-F, GIM-R, SIM-F, SIM-R, SPM-F and SPM-R primers and 0.625 U of *Taq* polymerase (Fermentas, USA) and 2 μl of DNA template. The condition of PCR was an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 50 seconds and a final extension at 72°C for 5 minutes.

1.4 Amplification of bla_{NDM-1}, bla_{KPC} and bla_{OXA-48} by multiplex PCR

The specific primers for bla_{NDM-1}, bla_{KPC} and bla_{OXA-48} were previously described by Poirel *et al.* [218]. The PCR was performed in final volume of 25 μl, containing 1X buffer, 2 mM of $MgCl_2$, 0.2mM of each dNTPs, 0.4 mM of KPC-F, KPC-R, NDM-F, NDM-R, OXA-48F and OXA-48R primers and 1.25 U of *Taq* polymerase and 2 μl DNA template. The PCR included conditions an initial denaturation step at 94°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 40 seconds, extension at 72°C for 50 seconds and a final extension at 72°C for 5 minutes.

1.5 Amplification of bla_{BIC}, bla_{AIM} and bla_{DIM} by multiplex PCR

The specific primers for bla_{BIC}, bla_{AIM} and bla_{DIM} were modified from previously described by Poirel *et al.*[218]. The PCR was performed in final volume of 25 μl containing 1X buffer, 2 mM of $MgCl_2$, 0.2mM of each dNTPs, 1 mM of BIC-F, BIC-R, AIM-F, AIM-R, DIM-F and DIM-R primers and 0.25 U of *Taq* polymerase and 2 μl DNA template. The PCR condition was an initial denaturation step at 94°C for 10 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 40 seconds, extension of 72°C for 50 seconds and final extension at 72°C for 5 minutes.

1.6 Analysis of PCR products

The PCR products were analyzed by electrophoresis on 1.2% agarose gel in 0.5 X Tris-Borate-EDTA buffer (TBE; 0.045M Tris-borate, 0.0001M EDTA pH 8.3± 1) and 0.5 µg/ml of ethidium bromide (Sigma, USA) was added to the solution before pouring the gel into a casting tray. The PCR products were mixed with 6X loading dye buffer (0.05% bromphenol blue and 20% ficoll and loaded into the gel in electrophoresis chamber containing of 0.5X TBE. Electrophoresis was run for 50 minutes at 100 volts. The PCR products were visualized by Gel Documentation System (Bio-Rad).

2. Detection of ESBL genes

A total of 19 Enterobacteriaceae isolates carring carbapenemase genes were determined for the presence of ESBL genes. Plasmid DNA extraction was performed as described in section 1.1.

2.1 Primers

ESBL genes, including bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM} and bla_{VEB}, were determined by multiplex PCRs. The specific primers for screening ESBL genes are listed in table 6

Table 6 Specific primers for amplification of ESBL genes

ESBL genes	Primers	Sequence (5'-3')	Product size (bp)	Reference
CTX-M	CTXM-A	5'-CGCTTTGCGATGTGCAG-3'	550	[219]
	CTXM-B	5'-ACCGCGATATCGTTGGT-3'		
OXA-1 group	OXA-F	5'-ATATCTCTAACTGTTGCATCTCC-3'	619	[220]
	OXA-R	5'-AAACCCTTCAAACCATCC-3'		
OXA-2 group	OXA-2-GR-F	5'-AAGAAACGCTACTCGCCTGC-3'	478	[221]
	OXA-2-GR-R	5'-CCACTCAACCCATCCTACCC-3'		
OXA-10 group	OXA-10-F	5'-GTCTTTCGAGTACGGCATTA-3'	720	[222]
	OXA-10-R	5'-ATTTTCTTAGCGGCAACTTAC-3'		
SHV group	SHV-F	5'-AGGATTGACTGCCTTTTTG-3'	392	[220]
	SHV-R	5'-ATTTGCTGATTTCGCTCG-3'		
TEM group	TEM-C	5'-ATCAGCAATAAACCAGC-3'	516	[223]
	TEM-H	5'-CCCCGAAGAACGTTTTC-3'		
VEB	VEB-A	5'-CCTTTTGCCTAAAACGTGGA-3'	216	[224]
	VEB-B	5'-TGCATTTGTTCTTCGTTTGC-3'		

2.2 Amplification of *bla*$_{OXA-1}$, *bla*$_{SHV}$ and *bla*$_{TEM}$ by multiplex PCR

The nucleotide sequences of *bla*$_{OXA-1}$, *bla*$_{SHV}$ and *bla*$_{TEM}$ were reported previously by Colom *et al.* [225] and Mabilat *et al.* [226]. The reaction mixture was 25 µl per vial, containing 1X buffer, 2 mM of MgCl$_2$, 0.2mM of each dNTPs, 0.06 mM of OXA-F and OXA-R primers, 0.08 mM of SHV-F, SHV-R primers, 0.04 mM of TEM-C and TEM-H primers and 0.5 U of *Taq* polymerase, 3 µl of DNA template. The condition for DNA amplification included an initial denaturation step at 94℃ for 5 minutes, followed by 31 cycles of denaturation at 94℃ for 30 seconds, annealing at 54℃ for 30 seconds and extension 72℃ for 1 minute and final extension at 72℃ for 10 minutes.

2.3 Amplification of *bla*$_{CTX-M}$ and *bla*$_{VEB}$ by multiplex PCR

The specific primers for *bla*$_{CTX-M}$ and *bla*$_{VEB}$ were reported previously by Bonnet *et al.* [219] and Udomsantisuk *et al.* [224]. The reaction mixture was 25 µl per vial, containing 1X buffer, 2 mM MgCl$_2$, 0.2mM of each dNTPs, 0.01mM of CTX-F and CTX-R primers, 0.06 mM of VEB-F and VEB-R primers, 0.5 U of *Taq* polymerase and 3 µl of DNA template. PCR condition for DNA amplification was as described in section 2.3.

2.4 Amplification of *bla*$_{OXA-2}$ and *bla*$_{OXA-10}$ by multiplex PCR

The specific primers for *bla*$_{OXA-2}$ and *bla*$_{OXA-10}$ were reported previously by Bert *et al.* [227] and Alipour *et al* [228]. The reaction mixture were 25 µl per vials, containing 1X buffer, 2 mM of MgCl$_2$, 0.2 mM of each dNTPs, 0.4 mM of OXA-2-GR-F, OXA-2-GR-R, OXA-10-F and OXA-10-R primers and 1.25 U *Taq* polymerase and 2 µl DNA template. PCR condition for DNA amplification was as described in section 2.3.

3. Detection of AmpC beta-lactamase genes

A total of 19 Enterobacteriaceae isolates carring carbapenemase genes were determined for the presence of AmpC beta-lactamase genes.

3.1 Primers

AmpC beta-lactamase genes, including bla_{MOX}, bla_{CIT}, $bla_{DHA,}$ $bla_{EBC,}$ bla_{ACC} and bla_{FOX}, were determined by multiplex PCRs. The specific primers for screening AmpC beta -lactamase genes are listed in table 7.

Table 7 Specific primers for amplification of AmpC beta-lactamase genes

AmpC genes	Primers	Sequence (5'-3')	Product size (bp)	Reference
ACC	ACCMF	5'-AACAGCCTCAGCAGCCGGTTA-3'	346	[229]
	ACCMR	5'-CCCCGAAGAACGTTTTC-3'		
CIT	CITMF	5'-TGGCCAGAACTGACAGGCAAA-3'	462	[229]
	CITMR	5'-TTTCTCCTGAACGTGGCTGGC-3'		
DHA	DHAMF	5'-AACTTTCACAGGTGTGCTGGGT-3'	405	[229]
	DHAMR	5'-CCGTACGCATACTGGCTTTGC-3'		
EBC	EBCMF	5'-TCGGTAAAGCCGATGTTGCGG-3'	302	[229]
	EBCMR	5'-CTTCCACTGCGGCTGCCAGTT-3'		
FOX	FOXMF	5'-AACATGGGGTATCAGGGAGATG-3'	190	[229]
	FOXMR	5'-CAAAGCGCGTAACCGGATTGG-3'		
MOX	MOXMF	5'-GCTGCTCAAGGAGCACAGGAT-3'	520	[229]
	MOXMR	5'-CACATTGACATAGGTGTGGTGC-3'		

3.2 Amplification of bla_{ACC}, bla_{CIT}, bla_{DHA}, bla_{EBC}, bla_{FOX} and bla_{MOX} by multiplex PCR

The specific primers for bla_{ACC}, bla_{CIT}, bla_{DHA}, bla_{EBC}, bla_{FOX} and bla_{MOX} were previously described by Perez *et al.* [230]. The reaction mixture was 25 µl per vial, containing 1X buffer, 2 mM of MgCl$_2$, 0.2 mM of each dNTPs, 0.5mM of ACCMF and ACCMR primers, 0.4 mM of CITMF and CITMR primers 0.6mM of DHAMF and DHAMR primers, 0.3 mM of EBCMF and EBCMR primers, 0.8mM of FOXMF, FOXMR, MOXMF and MOXMR primers and 1.25 U *Taq* polymerase and 2 µl of DNA template. The condition for DNA amplification was an initial denaturation step at 94℃ for 3

minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 1 minute and final extension at 72°C for 10 minutes.

PART V ANALYSIS OF ENTIRE BLA GENES BY PCR AND DNA SEQUENCING

All carbapenemase produces harbouring *bla* genes were investigated for the entire *bla* genes by PCR and DNA sequencing.

1. **Primer for PCR and DNA sequencing**

The specific primers of *bla* genes, including carbapenemase and ESBL genes are listed in table 8.

Table 8 Specific primers for amplification of entire *bla* genes and DNA sequencing

Entire genes	Primers	Sequence (5'-3')	Product size (bp)	Reference
IMP	IMP-entire-F	5'-GCGGTTTTCATGGCTTGT3'	971	[231]
	IMP-entire-R	5'-TAAGTTGCGCGTTGTGGA-3'		
NDM	NDM-entire-F	5'-ATGGAATTGCCCAATATTATGCAC-3'	813	This study
	NDM-entire-R	5'-TCAGCGCAGCTTGTCGGC-3'		
OXA-48	OXA-48-entire-F	5'-CTTCGCGCATCTTGTTGTC-3'	970	[231]
	OXA-48-entire-R	5'-CGATCGAGCATCAGCATTT-3'		
CTX-M	CTX-M-entire-F	5'-TCTTCCAGAATAAGGAATCCC-3'	909	[232]
	CTX-M-entire-R	5'-CCGTTTCCGCTATTACAAAC-3'		
OXA-1 group	OXA-1-entire-F	5'-ATGAAAAACACAATACATATC-3'	831	[233]
	OXA-1-entire-R	5'-GAGTTATAAATTTAGTGTGTTTAG-3'		
OXA-10 group	OXA-10-entire-F	5'TTGAAGTGTTGACGCCTTTG-3'	1,069	[231]
	OXA-10-entire-R	5'-TACAAATGTACGGCCAGCAA-3'		
SHV	SHV-entire-F	5'-TGGTTATGCGTTATATTCGCC-3'	868	[234]
	SHV-entire-R	5'-GGTTAGCGTTGCCAGTGCT-3'		
TEM	TEM-entire-F	5'-TCCGCTCATGAGACAATAACC-3'	931	[232]
	TEM-entire-R	5'-TTGGTCTGACAGTTACCAATGC-3'		
VEB	VEB-entire-F	5'-GATAGGAGTACAGACATATG-3'	914	[235]
	VEB-entire-R	5'-TTTATTCAAATAGTAATTCCACG-3'		

2. **Amplification of entire carbapenemase genes**

2.1 **Amplification of entire *bla*$_{IMP}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$**

The specific primers of entire *bla*$_{IMP}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ were as previously described, as shown in Table 8. The reaction mixture was 50 µl per vial, contained 1X buffer, 2 mM of MgCl$_2$, 0.2 mM of each dNTPs, 0.4 mM of IMP-F, IMP-R, NDM-F,

NDM-R, OXA-48-F and OXA-48-R primer and 1.25 U *Taq* polymerase and 2 µl DNA template. The conditions for DNA amplification were an initial denaturation step at 95℃ for 2 minutes followed by 25 cycles of denaturation at 95℃ for 1minute, annealing at 55℃ for 1minute and extension at 72℃ for 1 minute and final extension at 72℃ for 10 minutes.

    3. **Amplification of entire ESBL genes**

        3.1 Amplification of entire *bla*$_{CTX-M}$, *bla*$_{TEM,}$ *bla*$_{SHV}$, *bla*$_{VEB}$ and *bla*$_{OXA-10}$ group

The specific primers of entire *bla*$_{CTX-M}$, *bla*$_{TEM,}$ *bla*$_{SHV}$, *bla*$_{VEB}$ and *bla*$_{OXA-10}$ group as previously described, as shown in Table 8. The reaction mixture was 50 µl per vial, contained 1X buffer, 2 mM of MgCl$_2$, 0.2 mM of each dNTPs, 0.8 mM of CTX-F, CTX-R, TEM-F, TEM-R, SHV-F, SHV-R, VEB-F, VEB-R, OXA-10-F and OXA-10-R and 1.25 U Taq polymerase and 2 µl DNA template. The condition for DNA amplification was an initial denaturation step at 95℃ for 2 minutes followed by 30 cycles of denaturation at 95℃ for 1minute, annealing at 55℃ for 1minute and extension at 72℃ for 1 minute and final extension at 72℃ for 10 minutes.

        3.2 Amplification of entire *bla*$_{OXA-1}$ group

The specific primers of entire *bla*$_{OXA-1}$ group were as previously described as shown in Table 8. The reaction mixture was 50 µl per vial, contained 1X buffer, 2 mM of MgCl$_2$, 0.2 mM of each dNTPs, 0.4 mM of OXA-1-F, OXA-1-R and 1.25 U *Taq* polymerase and 2 µl DNA template. The condition for DNA amplification was an initial denaturation step at 95℃ for 2 minutes followed by 30 cycles of denaturation at 95℃ for 1minute, annealing at 55℃ for 1minute and extension at 72℃ for 1 minute and final extension at 72℃ for 10 minutes.

4. **Purification of PCR products**

HiYield^{TM} Gel/PCR Fragments Extraction Kit (RBC Bioscience's product, Taiwan) was used to purify the entire *bla* gene products. Five volumes of DF buffer was added into one volume of PCR product and mixed by vortexing. The mixture was poured into DF column and centrifuged at 13,000 rpm for 30 seconds. Then, DF column was washed with 600 µl of wash buffer and centrifuged at 13,000 rpm for 30 seconds. The column matrix was dried the centrifugation at 13,000 rpm for 2 minutes. After that, the DF column was put into a sterile microcentrifuge tube and 30 µl of elution buffer were added into the column matrix. Finally, purified DNA was eluted by centrifugation at 13,000 for 2 minutes.

5. **Sequencing reaction preparation**

Automated DNA sequencing was determined at the 1stBASE Inc, Singapore. Forward and reverse primers of entire bla_{OXA-1} group, bla_{OXA-10} group, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{NDM}, bla_{IMP} and bla_{OXA-48} are shown in Table 8. Sequencing reaction was conducted under BigDye® terminator v3.1 cycle sequencing kit chemistry conditions.

6. **Sequencing analysis**

The nucleotide and protein sequences were analyzed by the online software available at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) and ExPASy (www.expasy.org/). Multalin (http://multalin.toulouse.inra.fr/multalin/) was used to analyze the alignment of multiple sequences.

PART VI THE TRANSFER OF RESISTANCE GENES BY CONJUGATION

All of the 19 Enterobacteriaceae isolates, which carried bla_{IMP}, bla_{OXA-48} and bla_{NDM}, were investigated for the transfer of carbapenemase genes by conjugation, using filter mating assay in aerobic atmosphere, as described by McDougal *et al.*[225]. The donor strains were Enterobacteriaceae, carrying bla_{IMP}, bla_{OXA-48}, bla_{NDM} and the recipient was a sodium azide-resistanct *E. coli* (UB1637Az^R).

The bacteria were cultured on tryptic soy agar at 37° C for 16-18 hours. Then, The colonies of donor and recipient were separately inoculated to 2 ml of tryptic soy broth and incubated at 37°C for 16-18 hours. The 200 μl of bacterial suspensions were transferred to 5 ml of tryptic soy broth and incubated in shaking water bath at 37°C until the final turbidity was 0.3-0.5 at OD600. Then, 200 μl of donor strain suspension were mixed with 1.8 ml of recipient strain suspension. The mating mixture was poured onto 0.45-mm-pore-size nitrocellulose membrane filters (Gelman sciences Inc., USA) and were placed onto Mueller-Hinton agar plates. The plate was incubated at 37°C for 4-6 hours. After that, cells were washed from the filters with 500 μl of TSB, and 100 μl of cells suspension was spread onto MacConkey agar plates containing meropenem (0.06 μg/ml) and sodium azide (150 μg/ml). Finally, the colonies of transconjugants were confirmed by MIC testing and PCR amplification.

PART VII CLONALITY OF CARBAPENEMASE PRODUCERS

Pulse Field Gel Electrophoresis (PFGE)

Genetic relationships among carbapenemase producers were determined by PFGE performed by using the PulseNet International protocol (http://www.pulsenetinternational.org/protocols/pages/default.aspx).

1. Preparation of PFGE plugs

Enterobacteriaceae isolates were cultured on tryptic soy agar at 37° C for 16-18 hours. Bacterial colonies were suspended in 2 ml cell suspension buffer. The cell suspensions were adjusted to 0.8-1.0 at OD 610. Then, 400 µl of the adjusted cell suspension were mixed with 20 µl of proteinase K (20 mg/ml stock) and 400 µl of 1% melted Megabase agarose (Bio-Rad). The mixture was poured into wells of reusable plug mold and allowed to rigidity at room temperature for 10 minutes or at 4° C for 5 minutes.

2. Lysis of bacterial cells in agarose plugs

The bacterial cells in agarose plug were lysed with cell lysis/proteinase K buffer at $54\text{-}55^\circ$ C for 1.5-2 hours. The plugs were washed twice with pre-heated ($54\text{-}55^\circ$ C) sterile ultrapure water and 4 times with pre-heated ($54\text{-}55^\circ$ C) sterile TE buffer at $54\text{-}55^\circ$ C for 10-15 minutes.

3. Restriction digestion of DNA in the plugs with *Xba*I

The plugs were cut into a 2 mm-wide slice and digested with *Xba*I at 37° C for 4 hours. Then, the enzyme/buffer mixture was removed and 200 µl of 0.5X TBE were added. The plugs were incubated at room temperature for 5 minutes.

4. Casting agarose gel

The plugs were sealed on a comb with 1% Megabase Gold agarose. The 1% agarose in 0.5X TBE was poured into the gel stray and the comb was removed from the solidified gel.

5. Electrophoresis condition

The casted gel was placed into electrophoresis chamber. Electrophoresis condition on CHEF DR III (Bio-Rad, USA) was as follows : initial time 6.7 sec, final time 35.4 sec, voltage 6 V/cm, included angle 120° and run time 23 hours.

6. Staining and analysis of PFGE

The agarose gel was stained in 1 µg/ml of ethidium bromide for 20-30 min in a covered container. The gel was destained in double distilled water (ddH$_2$O) for 60-90 min. The water was changed every 20 min. The bands of DNA were visualized and photographed under UV light transilluminator. The gel images were analyzed with InfoQuest FP software version 4.5.

PART VIII PLASMID ANALYSIS OF CARBAPENEMASE PRODUCERS

Southern hybridization

1. Preparation of PFGE plugs

Enterobacteriaceae isolates were cultured on tryptic soy agar at 37° C for 16-18 hours. Bacterial colonies were suspended in 2 ml cell suspension buffer. The cell suspension were adjusted to 0.8-1.0 at OD 610. Then, 400 µl of the adjusted cell suspension were mixed with 20 µl of proteinase K (20 mg/ml stock) and 400 µl of 1% melted Megabase agarose (Bio-Rad). The mixture was poured into well of reusable plug mold and allowed to rigidity at room temperature for 10 minutes or at 4° C for 5 minutes.

2. Lysis of bacterial cell in agarose plugs

The bacterial cells in agarose plug was lysed with cell lysis/proteinase K buffer at 54-55° C for 1.5-2 hours. The plugs were washed twice with pre-heated (54-55° C) sterile ultrapure water and 4 times with pre-heated (54-55° C) sterile TE buffer at 54-55°C for 10-15 minutes.

3. Restriction digestion of plasmids in the plugs with S1nuclease

The plugs were washed twice with sterile ultrapure water and add 100 µl of 1X S1-nuclease buffer (Fermentas, USA) at 37°C for 15 minutes. The plugs into 200 µl of the mixture reagent, containing 40 µl of 1X S1-nuclease buffer, 1 µl of 10U S1-nuclease and 159 µl of sterile ultrapure water and were incubated at 37°C for 45 minutes. Then, the reaction was stopped by adding 0.5XTBE and incubated on ice for 20 minutes twice.

4. Casting agarose gel

The plugs were sealed on a comb with 1% Megabase Gold agarose. The 1% agarose in 0.5X TBE was poured into the gel stray and the comb was removed from the solidified gel.

5.Electrophoresis condition

The casted gel was placed into electrophoresis chamber. Electrophoresis condition on CHEF DR III (Bio-Rad, USA) was as follow: initial time 5 sec, final time 45 sec, voltage 6 V/cm, included angle 120° and run time 20 hours on CHEF DR III.

6. Preparation of DNA gel for transferring on membrane

The DNA gel was washed with depurination buffer for 30 minutes twice at room temperature with shaking. The reagent was removed and rinsed with DDW. The gel was washed with denaturing buffer for 30 minutes at room temperature with shaking. The reagent was removed and rinsed with DDW. Finally, the gel was shaken in neutralizing buffer at room temperature for 15 minutes.

7. DNA transfer method

The transfer of DNA gels was performed by capillary transfer method with 20XSSC on nylon membrane, according to Sambrook *et al.*

8. DNA fixation by UV-cross linking

The membrane was placed on Whatman 3 MM-paper soaked with 10XSSC and UV-cross linking. After that, the membrane was rinsed in DDW, allowed to air-dry and store at 2-8°C.

9. DNA-labeling

DIG High Prime DNA Labeling and Detection Starter Kit I, Roche Applied Science, Germany was used for DNA labeling and detection. A reaction vial contained 4 μl of template DNA (1 μg) and 12 μl of DDW. The DNA was boiled for 10 minutes for denaturation and was chilled on ice. The 4 μl of DIG-High Prime were added, mixed by centrifugation and incubated at 37°C for 20 hours. Then, the reaction was stopped by 2 μl of 0.2 M EDTA, pH 8.0.

10. Determination of labeling efficiency

The DIG-labeled control DNA and labeled probes were diluted to 1 ng/μl, as shown in table 9. Then, 1 μl of the DIG-labeled control DNA and labeled probes from tubes 2-9 were added on to the nylon membrane (table 9). The nucleic acid were fixed on the membrane with UV-cross linking for 30 seconds and the membrane was transferred to the plastic container, containing 20 ml of maleic acid buffer and incubated under shaking at 25°C for 2 minutes. The reagent was removed and 10 ml of blocking solution were added under shaking at 25°C for 30 minutes. The reagent was removed and 10 ml of antibody solution were added under shaking at 25°C for 30 minutes. The membrane was washed with 10 ml of washing buffer for 15 minutes twice and equilibrated in 10 ml of detection buffer for 5 minutes. The

membrane was incubated in 2 ml of color substrate solution in the dark for 5-10 minutes. After the spot was shown, the reaction was stopped with 50 ml of DDW for 5 minutes.

Table 9 Preparation of a dilution series of DIG-labeled control DNA and labeled probes

Tube	DNA (μl)	From tube	DNA Dilution Buffer (μl)	Dilution	Final concentration
1		diluted original			1 ng/μl
2	2	1	198	1:100	10 pg/μl
3	15	2	35	1:3.3	3 pg/μl
4	5	2	45	1:10	1 pg/μl
5	5	3	45	1:10	0.3 pg/μl
6	5	4	45	1:10	0.1 pg/μl
7	5	5	45	1:10	0.03 pg/μl
8	5	6	45	1:10	0.01 pg/μl
9	0	-	50	-	0

11. Hybridization

The membrane was incubated with 20 ml of pre-heated DIG Easy Hyb at 42°C for 30 minutes in shaking water bath. Then, the mixture of 4 μl of denatured DIG-labeled DNA probe and 7 ml of pre-heated DIG Easy Hyb were poured to the membrane and incubated at 42°C, overnight. The post-hybridization was performed by washing the membrane twice with the mixture of 2XSSC and 0.1%SDS for 5 minutes at 25°C. The membrane was washed twice with the mixture of 0.5 XSSC and 0.1%SDS for 15 minutes at 68°C.

12. Immunological detection

The membrane was washed with washing buffer for 5 minutes at 25°C and incubated in 100 ml of blocking solution for 30 minutes. The membrane was incubated for 30 minutes in 20 ml of antibody solution, washed twice with 100 ml of washing buffer for 15 minutes and equilibrated in 20 ml of detection buffer for 5 minutes. The membrane was incubated with color substrate solution in container in the dark for overnight and the reaction was stopped by washing the membrane with 50 ml DDW for 5 minutes.

CHAPTER V

RESULTS

PART I ENTEROBACTERIACEAE ISOLATES

A total of 3,854 Enterobacteriaceae were recovered from different patients in King Chulalongkorn Memorial Hospital during August 2012 to January 2013. These isolates were obtained from urine (48%), sputum (15%), pus and wound (10%), body fluid (9%), blood (9%) and others (9%). *E. coli* (53.06%) and *K. pneumoniae* (27.93%) were the major Enterobacteriaceae isolates. Carbapenemase-producing isolates were screened by plating all 3,854 isolates onto MacConkey agar containing ertapenem, cloxacillin and $ZnSO_4$. The results are shown in figure 12.
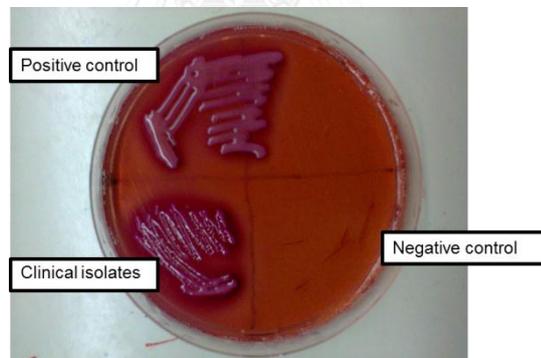
Figure 12 The detection of carbapenemase-producing Enterobacteriaceae on

MacConkey agar containing ertapenem, cloxacillin and $ZnSO_4$.

Positive control : OXA-48-producing *K. pneumoniae*. Negative control :

E. coli ATCC 25922.

Table 10 Types of clinical specimens of the 104 Enterobacteriaceae isolates

Sites of specimen	Types of specimen	No. of isolates	% of isolates
Sterile	Bile	6	5.77
	Blood	8	7.69
	Body fluid	7	6.73
	Tissue	1	0.96
Non-sterile	Pus	8	7.69
	Sputum	24	23.08
	Urine	42	40.38
	Stool	4	3.85
	Others	4	3.85

Table 11 The 104 Enterobacteriaceae positive on MacConkey agar containing ertapenem, cloxacillin and ZnSO$_4$

Organisms	No.	% Isolates
C. freundii	2	2%
E. aerogenes	2	2%
E. cloacae	24	23%
E. coli	22	21%
K. pneumoniae	51	49%
M. morganii	1	1%
Providencia spp.	1	1%
P. mirabilis	1	1%
Total	104	100%

A total of 104 Enterobacteriaceae were positive on screening medium for carbapenemase-producing isolates. These isolates were obtained from both sterile and non-sterile sites. Urine and sputum were the major types of specimen (table 10). The most common organisms included *K. pneumoniae* (49 %), *E. cloacae* (23 %) and *E. coli* (21 %) (table 11).

PART II ANTIMICROBIAL SUSCEPTIBILITY TEST

The MIC ranges, MIC_{50}, MIC_{90} and susceptibility rates of all 104 clinical isolates are shown in table 12. The resistance rate of ertapenem was 96.2 %. The MIC range was from 0.03 to >256 µg/ml. The MIC_{50} and MIC_{90} were 8 µg/ml and 64 µg/ml, respectively. The resistance rate of imipenem was 37.5 %. The MIC range was from 0.06 to >256 µg/ml. The MIC_{50} and MIC_{90} were 1 µg/ml and 16 µg/ml, respectively. The resistance rate of meropenem was 46.2 %. The MIC range was from 0.06 to >256 µg/ml. The MIC_{50} and MIC_{90} were 2 µg/ml and 32 µg/ml, respectively. The resistance rate of cefotaxime, ceftazidime and ceftriaxone were 98 %. The MIC_{50} and MIC_{90} of cefotaxime, ceftazidime and ceftriaxone were >256 µg/ml and >256 µg/ml, respectively. The MIC range of cefotaxime was from 1 to >256 µg/ml. The MIC range of ceftazidime was from 4 to >256 µg/ml. The MIC range of ceftriaxone was from 2 to >256 µg/ml. The resistance rate of cefoxitin was 95.2 %. The MIC range was from 8 to >256 µg/ml. The MIC_{50} and MIC_{90} were >256 µg/ml and >256 µg/ml, respectively.

The distributions of MICs for antimicrobial agents among 104 Enterobacteriaceae isolates are shown in figures 13-19.

Table 12 The susceptibility of ertapenem, imipenem, meropenem, cefotaxime, ceftazidime, ceftriaxone and cefoxitin against 104 Enterobacteriaceae isolates

Antimicrobial agents	MIC (µg/ml)			No.susceptibility (%)		
	Range	MIC50	MIC90	S	I	R
Ertapenem	0.03 - >256	8	64	4 (3.8)	0 (0)	100 (96.2)
Imipenem	0.06 - >256	1	16	56 (53.8)	9 (8.7)	39 (37.5)
Meropenem	0.06 - >256	2	32	44 (42.3)	12 (11.5)	48 (46.2)
Cefotaxime	1 - >256	>256	>256	1 (1)	1 (1)	102 (98)
Ceftazidime	4 - >256	>256	>256	1 (1)	1 (1)	102 (98)
Ceftriaxone	2 - >256	>256	>256	0 (0)	2 (2)	102 (98)
Cefoxitin	8 - >256	>256	>256	1 (1)	4 (3.8)	99 (95.2)
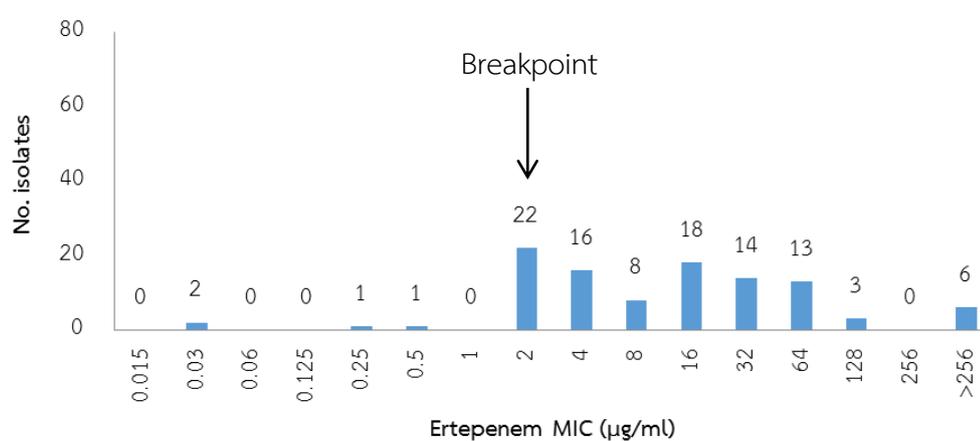
Figure 13 The distribution of ertapenem MICs among 104 Enterobacteriaceae isolates
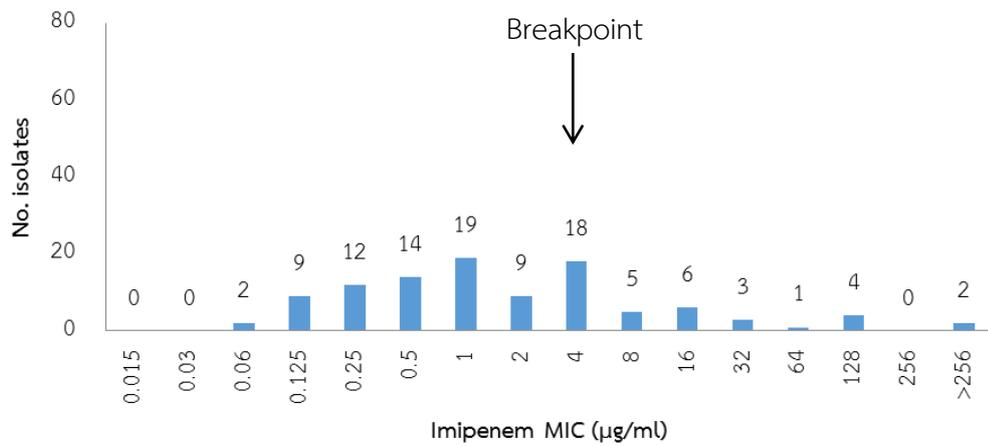
Figure 14 The distribution of imipenem MICs among 104 Enterobacteriaceae isolates
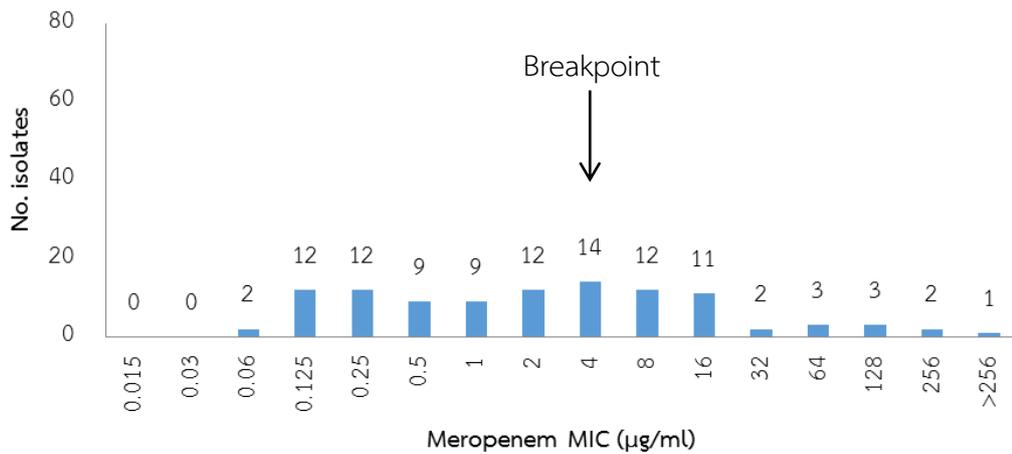
Figure 15 The distribution of meropenem MICs among 104 Enterobacteriaceae
isolates
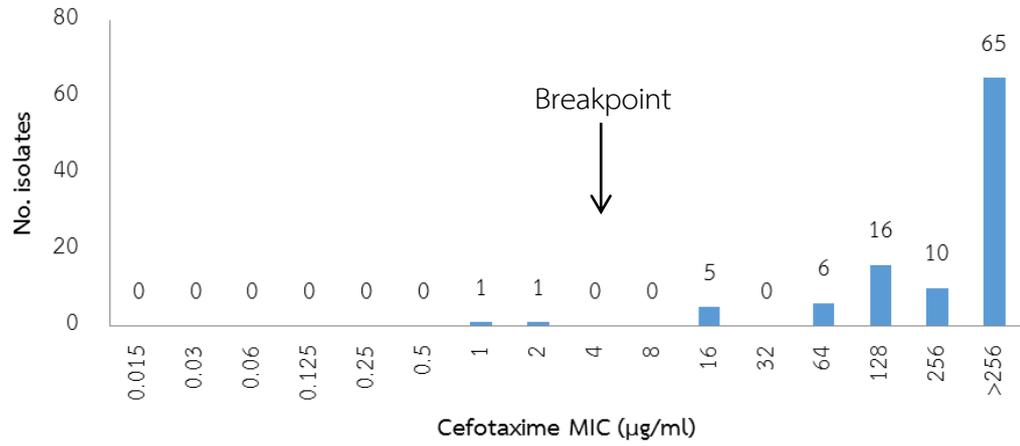
Figure 16 The distribution of cefotaxime MICs among 104 Enterobacteriaceae isolates
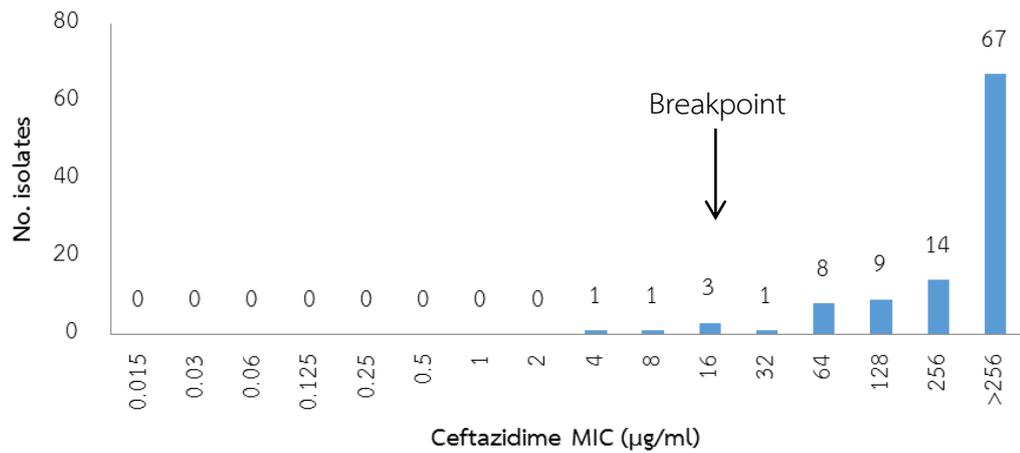
Figure 17 The distribution of ceftazidime MICs among 104 Enterobacteriaceae isolates
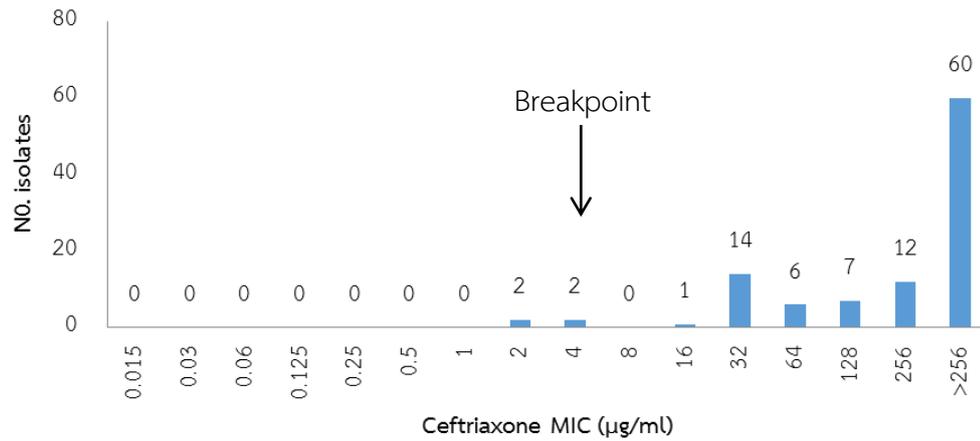
Figure 18 The distribution of ceftriaxone MICs among 104 Enterobacteriaceae
isolates
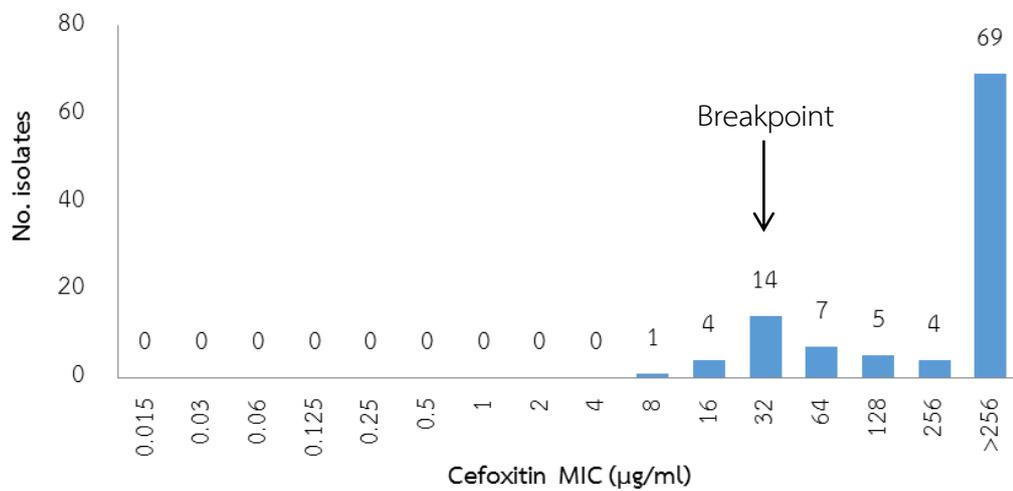
Figure 19 The distribution of cefoxitin MICs among 104 Enterobacteriaceae isolates

PART III DETECTION OF CARBAPENEMASE PRODUCERS

A total of 104 Enterobacteriaceae isolates were examined for the carbapenemase activity by modified Hodge test (MHT). The results are shown in figures 20-22.
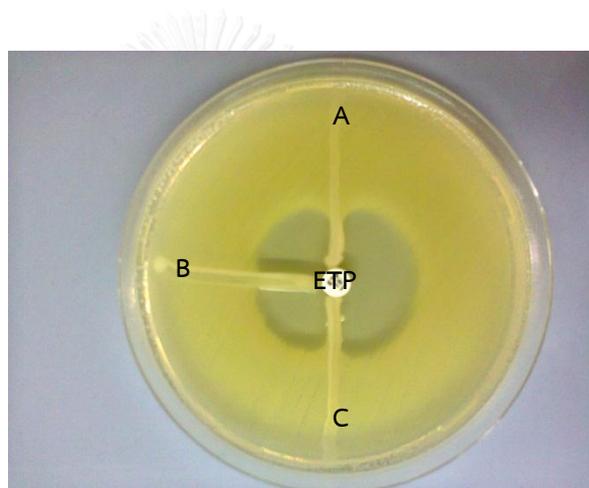
Figure 20 The detection of carbapenemase phenotypes by MHT with ertapenem
(A) positive control: NDM-1-producing *K. pneumoniae*, (B) negative control:
E. coli ATCC 25922, (C) *E. coli* isolate carrying *bla*$_{NDM}$ (Isolate S 212)
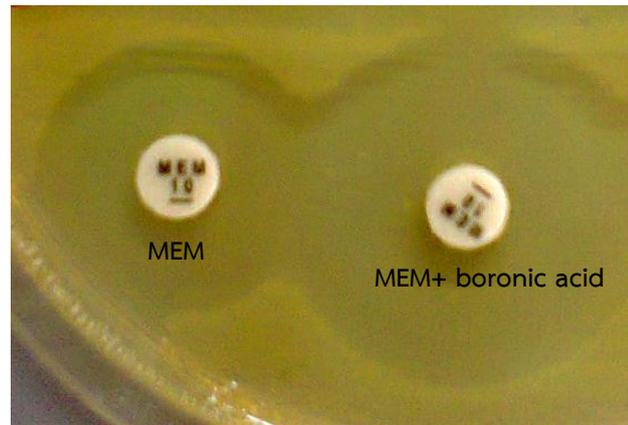
Figure 21 The positive result of class A carbapenemase phenotype by the boronic

acid-based inhibition test

The figure are show the difference of inhibition zone ≥ 5 mm between zone

of meropenem disk containing phenylboronic acid and meropenem disk

alone in *K. pneumoniae* carrying bla_{KPC}
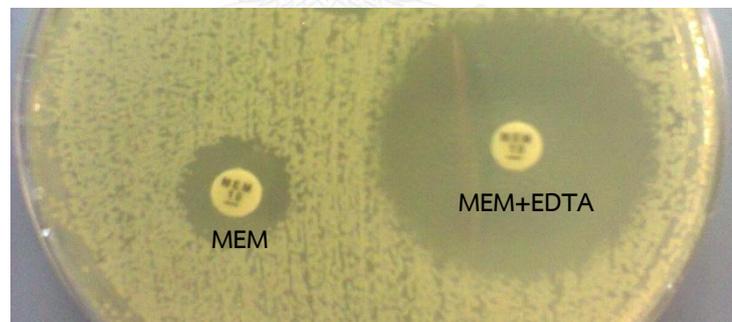
Figure 22 The positive result of class B carbapenemase phenotype by the EDTA-

meropenem combined-disk test

The figure are show the difference of inhibition zone ≥ 5 mm between zone

of meropenem disk containing EDTA and meropenem disk alone in *E. coli*

carrying bla_{NDM} (Isolate S 212).

Table 13 The carbapenemase phenotypes of the 104 Enterobacteriaceae isolates

Organism	Carbapenemase phenotypes		
	MHT	Boronic acid-based inhibition test	EDTA-meropenem combined-disk test
C. freundii	2 (4.55%)	0 (0%)	2 (5.56%)
E. cloacae	13 (29.55%)	0 (0%)	7 (15.90%)
E. coli	6 (13.64%)	0 (0%)	3 (6.82%)
K. pneumoniae	22 (50%)	2 (4.54%)	21 (47.72%)
Providencia spp.	1 (2.27%)	0 (0%)	0 (0%)
Total	44	2	33

Of the 104 Enterobacteriaceae isolates, 44 were positive for modified Hodge test (MHT). The results are shown in table 13. Of the 44 MHT-positive isolates, two isolates (2/44, 4%) were positive for boronic acid-based inhibition test and 33 (33/44, 75%) isolates were positive for the EDTA-meropenem combined-disk test. *K. pneumoniae* isolates were the most common bacteria positive for carbapenamase activity, followed by *E. cloacae* and *E. coli*. The majority of isolates had metallo-beta-lactamase activity which was inhibited by EDTA.

PART IV DETECTION OF CARBAPENEMASE GENES

Detection of carbapenemase genes by multiplex PCR

All of the 104 Enterobacteriaceae isolates were determined for carbapenemase genes by 2 multiplex PCRs. Carbapenemase genes included bla_{IMP} , bla_{VIM} , bla_{GIM} , bla_{SIM} , bla_{SPM} , bla_{NDM}, bla_{OXA-48}, bla_{KPC}, bla_{AIM} , bla_{DIM} and bla_{BIC}. The results are shown in table 14. The PCR products of carbapenemase genes are shown in figure 23.
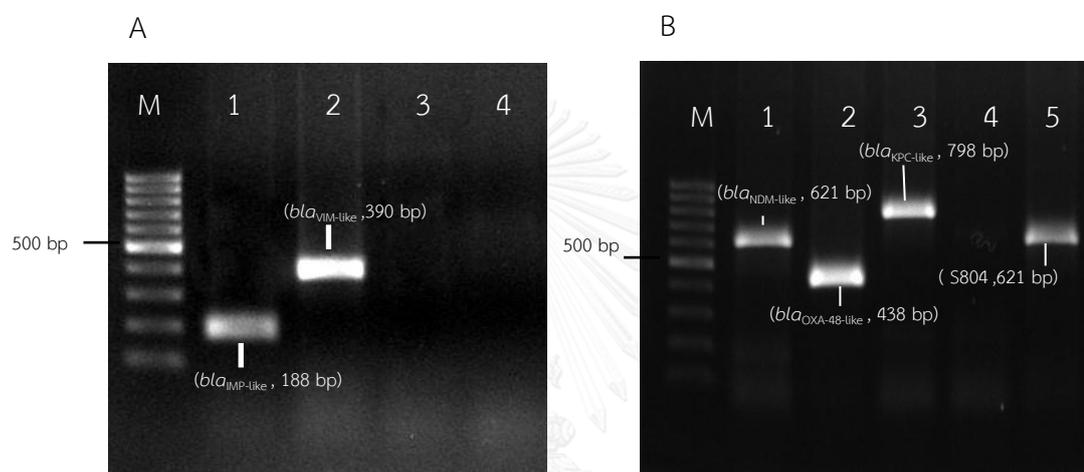
Figure 23 The multiplex PCR of carbapenemase genes

(A) The multiplex PCR of bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM} and bla_{SPM}

. Lane 1, Template $bla_{IMP-like}$ (188 bp); Lane 2, Template $bla_{VIM-like}$ (390 bp); Lane 3, isolate S804; Lane 4, negative control (sterile DDW).

(B) Results of the multiplex PCR detecting bla_{OXA-48} , bla_{KPC} and bla_{NDM} genes. Lane 1, Template $bla_{NDM-like}$ (621 bp); Lane 2, Template $bla_{OXA-48-like}$ (438 bp); Lane 3, Template $bla_{KPC-like}$ (798 bp); Lane 4, negative control (sterile DDW); Lane 5, isolate S804; M, 100 bp plus DNA Ladder

Table 14 Carbapenemase genes in 19 Enterbacteriaceae isolates

Carbapenemase gene	Organism	No.	% Isolates
NDM	*K. pneumoniae*	10	52.6
	E. coli	3	15.8
	E. cloacae	2	10.5
	C. freundii	2	10.5
IMP	*E. cloacae*	1	5.3
OXA-48	*E. cloacae*	1	5.3
	Total	19	100

There were 19 Enterobacteriaceae isolates habouring carbapenemase genes, including IMP, OXA-48 and NDM genes (table 14). The bla_{NDM} was detected in 17 isolates (89.4%). The bla_{IMP} gene was found in one *E. cloacae* isolate (5.3%). The bla_{OXA-48} gene was present in one *E. cloacae* isolate (5.3%). The bla_{NDM} gene was the most prevalent carbapenemase gene in Enterobacteriaceae, especially in *K. pneumoniae*. The inhibition zone of meropenem disk, meropenem disk containing phenylboronic acid and meropenem disk containing EDTA in 19 Enterobacteriaceae are shown in table 15.

Table 15 The inhibition zone of meropenem disk, meropenem disk containing phenylboronic acid and meropenem disk containing EDTA in 19 Enterobacteriaceae

Isolate no.	Organism	Carbapenemase genes	MHT	Zone size of inhibition zone (mm)		
				MEM	MEM/Boronic acid	MEM/EDTA
T 197	*K. pneumoniae*	NDM	+	6	6	27
T 458	*C. freundii*	NDM	+	12	12	28
T 520	*E. coli*	NDM	+	16	16	30
T 632	*C. freundii*	NDM	+	15	15	30
T 1179	*E. cloacae*	OXA-48	+	24	24	24
T 1469	*k. pneumoniae*	NDM	+	16	29	28
T 1580	*E. coli*	NDM	+	11	12	30
T 1830	*E. cloacae*	NDM	+	15	15	28
T 2969	*K. pneumoniae*	NDM	+	12	12	25
T 3367	*K. pneumoniae*	NDM	+	13	14	27
T 3525	*E. cloacae*	NDM	+	11	10	29
T 3689	*K. pneumoniae*	NDM	+	6	6	26
M 38	*E. cloacae*	IMP	+	17	17	25
M 57	*K. pneumoniae*	NDM	+	17	17	28
M 84	*K. pneumoniae*	NDM	+	6	6	28
S 212	*E. coli*	NDM	-	13	13	27
S 804	*K. pneumoniae*	NDM	+	10	11	30
S 992	*K. pneumoniae*	NDM	+	15	16	32
S 1596	*K. pneumoniae*	NDM	+	13	15	33

MEM ; meropenem

PART V DETECTION OF ESBL AND AMPC BETA-LACTAMASE PRODUCERS

All of the 19 Enterobacteriaceae isolates carrying carbapenemase genes were examined for ESBL production by combination disk test. AmpC production producers were determined by MHT with cefoxitin. The positive results of ESBL and AmpC beta-lactamase productions are shown in figure 24 and figure 25. Summary of positive screening for ESBL and AmpC beta-lactamase producers are shown in table 16.

Nine out of 19 (47.4%) carbapenemase-producing isolates were positive for ESBL phenotypes by combination disk test and 2 of 19 (10.5%) clinical isolates were positive for AmpC beta-lactamase phenotypes by MHT.
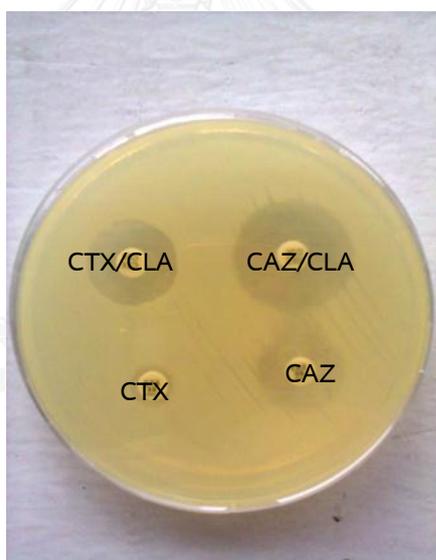
Figure 24 The positive result of ESBL phenotype by combination disk test.
E. coli carrying bla_{CTX-M} (isolate T520) showed ≥ 5 mm of inhibition zone between ceftriaxone/ceftazidime with clavulanic acid and ceftriaxone/ceftazidime alone.
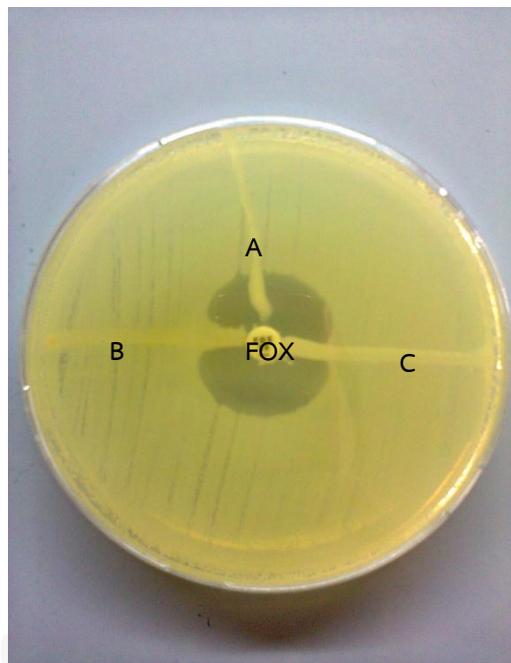
Figure 25 The detection of AmpC beta-lactamase phenotypes by MHT with cefoxitin
(A) negative control, (B) positive control, (C) *K. pneumoniae* carrying bla_{TEM}
(strain T3689)

Table 16 The summary of carbapenemase, ESBL and AmpC phenotypes in Enterobacteriaceae isolates harbouring carbapenemase genes

Code	organism	Carbapenemase genes	carbapenemase phenotypes			ESBL phenotypes	AmpC phenotypes
			Modified Hodge test	Boronic acid-based inhibition test	EDTA-meropenem combined-disk test	Combination disk test	Modified Hodge test
T 197	*K. pneumoniae*	NDM-1	+	-	+	-	-
T 458	*C. freundii*	NDM-1	+	-	+	-	-
T 520	*E. coli*	NDM-1	+	-	+	-	-
T 632	*C. freundii*	NDM-1	+	-	+	-	-
T 1179	*E. cloacae*	OXA-48	+	-	-	+	-
T 1469	*K. pneumoniae*	NDM-1	+	+	+	-	-
T 1580	*E. coli*	NDM-1	+	-	+	-	-
T 1830	*E. cloacae*	NDM-1	+	-	+	-	-
T 2969	*K. pneumoniae*	NDM-1	+	-	+	+	-
T 3367	*K. pneumoniae*	NDM-1	+	-	+	-	-
T 3525	*E. cloacae*	NDM-1	+	-	+	-	+
T 3689	*K. pneumoniae*	NDM-1	+	-	+	-	-
M 38	*E. cloacae*	IMP-14a	+	-	+	+	+
M 57	*K. pneumoniae*	NDM-1	+	-	+	+	-
M 84	*K. pneumoniae*	NDM-1	+	-	+	+	-
S 212	*E. coli*	NDM-4	-	-	+	+	-
S 804	*K. pneumoniae*	NDM-1	+	-	+	+	-
S 992	*K. pneumoniae*	NDM-1	+	-	+	+	-
S 1596	*K. pneumoniae*	NDM-1	+	-	+	+	-

PART VI DETECTION OF ESBL AND AMPC GENES

1. **Detection of ESBL genes by multiplex PCR**

All 19 Enterobacteriaceae isolates habouring carbapenemase genes were detected for ESBL genes by multiplex PCR. ESBL genes included bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM} and bla_{VEB}. ESBL genes detected in Enterobacteriaceae carrying carbapenemase genes are shown in figures 26-27. The summary of carbapenemase gene and patterns of 19 Enterobacteriaceae carrying carbapenemase genes are shoen in table 17.
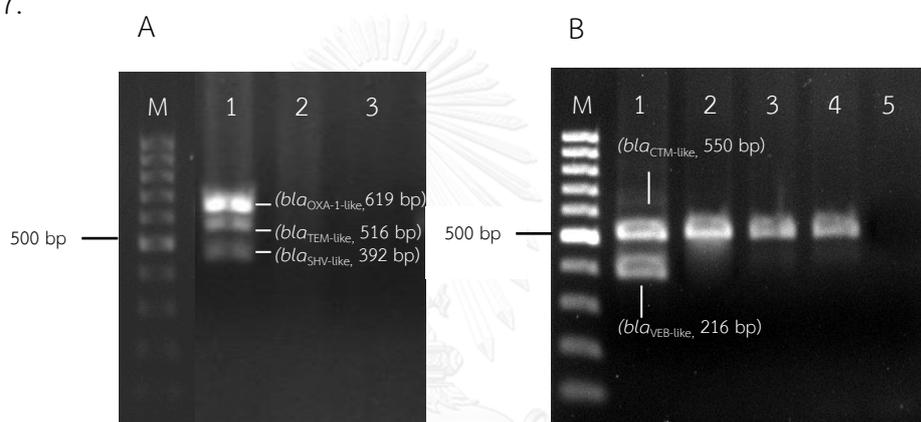
Figure 26 The multiplex of ESBL genes

(A) The multiplex PCR of the bla_{SHV}, bla_{TEM} and bla_{OXA-1}
Lane 1, Template $bla_{SHV-like}$ (392 bp), Template $bla_{TEM-like}$ (516 bp), Template $bla_{OXA-1-like}$ (619 bp); Lane 2, isolate T1830; Lane 3, negative control (sterile DDW).

(B) The multiplex PCR of the bla_{CTX-M} and bla_{VEB} genes. Lane 1, Template $bla_{CTX-M-like}$ (550 bp), Template $bla_{VEB-like}$ (216 bp); Lane 2, isolate M84; Lane 3, isolate S992; Lane 4 isolate T1469; Lane 5, negative control (sterile DDW). M, 100 bp plus DNA Ladder
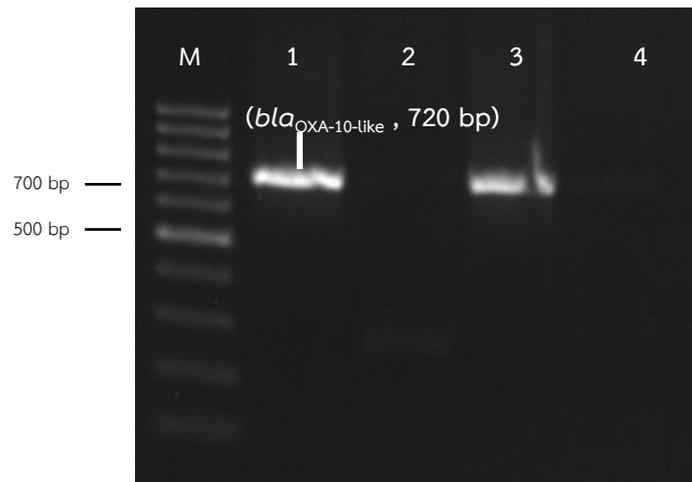
Figure 27 The multiplex PCR of the bla_{OXA-10}.

Lane 1, Template $bla_{OXA-10-like}$ (720 bp); Lane 2, isolate no.T197; Lane 3, isolate M38; Lane 4, negative control (sterile DDW) ;M, 100 bp plus DNA Ladder

Table 17 The summary of carbapenemase gene and patterns of 19

Enterobacteriaceae carrying carbapenemase genes

Bla genes pattern	No of isolates	% isolates	ESBL phenotype by combination disks		ESBL phenotype by EDTA disk	
			Positive No.	Negative No.	Positive No.	Negative No.
NDM, CTX-M	6	35.3	3	3	6	0
NDM, CTX-M , OXA-1	1	5.9	1	1	1	0
OXA-48, CTX-M , OXA-1	1	5.9	1	0	NT	NT
NDM, CTX-M , SHV	1	5.9	1	0	NT	NT
IMP, CTX-M , OXA-10 , TEM	1	5.9	1	0	NT	NT
NDM, CTX-M , OXA-1 , TEM	1	5.9	1	0	NT	NT
NDM, CTX-M , TEM , SHV	1	5.9	0	1	1	0
NDM, OXA-1	2	11.8	0	2	2	0
NDM, OXA-1 , SHV	1	5.9	1	0	NT	NT
NDM, OXA-1 , TEM, SHV	1	5.9	0	1	1	0
NDM, TEM	1	5.9	0	1	1	0
Total	17	100				

NT: not tested

The bla_{CTX-M} genes (36.8 %) were the most common ESBLs and were frequently present in *K. pneumoniae*. DNA sequencing of the entire genes were further investigated to confirm the presence of ESBL genes.

2. Detection of AmpC genes by multiplex PCR

AmpC beta-lactamases genes were examined in 19 Enterobacteriaceae isolates carrying carbapenemase genes by multiplex PCR. These included bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{EBC}, bla_{ACC} and bla_{FOX}. None of the isolates carried AmpC genes. The results of multiplex PCR for AmpC genes are shown in figure 28.
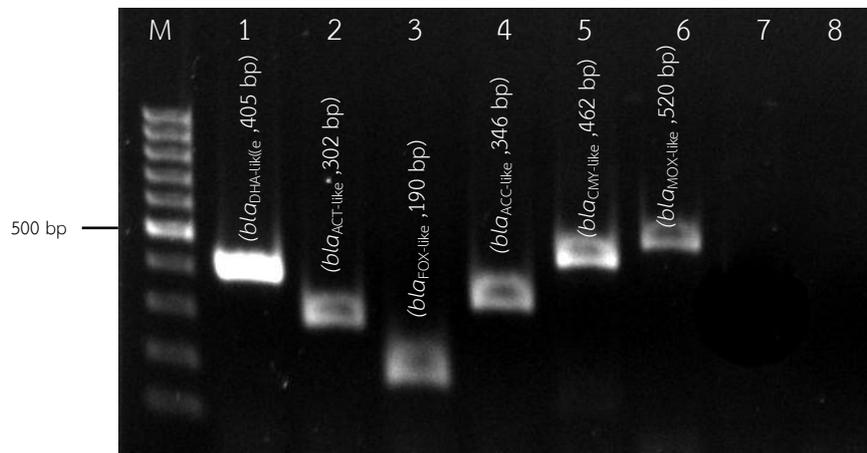
Figure 28 The multiplex PCR of AmpC beta-lactamase genes

The bla_{MOX} (520 bp), bla_{ACT} (302 bp), bla_{DHA} (405 bp), bla_{CMY} (392bp), bla_{ACC} (346 bp) and bla_{FOX} (190 bp). Lane 1 , Template $bla_{DHA-like}$ (405 bp); Lane 2, Template $bla_{ACT-like}$ (302 bp); Lane 3, Template $bla_{FOX-like}$ (190 bp); Lane 4, $bla_{ACC-like}$ (346 bp); Lane 5, Template $bla_{CMY-like}$ (462bp); Lane 6, Template $bla_{MOX-like}$ (520 bp); Lane 7, Clinical isolate no.S804; Lane 8, negative control (sterile DDW) ;M, 100 bp plus DNA Ladder

PART VII ANALYSIS OF ENTIRE BLA GENES BY PCR AND DNA SEQUENCING

Of the 19 Enterobacteriaceae isolates, the bla_{NDM-1} was found in 16 isolates including 2 each of *C. freundii, E. coli, E. cloacae* and 10 *K. pneumoniae*. Of the 2 *E. cloacae*, one isolate carried $bla_{IMP-14a}$ and the other harboured bla_{OXA-48}. (table 18). The bla_{NDM-4} was found in one *E. coli* isolate. The susceptibility of these isolates are shown in table 19. All isolates were resistant to ertapenem, cefotaxime, ceftazidime, ceftriaxone and cefoxitin. The MIC_{90} of all antimicrobial agents were >256 μg/ml except imipenem and meropenem were 32 and 128 μg/ml, respectively. The resistance gene patterns 19 Enterobacteriaceae are shown in table 20.

Table 18 Carbapenemase genes in the 19 Enterobacteriaceae isolates

Carbapenemase gene	Organism	No. of isolates (%)
IMP-14a	*E. cloacae*	1 (5.3)
OXA-48	*E. cloacae*	1 (5.3)
NDM-1	*C. freundii*	2 (10.5)
	E. cloacae	2 (10.5)
	E. coli	2 (10.5)
	K. pneumoniae	10 (52.6)
NDM-4	*E. coli*	1 (5.3)
	Total	19

Table 19 The susceptibility of the 19 Enterobacteriaceae carrying carbapenemase
genes

Antimicrobial agents	MIC			No.susceptibility (%)		
	Range	MIC_{50}	MIC_{90}	S	I	R
Ertapenem	4 - >256	32	>256	0 (0)	0 (0)	19 (100)
Imipenem	0.5 - >256	8	32	3 (15.8)	0 (0)	16 (84.2)
Meropenem	0.25 - >256	16	128	1 (5.3)	1 (5.3)	17 (89.4)
Cefotaxime	128 - >256	>256	>256	0 (0)	0 (0)	19 (100)
Ceftazidime	128 - >256	>256	>256	0 (0)	0 (0)	19 (100)
Ceftriaxone	256 - >256	>256	>256	0 (0)	0 (0)	19 (100)
Cefoxitin	>256	>256	>256	0 (0)	0 (0)	19 (100)

Table 20 The resistance gene patterns of the 19 isolates

Isolate No.	Organism	Carbapenemase production — Modified Hodge test	Boronic acid-based inhibition test	EDTA-meropenem combined-disk test	ESBLs production — combined-disk test	Combination disk test	AmpC beta-lactamases production — Modified Hodge test	MIC — Ertapenem	Imipenem	Meropenem	Cefotaxime	Ceftazidime	Ceftriaxone	Cefoxitin	Carbapenemase genes	Type of bla gene
T 197	K. pneumoniae	+	-	+	-	-	-	>256	32	128	>256	>256	>256	>256	NDM-1	CTX-M15
T 458	C. freundii	+	-	+	-	-	-	32	4	8	128	>256	>256	>256	NDM-1	TEM-1
T 520	E. coli	+	-	+	-	-	-	32	4	16	>256	>256	>256	>256	NDM-1	CTX-M15 , OXA-1
T 632	C. freundii	+	-	-	-	-	-	16	1	4	256	>256	>256	>256	NDM-1	OXA-1
T 1179	E. cloacae	+	-	+	+	+	-	4	0.5	0.25	128	128	256	>256	OXA-48	CTX-M15 , OXA-1
T 1469	k. pneumoniae	+	+	+	-	-	-	16	4	16	256	>256	>256	>256	NDM-1	CTX-M15
T 1580	E. coli	+	-	-	-	-	-	64	8	16	>256	>256	>256	>256	NDM-1	OXA-1
T 1830	E. cloacae	+	-	-	-	-	-	16	16	16	256	>256	>256	>256	NDM-1	
T 2969	K. pneumoniae	+	-	+	+	+	-	32	16	16	>256	>256	>256	>256	NDM-1	OXA-1 , SHV-11
T 3367	K. pneumoniae	+	-	+	-	-	-	64	32	64	128	>256	256	>256	NDM-1	
T 3525	E. cloacae	+	-	+	-	-	+	8	32	4	>256	>256	>256	>256	NDM-1	CTX-M15
T 3689	K. pneumoniae	+	-	+	-	-	-	>256	128	>256	>256	>256	>256	>256	NDM-1	OXA-1 , TEM-1 , SHV-11
M 38	E. cloacae	+	-	+	+	+	+	32	0.5	2	>256	>256	>256	>256	IMP-14a	CTX-M15 , OXA-10 , TEM-1
M 57	K. pneumoniae	+	-	+	+	+	-	32	16	16	>256	>256	>256	>256	NDM-1	CTX-M15
M 84	K. pneumoniae	+	-	+	+	+	-	>256	>256	256	>256	>256	>256	>256	NDM-1	CTX-M15 , OXA-1 , TEM-1
S 212	E. coli	-	-	+	+	+	-	64	8	16	>256	>256	>256	>256	NDM-4	CTX-M15
S 804	K. pneumoniae	+	-	+	+	+	-	64	16	32	>256	>256	>256	>256	NDM-1	CTX-M15 , TEM-1 , SHV-1
S 992	K. pneumoniae	+	-	+	+	+	-	32	4	8	>256	>256	>256	>256	NDM-1	CTX-M15, SHV-11
S 1596	K. pneumoniae	+	-	+	+	+	-	64	16	32	>256	>256	>256	>256	NDM-1	CTX-M15

An isolate carrying bla_{OXA-48} , T1179, was analyzed for entire $bla_{OXA-48.}$ DNA sequence analysis of the bla_{OXA-48} revealed 798 bp ORF, encoding 265 amino acids. T1179 had 100 % nucleotide identity and 100% amino acid identity to bla_{OXA-48} and OXA-48 (GenBank accession no. JN626286).

An isolate carrying bla_{IMP} , M38, was analyzed for entire $bla_{IMP.}$ DNA sequence analysis of the bla_{IMP} revealed 741 bp ORF, encoding 246 amino acids. This strain had 100 % nucleotide identity and 100% amino acid identity to $bla_{IMP-14a}$ and IMP-14a (GenBank accession no. GQ302617).

A total of 17 isolates carrying bla_{NDM} , including T197, T458, T520, T632, T1469, T1580, T1830, T2969, T3367, T3525, T3689, M57, M84, S212, S804, S992 and S1596, were analyzed for entire bla_{NDM}. DNA sequence analysis of the bla_{NDM} revealed 813 bp ORF, encoding 270 amino acids (figures 29-30). All strains except S212 had 100 % nucleotide identity and 100% amino acid identity to bla_{NDM-1} and NDM-1 (GenBank accession no. KC404829). S212 had 100 % nucleotide identity and 100% amino acid identity to bla_{NDM-4} and NDM-4 (GenBank accession no. JQ348841) (figures 29-32).

A total of 12 isolates carrying bla_{CTX-M} , including T197, T520, T1179, T1469, T3525, M38, M57, M84, S212, S804, S992 and S1596, were analyzed for entire bla_{CTX-M}. DNA sequence analysis of the bla_{CTX-M} revealed 876 bp ORF, encoding as 291 amino acids. All strains had 100 % nucleotide identity and 100% amino acid identity to $bla_{CTX-M-15}$ and CTX-M-15(GenBank accession no. KC513926)

An isolate carrying bla_{OXA-10}, M38, was analyzed for entire $bla_{OXA-10.}$ DNA sequence analysis of the bla_{OXA-10} revealed 801 bp ORF, encoding 266 amino acids. This strain 100 % nucleotide identity and 100% amino acid identity to bla_{OXA-10} and OXA-10 (GenBank accession no. KJ488983).

A total of 4 isolates carrying bla_{SHV} , including T2969, T3689, S804 and S992 were analyzed for entire bla_{SHV}. DNA sequence analysis of the bla_{SHV} revealed 861 bp ORF, encoding 286 amino acids. All strains except S804 had 100 % nucleotide identity and 100% amino acid identity to bla_{SHV-11} and SHV-11 (GenBank accession no. JN676837.1

JN676837.1). T2969, T3689 and S992 had 100 % nucleotide identity and 100% amino acid identity to bla_{SHV-1} and SHV-1 (GenBank accession no. GU064389).

A total of 7 isolates carrying bla_{OXA-1} , including T520, T632, T1179, T1580, T2969, T3689 and M84 were analyzed for entire bla_{OXA-1}. DNA sequence analysis of the bla_{OXA-1} revealed 831 bp ORF, encoding 276 amino acids. All strains had 100 % nucleotide identity and 100% amino acid identity to bla_{OXA-1} and OXA-1 (GenBank accession no. HQ386842).

A total of 5 isolates carrying bla_{TEM} , including T458, T3689, M38, M84 and S804 were analyzed for entire bla_{TEM-1}. DNA sequence analysis of the bla_{TEM-1} revealed 861 bp ORF, encoding 286 amino acids. All strains had 100 % nucleotide identity and 100% amino acid identity to bla_{TEM-1} and TEM-1 (GenBank accession no. JX997935).

Figure 29 The nucleotide sequence alignments bla_{NDM-1} from 16 Enterobacteriaceae isolates and bla_{NDM-4} from one *E. coli* isolate (S212)

```
                1                                                                                              130
NDM-1      MELPNIMHPV AKLSTALAAA LMLSGCMPGE IRPTIGQQME IGDQRFGDLV FRQLAPNVWQ HTSYLDMPGF GAVASNGLIV RDGGRVLVVD TAWTDDQTAQ ILNWIKQEIN LPVALAVVTH AHQDKMGGMD
T197       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T458       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T520       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T632       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T1469      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T1580      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T1830      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
I2969      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T3367      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T3525      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T3689      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
M57        .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
M84        .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S804       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S992       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S1596      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S212       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                131                                                                                            260
NDM-1      ALHAAGIATY ANALSNQLAP QEGMVAAQHS LTFAANGWVE FATAPNFGPL KVFYPGPGHT SDNITVGIDG TDIAFGGCLI KDSKAKSLGN LGDADTEHYA ASARAFGAAF PKASMIVMSH SAPDSRAAIT
T197       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T458       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T520       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T632       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T1469      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T1580      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T1830      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
I2969      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T3367      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T3525      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
T3689      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
M57        .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
M84        .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S804       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S992       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S1596      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S212       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......L.. .......... ..........
Consensus  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
```

Figure 30 The amino-acid sequence alignments of NDM-1 from 16 Enterobacteriaceae isolates and NDM-4 from one *E. coli* isolate (S212)

Figure 31 The nucleotide sequence alignments of bla_{NDM-4} from the *E. coli* isolate (S212), bla_{NDM-1} (Genbank accession no.KC404829)

and bla_{NDM-4} (Genbank accession no. JQ348841)

Figure 32 The amino-acid sequence aligments of NDM-4 from the *E. coli* isolate (S212), NDM-1 (Genbank accession no. KC404829) and NDM-4
(Genbank accession no. JQ348841)

PART VIII THE CONJUGATION OF RESISTANCE GENES

Of the 19 Enterobacteriaceae isolates carrying carbapenemase genes, 15 (78.9%) isolates carrying bla_{NDM-1} and one isolate carrying bla_{OXA-48} were successfully transferred carbapenemase genes to UB1637Az^R. Four (21.1%) isolates, including S212 (NDM-4 producer), M38 (IMP-14a producer), T1469 (NDM-1 producer) and T3689 (NDM-1 producer) were unable to transfer any resistance genes by conjugation. ESBL genes were not detected in any transconjugants.

The transconjugants carrying bla_{NDM-1} , bla_{OXA-48} had 16- to 128-fold increase in MICs of ertapenem, meropenem and imipenem compared with the recipient strain. All transconjugants were resistant to cefotaxime, ceftazidime, ceftriaxone, cefoxitin. The transconjugant carried bla_{OXA-48} had 16-fold increase in ertapenem MIC, 2-fold increase in meropenem MIC and 16-fold increase in imipenem MIC when compared with the recipient strain. The transconjugants carrying bla_{NDM-1} had 64- to 512-fold increase in MICs of ertapenem, 2- to 256-fold increase in MICs of meropenem and 32- to 128-fold increase in MICs of imipenem when compared with the recipient strain. The antimicrobial susceptibility of the transconjugants are shown in tables 21-22. The detection of carbapenemase genes in the transconjugants are shown in figure 33.

Table 21 The MIC of antimicrobial agents for the *E. cloacae* carrying bla_{OXA-48} (T1179) and its transconjugants (trc1179)

No.of isolates	Organism	Carbapenemase gene	ESBLs gene	MIC (µg/ml)						
				IPM	MEM	ETP	CAZ	CTX	CRO	FOX
UB1637Az^R	*E.coli*			0.06	0.03	0.015	0.03	0.03	0.015	2
T 1179	*E. cloacae*	OXA-48	CTX-M 15	0.5	0.25	4	128	128	256	>256
trc 1179		OXA-48	-	1	0.06	0.25	0.25	0.5	0.125	8

Table 22 The MIC of antimicrobial agents for 16 Enterobacteriaceae carrying bla_{NDM-1} and their transconjugants

No.of isolates	Organism	Carbapenemase gene	ESBLs gene	MIC (µg/ml)						
				IPM	MEM	ETP	CAZ	CTX	CRO	FOX
UB1637Az[R]	*E. coli*			0.06	0.03	0.015	0.03	0.03	0.015	2
T 197	*K. pneumoniae*	NDM-1	CTX-M 15	32	128	>256	>256	>256	>256	>256
trc 197		NDM-1		8	2	4	>256	256	>256	>256
T 2969	*K. pneumoniae*	NDM-1		2	16	32	>256	>256	>256	>256
trc 2969		NDM-1		2	1	1	>256	128	128	256
T 3367	*K. pneumoniae*	NDM-1		16	8	16	>256	128	256	>256
trc 3367		NDM-1		8	4	8	>256	256	256	>256
M 57	*K. pneumoniae*	NDM-1	CTX-M 15	8	8	32	>256	>256	>256	>256
trc 57		NDM-1		8	4	4	256	256	256	>256
M 84	*K. pneumoniae*	NDM-1	CTX-M 15	0.5	1	8	256	256	256	>256
trc 84		NDM-1		8	2	4	256	256	256	>256
S 804	*K. pneumoniae*	NDM-1	CTX-M 15	16	32	64	>256	>256	>256	>256
trc 804		NDM-1		8	4	8	>256	128	256	>256
S 992	*K. pneumoniae*	NDM-1	CTX-M 15	4	8	16	>256	>256	>256	>256
trc 992		NDM-1		4	2	4	256	256	256	>256
S 1596	*K. pneumoniae*	NDM-1	CTX-M 15	16	32	32	>256	>256	>256	>256
trc 1596		NDM-1		8	2	4	256	256	256	>256
T 1830	*E. cloacae*	NDM-1		8	8	8	>256	256	>256	>256
trc 1830		NDM-1		8	4	8	>256	256	256	>256
T 3525	*E. cloacae*	NDM-1	CTX-M 15	16	16	32	>256	>256	>256	>256
trc 3525		NDM-1		8	4	8	>256	256	256	>256
T 458	*C. freundii*	NDM-1		8	16	8	>256	256	>256	>256
trc 458		NDM-1		8	8	4	256	256	>256	>256
T 632	*C. freundii*	NDM-1		2	4	8	>256	>256	>256	>256
trc 632		NDM-1		8	4	4	256	256	>256	>256
T 520	*E. coli*	NDM-1	CTX-M 15	8	16	64	>256	>256	>256	>256
trc 520		NDM-1		8	1	4	>256	256	>256	>256
T 1580	*E. coli*	NDM-1		8	16	64	>256	>256	>256	>256
trc 1580		NDM-1		8	8	4	>256	256	>256	>256
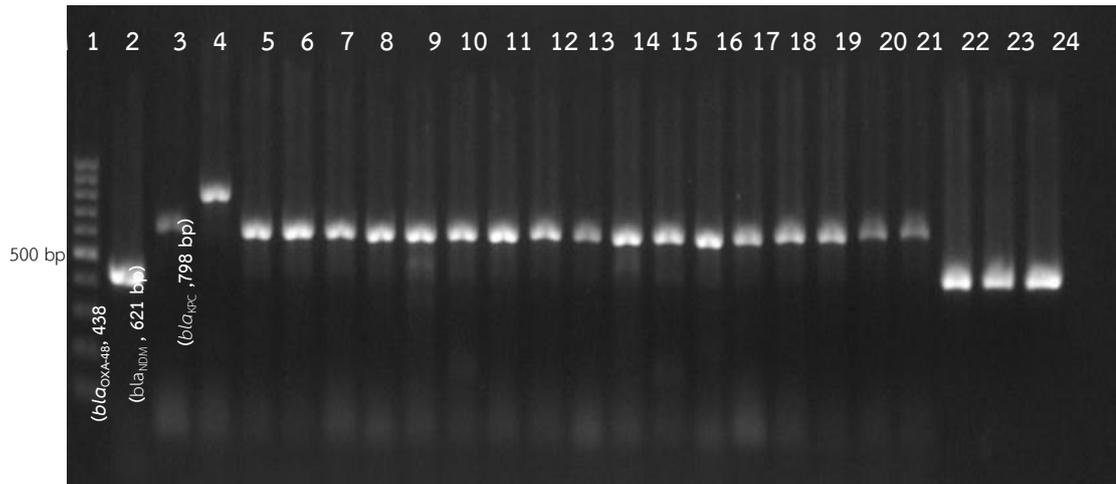
Figure 33 Detection of carbapenemase genes in the transconjugants.

Lane 1, Template bla_{OXA-48} (438 bp); Lane 2, Template bla_{NDM} (621 bp); Lane 3, Template bla_{KPC} (798 bp); Lane 4-20, Transconjugant 57, trc84, trc197, trc458, trc520, trc632, trc804 , trc992, trc1580, trc1596, trc1830, trc2969, trc3367, trc3525, trc3969, respectively. Lane 21-23, trc1179.; Lane 24, negative control (sterile DDW) ; M, 100 bp plus DNA Ladder

PART IX CLONALITY OF BETA LACTAMASE PRODUCER STRAINS

Genetic relationships among 19 Enterobacteriaceae isolates carrying carbapenemase genes were determined by pulse field gel electrophoresis (PFGE). The InfoQuestFP software was used to create a dendrogram by using the unweighted pair group method with arithmetic averages (UPGMA) and with the Dice similarity coefficient at a 1.5 % tolerance and 1.5 % optimize in band position. An isolate is considered to be closely related when the banding patterns were ≥ 80% similarity and define as PFGE type. The PFGE patterns are shown in figures 34-37. The four isolates of *E. cloacae* had 4 PFGE types. The results showed that all isolates were genetically different (71.8, 68.3, 61.3 % similarity) (figure 34). They were isolated from different wards and had different resistance gene patterns. Similar, 2 isolates of *C. freundii* crrying bla_{NDM-1} were genetically different with 61.3 % similarity (figure 35). These isolates were isolated from patients in different wards. Of the three bla_{NDM} carrying *E. coli*, 2 isolates (T520 and T1580) had 100% similarity (figure 36). T520 carried both bla_{NDM-1} together with $bla_{CTX-M-15}$ and were isolated from patients in different wards and about a month apart. T1580 had only bla_{NDM-1}. The results demonstrated that these 2 isolates were associated with clonal spread and horizontal gene transfer. The other isolate, S212, harbouring bla_{NDM-4} and $bla_{CTX-M-15}$ had 83.3 % similarity, which was closely related with T520 and T1580. The PFGE patterns of 10 *K. pneumoniae* isolates carrying bla_{NDM} had 8 PFGE types (figure 37). PFGE type C and G had 2 members each with 89.7 % and 91.9% similarity,

respectively. Both T1596 and T992 carried both bla_{NDM-1} and $bla_{CTX-M-15}$, and had PFGE type G. They were isolated from patients at the same ward and about a month apart. M 57 and T197 harboured both bla_{NDM-1} and $bla_{CTX-M-15}$ and had PFGE type C. Both of the isolates were collected from patients in different wards and five months apart.

The PFGE patterns showed that the spread of bla_{NDM} and $bla_{CTX-M\ 15}$ genes in *E. coli* and *K. pneumoniae* isolates from King Chulalongkorn Memorial Hospital, was associated with clonal spread.

PFGE type	Collection date	Ward	Pattern genes	Isolate No
A	15/1/2013	W2	IMP-14a, CTX-M15, OXA-10	M 38
B	1/1/2014	J	NDM-1, CTX-M15	T 3525
C	20/9/2013	MICU2	OXA-48, CTX-M15	T 1179
D	19/10/2013	M 3	NDM-1	T1830
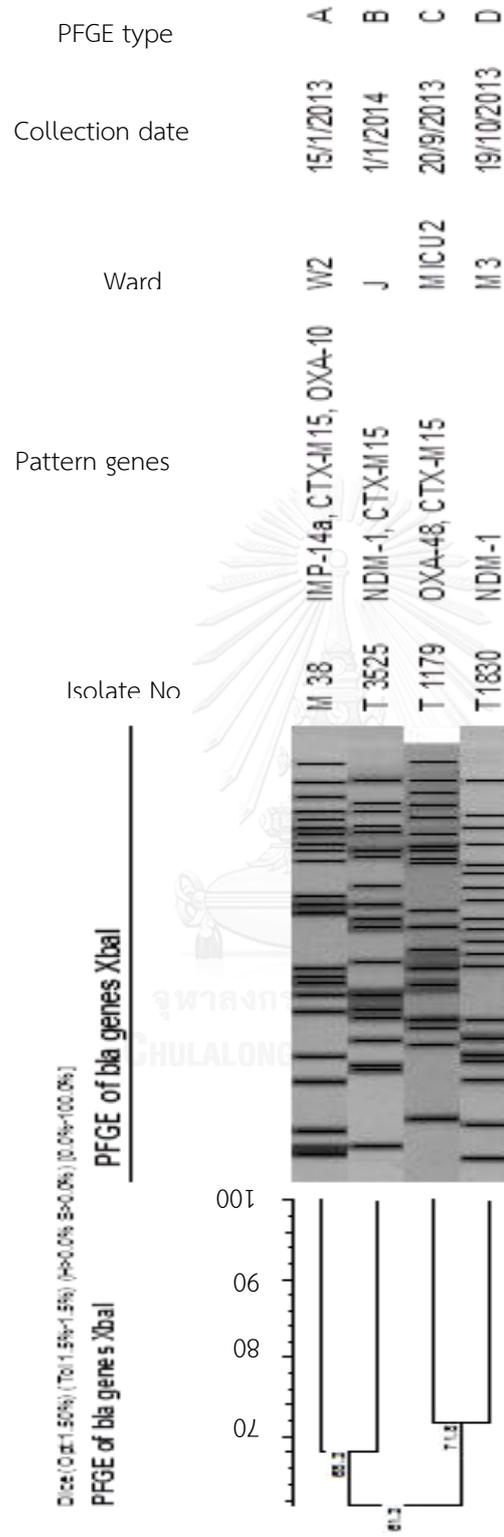
Figure 34 Genetic relatedness among 4 *E. cloacae* isolates by PFGE
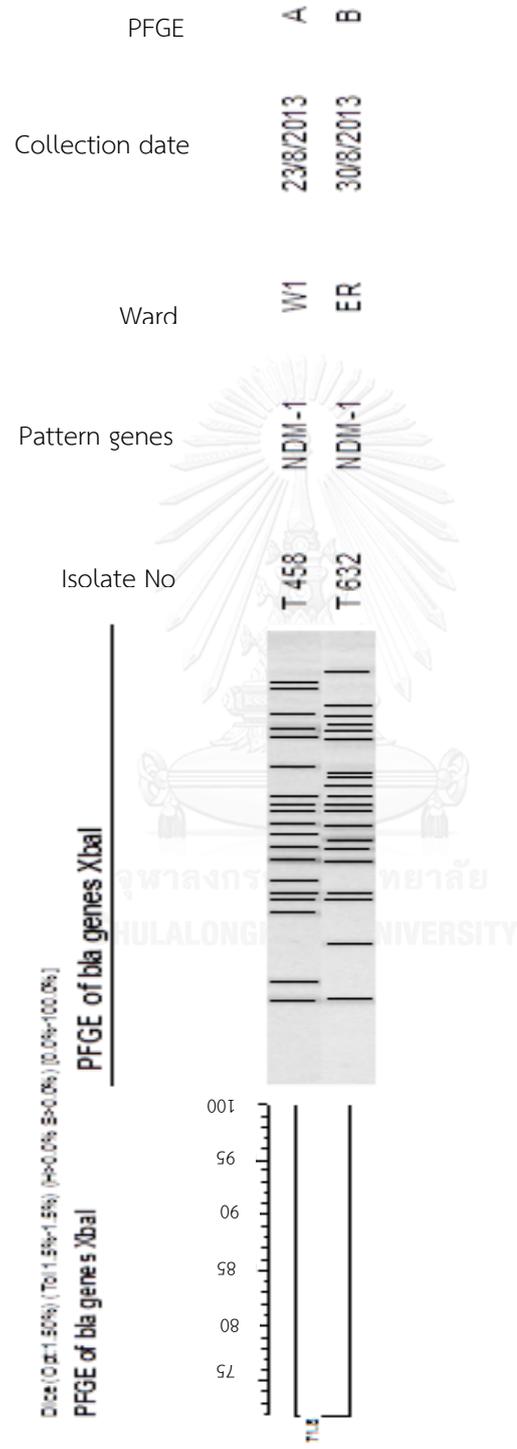
Figure 35 Genetic relatedness among 2 *C. freundii* isolates by PFGE

Isolate No	Pattern genes	Ward	Collection date	PFGE
T 520	NDM -1, CTX-M 15	J	28/8/2013	A
T 1580	NDM -1	OPD	8/10/2013	A
S 212	NDM -4, CTX-M 15	OPD	27/3/2013	A
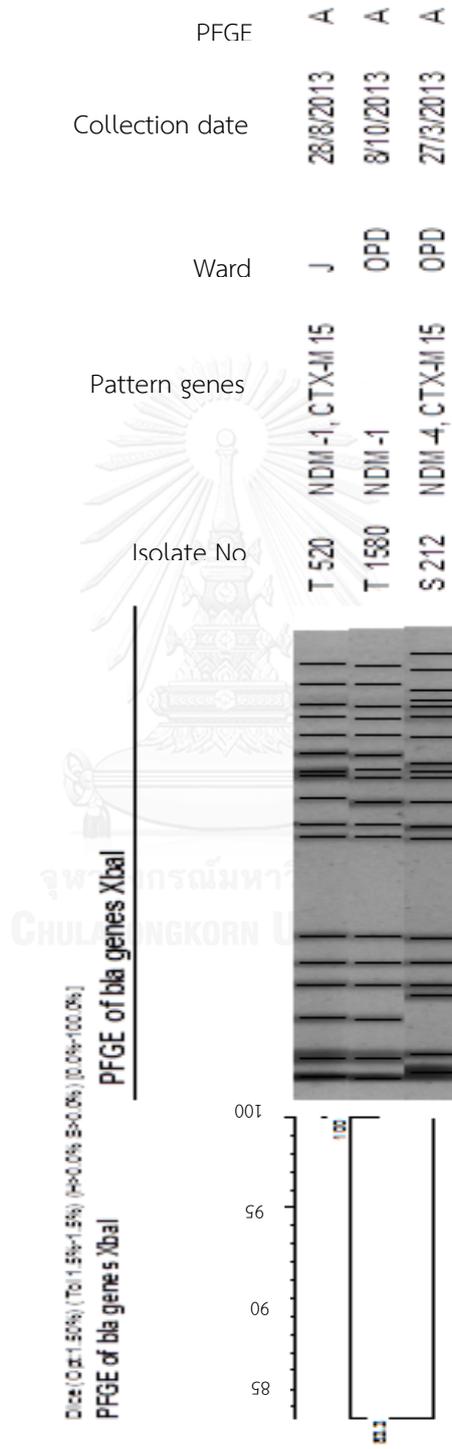
Figure 36 Genetic relatedness among 3 *E. coli* isolates by PFGE

PFGE	Collection date	Ward	Pattern genes	Isolate No
A	5/10/2013	S18	NDM-1, CTX-M15	T 1469
B	3/5/2013	P	NDM-1, CTX-M15	M 84
C	5/3/2013	J	NDM-1, CTX-M15	M 57
C	15/8/2013	P	NDM-1, CTX-M15	T 197
D	14/5/2013	W1	NDM-1, CTX-M15	S 804
E	26/12/2013	W2	NDM-1	T 3367
F	7/1/2014	OPD	NDM-1	T 3689
G	22/6/2013	MICU2	NDM-1, CTX-M15	S 1596
G	21/5/2013	MICU2	NDM-1, CTX-M15	S 992
H	7/12/2013	P	NDM-1	T 2969
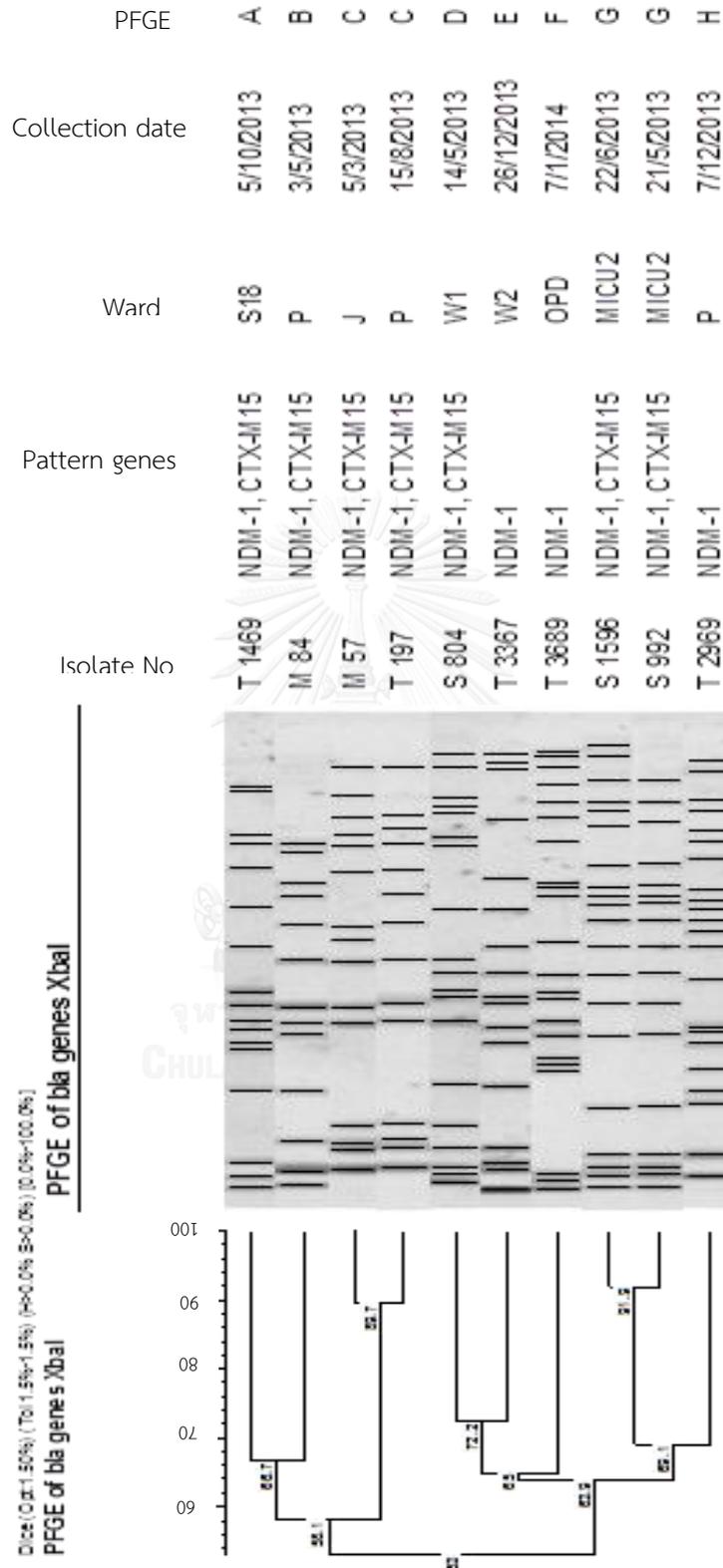
Figure 37 Genetic relatedness among 10 *K. pneumoniae* isolates by PFGE

PART X PLASMID ANALYSIS OF CARBAPENEMASE PRODUCERS

All 16 Enterobacteriaceae carrying carbapenemase genes were analyzed for the presence of resistance genes on plasmids by Southern blot hybridization. The plasmids were cut by S1-nuclease and were analyzed by PFGE. The gel was transfered to nylon membrane and hybridized with specific probes for carbapenemase genes including bla_{NDM} and bla_{OXA-48}. The T1179 (figure 39) had a plasmid carrying with bla_{OXA-48} with the size of approximately 51 kb. All 13 Enterobacteriaceae including M57, M84, S804, S992, S1596, T197, T458, T520, T632, T1580, T1830, T3525 and T3367 had a plasmid harbouring bla_{NDM-1} with the size of approximately 24 kb (figure 38). Only T2969 had bla_{NDM-1} on plasmid with different size (approximately 159 kb).

The $bla_{CTX-M\ 15}$ gene was not detected in the Southern blot hybridization experiment.
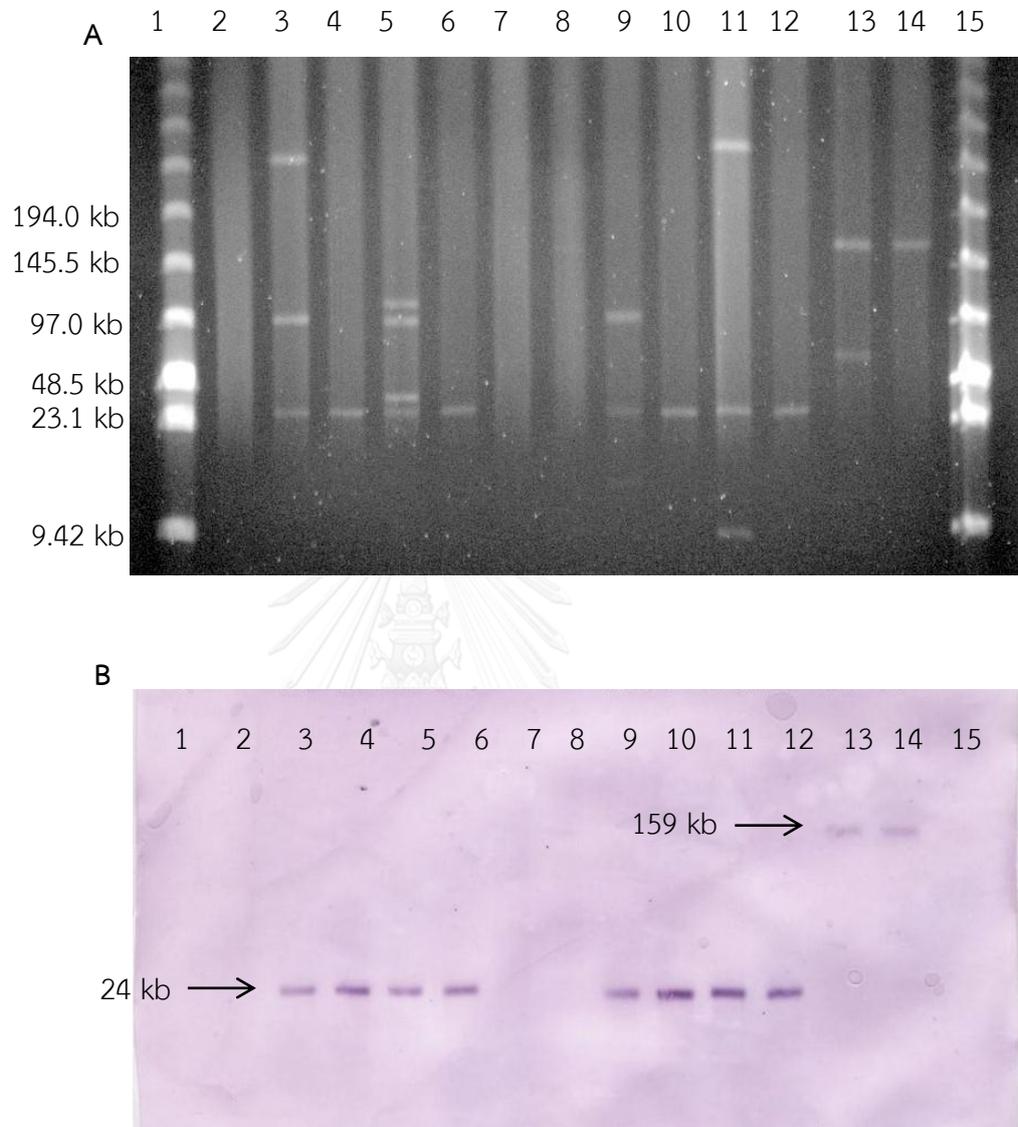
Figure 38 S1-PFGE and Southern blot hybridization with NDM probe

    (A) PFGE of S1-digested plasmid DNA

    (B) Southern blot hybridyzation with NDM probe

        Lane 1, Low range PFGE marker (kb); Lane 2, *E. coli* UB1637 (negative control); Lane 3, Donor, M84; Lane 4, trc84; Lane 5,Donor, T520; Lane 6 trc520; Lane 7, 8, no sample; Lane 9, Donor, S1596;Lane 10, trc1596; Lane 11, Donor, T1830;Lane 12, trc1830; Lane13, Donor,T2969; Lane 14, trc2969; Lane 15, Low range PFGE marker (kb)

Figure 39 S1-PFGE and Southern blot hybridization with OXA-48 probe

    (A)   PFGE of S1-digested plasmid DNA

    (B)   Southern blot hybridyzation with OXA-48 probe

           Lane 1, Donor, T1179; Lane 2, trc1179; Lane 3, Low range PFGE marker

(kb)

CHAPTER VI

DISCUSSION

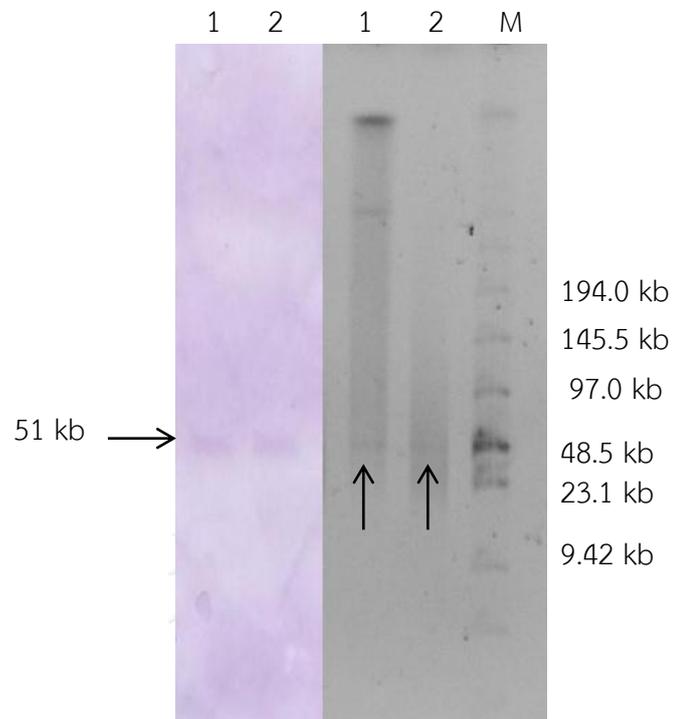The emergence of carbapenemase-producing Enterobacteriaceae has been increasingly reported worldwide. Most of them were *Klebsiella* spp. and *E. coli* [217]. In this study, we characterized carbapenemase-encoding genes in Enterobacteriaceae from patients in King Chulalongkorn Memorial Hospital during August 2012 to January 2013. Similar to previous studies, the results showed that the majority of carbapenemase-producing Enterobacteriaceae were *K. pneumoniae* (52.8%) and *E. coli* (15.6%). A total of 104 clinical isolates were positive on MacConkey agar containing ertapenem , cloxacillin and $ZnSO_4$ [218]. Drigalski agar-based containing cloxacillin, ertapenem and zinc sulfate, namely Supercarba medium, was reported to be used for the detection of carbapenemase producers in Enterobacteriaceae. This medium had high sensitivity (95.6%) and high specificity (82.2%). In this study, MacConkey agar was used instead of Drigalski agar with the same concentration of cloxacillin, ertapenem and zinc sulfate. The low concentration of ertapenem was useful for the detection of carbapenemase producers, particularly OXA-48 producers. $ZnSO_4$ was used for detection of metallo-beta-lactamase (MBL, class B carbapenemase) and cloxacillin inhibited growth of overexpression of AmpC beta-lactamase-producing Enterobacteriaceae, usually detected in *E. cloacae*, *E. aerogenes*, *M. morgannii,* and *S. marcescens* [218]. Of the 104 Enterobacteriaceae isolates positive on the screening medium, only 19 isolates (18.9%) were carbapenemase producers. The false-positive results may be due to the presence of ESBL/AmpC or alteration of PBPs or the efflux pump or the decrease of outer membrane permeability. All of these carbapenemase producers carrying carbapenemase genes, including bla_{IMP}, bla_{NDM} and bla_{OXA-48}, were able to grow on

this screening medium. Similar to previous report, this medium can detect OXA-48 producers which usually have low-level resistance to carbapenems.

The MICs of carbapenems in carbapenemase-producing Enterobacteriaceae were reported to be highly variable and often low level [51, 220]. Nordmann *et al.* reported that the MIC range of imipenem, meropenem and ertapenem of OXA-48-producing Enterobacteriaceae was 0.5->64, 0.5->64 and 0.25->64 µg/ml, respectively. The MBL-producing Enterobacteriaceae had the MIC range of imipenem, meropenem, ertapenem as 1->64, 0.25->64 and 0.5->64 µg/ml, respectively. Similarly, our results showed the wide range of carbapenem MIC, including 4->256, 0.5->256 and 0.25->256 µg/ml for ertapenem, imipenem and meropenem, respectively.

Modified Hodge test (MHT) with ertapenem was recommended by the CLSI (2013) to be used as a screening test for carbapenemase producer in Enterobacteriaceae. This method had low sensitivity to NDM producer in contrast to KPC and OXA-48 producers [219, 221, 222]. The false-negative results were due to the interpretation of MHT was difficult. In our study, 44 isolates were positive on MHT. The false-positive results of MHT may be due to overexpression of AmpC cephalosporins or porin loss or coproduction of ESBL/AmpC [222] [223, 225].

All 19 carbapenemase producers were detected for screening ESBL phenotypes by combination disk test and AmpC beta-lactamase by MHT. In this study, carbapenemase producers showed false-negative results in the detection of ESBLs by combination disk, suggesting that metallo-beta-lactamases interfered the interpretation of the results. The addition of EDTA, the inhibitor of metallo-beta-lactamases, in cefotaxime disk, cefotaxime containing clavulanic acid disk, ceftazidime disk, ceftazidime containing clavulanic acid disk could detect ESBL phenotype.

Four carbapenemase genes, including NDM-1, NDM-4, IMP-14a and OXA-48 genes were detected in carbapenemase-producing isolates. Similar to the report from Thailand, IMP-14a and NDM-1 producers were detected from Enterobacteriaceae isolates [161]. The most common carbapenemases-producing Enterobacteriaceae have been reported to be *K. pneumoniae* and *E. coli*. Our results showed that *K. pneumoniae* was the most common carbapenemase producers, followed by *E. cloacae*, *E. coli* and *C. freundii*. The results were similar to the report from China [226]. The bla_{NDM-1} was the most common carbapenemase gene and none of the isolate was co-harboured with the other carbapemase genes. Recently, NDM-1 was found to be co-produced with other carbapenemases, such as OXA-48 and OXA-181 [227-229]. The first report of NDM-4 was found in India. It differed from NDM-1 gene by substitution of amino acid at position 154 (Met154Leu) [172]. Similar to this study, Nordmann *et.al.* showed that the MIC of ertapenem and imipenem of isolates carrying bla_{NDM-4} was higher than the isolates carrying bla_{NDM-1} [172]. NDM-4 was reported in *E. coli* and *K. pneumoniae* in Cameroon, Italty and Vietnam [153, 171, 173]. In the present study, carbapenemase producers co-produced CTX-M-15, the most common ESBL. The bla_{NDM-1} and $bla_{CTX-M-15}$ were carried on a different plasmid. In contrast to our results, Poirel *et al.* showed that NDM-1 producer was found to co-produce CTX-M-15 and both bla_{NDM-1} and $bla_{CTX-M-15}$ were carried on the same plasmid.

Similar to previous report, PFGE showed that *E. coli* and *K. pneumoniae* carrying bla_{NDM-1} and $bla_{CTX-M-15}$ was associated with clonal spread in the hospital.

The plasmids carrying carbapenemase genes were reported to be transferred by conjugation. The MICs of carbapenems for transconjugants were lower than the MIC of their donors, suggesting that other mechanisms involved in carbapenamase resistance [154]. In this study, 4 out of 19 clinical isolates were failed in conjugation

experiment. This suggested that the isolate had large plasmid or located on chromosome or non-conjugative plasmid [162]. However, the plasmids were successfully transformed by using electrotransformation in 2 isolates, including *E. coli* carrying bla_{NDM-4} (S212) and *K. pneumoniae* carrying bla_{NDM-1} (T3689).

The bla_{NDM-1} was reported on various size of plasmids, such as 130 kb, 150 kb in Switzerland [154] 140 kb, 180 kb in India [230], 55 kb – 360 kb in China [226] and 150 kb in Turkey [231]. NDM-4 carried on plasmid with the size of 70 kb and 120 kb [153, 172]. Southern blot hybridization showed that NDM-1 gene was located on a single plasmid with size of 24 kb.

In 2009 the United State, the HICPAC (Healthcare Infection Control Practices Advisory Committee) and CDC recommended to control carbapenem-resistant Enterobacteriaceae and carbapenemase-producing Enterobacteriaceae for acute care facilities because of the prevalence of CRE and CPE is increasingly in United State and associated with high mortality and morbidity [217]. In Pakistan, the prevalence of faecal carriage of multidrug-resistant Enterobacteriaceae-producing NDM-1 in Rawalpindi was high (18.5%) [232]. Nosocomial outbreak of OXA-48-producing *E. cloacae* in the gut flora has been reported [233]. In Thailand, the prevalence of carbapenemase-producing Enterobacteriaceae was 0.17% from report of Rimrang *et al.* [179]. A little increasing prevalence (0.49 %) was found in this study. The KPC enzymes were commonly found in the United States and then worldwide [234]. However, KPC was not detected in this study. This is the first report of NDM-4 in *E. coli* in Thailand.

CHAPTER VII

CONCLUSION

A total of 3,854 Enterobacteriaceae recovered from clinical samples of different patients in King Chulalongkorn Memorial Hospital during August 2012 to January 2013. There were 104 Enterobacteriaceae isolates positive on screening medium containing ertapenem (0.25 μg/ml), cloxacillin (250 μg/ml) and $ZnSO_4$ (70 μg/ml) for carbapenemase producers. Resistance rates of ertapenem, imipenem, meropenem, cefotaxime, ceftriazone, ceftazidime and cefoxitin were 96.2%, 37.5%, 46.2%, 98%, 98%, 98% and 95.2%, respectively. The prevalence of carbapenem resistace in Enterobacteriaceae was 2.7%.

The prevalence of carbapenemase-encoding genes in Enterobacteriaceae are 0.49% (19/3,854). There were 19 Enterobacteriaceae isolates habouring carbapenemase genes, including IMP, OXA-48 and NDM genes. All of them had carbapenemase activity.

DNA sequence analysis revealed that carbapenemase genes were bla_{NDM-1}, bla_{NDM-4}, $bla_{IMP-14a}$, and bla_{OXA-48}. The bla_{NDM-1} was the most common carbapenemase gene, which was found in 16 enterobacteriaceae isolates, including 2 each of *C. freundii*, *E. coli*, *E. cloacae* and 10 *K. pneumoniae*. One *E. coli* isolate carried the bla_{NDM-4}. Of the 2 *E. cloacae*, one isolate carried $bla_{IMP-14a}$ and the other carried bla_{OXA-48}.

Of the 19 Enterobacteriaceae isolates carrying carbapenemase genes, 15 (78.9%) isolates carrying bla_{NDM-1} and one isolate carrying bla_{OXA-48} were successfully transferred carbapenemase genes to $UB1637Az^R$. Four (21.1%) isolates, including S212 (NDM-4 producer), M38 (IMP-14a producer), T1469 (NDM-1 producer) and T3689

(NDM-1 producer) were unable to transfer any resistance genes by conjugation. All 16 Enterobacteriaceae carrying carbapenemase genes were analyzed for the presence of resistance genes on plasmids by Southern blot hybridization. The T1179 had a plasmid carrying with bla_{OXA-48} with the size of approximately 51 kb. All 13 Enterobacteriaceae isolates, including M57, M84, S804, S992, S1596, T197, T458, T520, T632, T1580, T1830, T3525 and T3367, had a plasmid harbouring bla_{NDM-1} with the size of approximately 24 kb. Only T2969 had bla_{NDM-1} on a plasmid with different size (approximately 159 kb). The PFGE patterns showed that the spread of bla_{NDM} and $bla_{CTX-M-15}$ genes in isolates from King Chulalongkorn Memorial Hospital, was associated with clonal spread.

The results showed that NDM-1 was the most common carbapenemase among Enterobacteriaceae isolates, including *K. pneumoniae*, *E. coli* and. This is the first report of *E. coli* carrying bla_{NDM-4} in Thailand.

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APPENDIX

APPENDIX A

SCREENING MEDIA PREPARATION

1. MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, England)

Suspend 51.5 grams of the medium in 1 L of distilled water. Mix and boiling to suspension. Sterilize in an autoclave at 121 $^\circ$C for 15 minutes. Cool to 60 $^\circ$C and pour into petri dishes, 20ml in each dish. Allow to solidify and keep the dishes at 4 $^\circ$C

2. Stock solution for Ertapenem (0.25 µg/ml) (LABORATORIES MERCK SHARP & DOHME-CHIBRET, France)

Suspend 0.0005 grams of ertapenem in 1 ml of distilled water and mix with 9 ml of distilled water until the final volume is 10 ml and mix well until dissolve at roomtemperature.

3. Stock solution for Cloxacillin (250 µg/ml) (M&H Manufacturing, Thailand)

Suspend 0.0005 grams of cloxacillin in 1 ml of distilled water and mix well until dissolve at roomtemperature.

4. Stock solution for $ZnSO_4$ (70 µg/ml) (SIGMA-ALDRICH, St.Louis, MO, USA)

Suspend 0.014 grams of $ZnSO_4$ in 1 ml of distilled water and mix well until dissolve.

5. MacConkey agar containing ertapenem (0.25 µg/ml), cloxacillin (250 µg/ml) and $ZnSO_4$ (70 µg/ml)

Prepare MacConkey media 170 ml and mix with ertapenem 1 ml from stock, cloxacillin 1 ml from stock and $ZnSO_4$ 1ml from stock. Pouring the mixing media on plates and allow to solidify.

APPENDIX B

MEDIA PREPARATION

1. Muller-Hinton II agar (BBL, USA)

Suspend 38 grams of the dehydrated medium in 1,000 ml of distilled water. Dissolve by heating with frequent agitation until complete dissolution. Adjust final volume to 1,000 ml. Sterilize at 121°C (15 lbs. sp) for 15 minutes. Once the medium is prepared, store at 4°C.

2. Tryptic soy broth (BBL, USA)

Suspend 30 grams in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121oC for 15 minutes. Once the medium is prepared, store at 4oC.

3. LB broth (Pronadisa, Spain)

Suspend 20 grams of the dehydrated medium in 900 ml of distilled water. Dissolve by heating with frequent agitation until complete dissolution. Adjust final volume to 1,000 ml. Sterilize at 121oC (15 lbs. sp) for 15 minutes. Once the medium is

prepared, store at 4°C.

4. Trytic soy agar (BBL, USA)

Suspend 40 grams in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121oC for 15 minutes. Once the medium is prepared, store at 4°C.

5. Sterile 0.85% NaCl (Merck, Germany)

NaCl 8.5 grams in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121oC for 15 minutes. Once the medium is prepared, store at room temperature.

6. Antibiotic solution preparation

Cefoxitin, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0269 g in 5 ml sterile distilled water

Ceftazidime, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 400 μL of 0.1 N NaOH and

4.6 ml sterile distilled water

Cefotaxime, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 5 ml sterile distilled water

Ceftriaxone, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 5 ml sterile distilled water

Ertapenem, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 5 ml sterile distilled water

Meropenem, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 5 ml sterile distilled water

Imipenem, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 2.5 ml sterile distilled water

APPENDIX C

REAGENTS PREPARATION

1. 10x Tris-Borate buffer (TBE)

Tris base	108 g/L
Boric acid	55 g/L
0.5 M EDTA (pH 8.0)	40 ml

Adjust volume to 1 liter with distilled water. The solution was mixed and sterilized by autoclaving at 121oC for 15 min.

2. 0.5 M EDTA (pH 8.0)

Disodium ethylene diamine tetra-acelate 2H$_2$O	186.1 g/L
Distilled water	1 L

Adjust pH to 8.0 and volume to 1 liter. Store at room temperature for no longer than 1 year.

3. 10x TE buffer

Tris	12.11 g/L
0.5 M EDTA	20 ml

Adjust to pH 8.0 by adding conc. HCl. Adjust volume to 1,000 ml and sterilized by autoclaving at 121oC for 15 min.

4. 1.5 % Agarose gel

Agarose	0.6 g
1x TBE	40 ml

Dissolve by heating in microwave oven and occasional mix unit no granules of agarose are visible.

5. 6X Loading buffer 100 ml

Tris HCl	0.6 g
EDTA	1.68 g
SDS	0.5 g
Bromphenol Blue	0.1 g
Sucrose	40 g

Adjust volume to 100 ml with distilled water. Mix the solution, aliquot into 1.5

microtubes and store at 4°C.

6. Reagent for DNA extraction

6.1 Protease K

Reconstituted of protease K (lyophilized) with 1.25 ml protease solvent,

stored at -20oC

6.2 Buffer AL (Ready to used)

6.3 Buffer AW1

Buffer AW1 is supplied as a concentrate. Before using for the first time, add the 25 ml

of ethanol (96-100%) to buffer AW1 concentrate as indicated on the bottle.

6.4 Buffer AW2

Buffer AW2 is supplied as a concentrate. Before using for the first time, add the 30 ml

of ethanol (96-100%) to buffer AW2 concentrate as indicated on the bottle.

6.5 Buffer AE (Ready to used)

7. Reagent for PCR product purification

7.1 Buffer PB (Ready to used)

7.2 Buffer PE

Buffer PE is supplied as a concentrate. Before using for the first time, add the 55 ml

of ethanol (96-100%) to buffer PE concentrate as indicated on the bottle.

8. Denature solution

     1.5 M NaCl   87.66 g

     0.5 M NaOH   20 g

     Double distilled water 1,000 g

The solution was mixed and sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes

9. Detection solution

     NaCl   58 g

     Tris-base   12.1 g

Adjust to pH 9.5 by adding conc. HCl. Adjust volume to 1,000 ml with Double

distilled water and sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes

10. Depurination buffer

     Conc. HCl   20.8 ml

     Double distilled water up to 1,000 ml

The solution was sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes

11. 1M EDTA (pH 8.0)

Disodium ethylene diamine tetra-acelate 2H$_2$O   186.1 g

Adjust to pH 8.0 by adding NaOH. Adjust volume to 500 ml with Double distilled

water and sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes

12. Maleic acid

Maleic acid   11.6 g

Nacl   8.76 g

Adjust to pH 7.5 by adding conc. HCl. Adjust volume to 1,000 ml with Double

distilled water and sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes

13. Neutralization solution

1.5 M NaCl   87.66 g

0.5 M Tris-base , pH 7.2   60.57 g

Adjust to pH 7.5 by adding conc.HCl. Adjust volume to 1,000 ml with Double distilled

water and sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes

14. 1M NaOH

NaOH   20 g

Adjust volume to 500 ml with distilled water and sterilized by autoclaving at 121 $^{\circ}$C

for 15 minutes

15. 10% SDS

SDS   30 g

Double distilled water 300 ml

The solution was mixed and sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes

16. 20X SSC

Trisodium citrate (citric acid) 88.2 g

NaCl   175.3 g

Double distilled water 1,000   ml

The solution was mixed and sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes

17. Tris-EDTA

Tris-base   1.58 g

Disodium ethylene diamine tetra-acelate 2H$_2$O   0.38 g

Adjust to pH 8.0 by adding conc.HCl. Adjust volume to 1,000 ml with Double distilled water

and sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes.

18. Washing solution

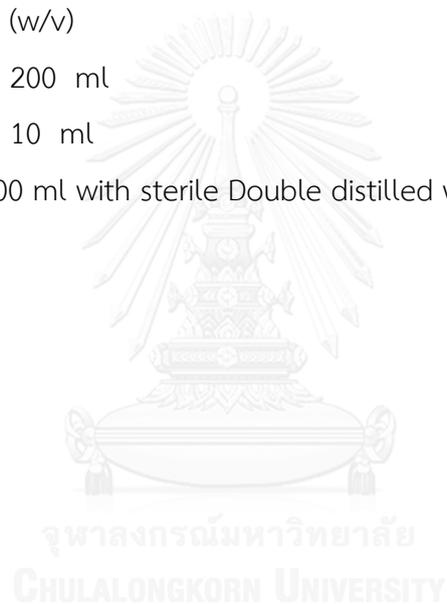   Maleic acid   11.6 g

      NaCl   8.76 g

      Adjust to pH 7.5 by adding conc.HCl. Adjust volume to 1,000 ml with with Double distilled water and sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes. After sterilized add 3 ml of tween 20.

19. 0.2 NaOH, 0.1 SDS (w/v)

      1M NaOH   200 ml

      10% SDS   10 ml

Adjust volume to 1,000 ml with sterile Double distilled water.

APPENDIX D

The results of 104 clinical isolates were positive on screening MacConkey agar

No.	Code	Organism	carbapenemase phenotypes			ESBLs phenotypes	AmpC β-lactamases phenotype
			Modified Hodge test	Boronic acid-based inhibition test	EDTA-meropenem combined-disk test	Combination disk test	Modified Hodge test
1	T 115	E.coli	-	-	-	+	-
2	T 197	K.pneumoniae	+	-	+	-	-
3	T 458	C.freundii	+	-	+	-	-
4	T 491	K.pneumoniae	-	-	-	-	-
5	T 520	E.coli	+	-	+	-	-
6	T 539	K.pneumoniae	-	-	-	+	-
7	T 597	E.cloacae	-	-	-	-	-
8	T 623	Pt.mirabilis	-	-	-	-	-
9	T 632	C.freundii	+	-	+	-	-
10	T 739	E.coli	-	-	-	-	-
11	T 943	E.cloacae	-	-	-	+	-
12	T 1003	Providencia spp.	+	-	-	-	-
13	T 1005	E.cloacae	-	-	-	+	-
14	T 1006	E.cloacae	-	-	-	+	-
15	T 1080	E.cloacae	+	-	+	-	+
16	T 1179	E.cloacae	+	-	-	+	-
17	T 1195	E.coli	-	-	-	+	-
18	T 1275	E.coli	-	-	-	+	-
19	T 1286	K.pneumoniae	-	-	-	+	-
20	T 1469	k.pneumoniae	+	+	+	-	-
21	T 1580	E.coli	+	-	+	-	-
22	T 1605	E.cloacae	+	-	+	-	+
23	T 1696	K.pneumoniae	+	-	+	+	-
24	T 1824	E.cloacae	-	-	-	+	-
25	T 1830	E.cloacae	+	-	+	-	-
26	T 1847	K.pneumoniae	-	+	+	-	-
27	T 1882	E.cloacae	+	-	+	-	+
28	T 1958	E.coli	-	-	-	+	-
29	T 1975	K.pneumoniae	+	-	+	-	+
30	T 2026	K.pneumoniae	+	-	+	-	-
31	T 2168	K.pneumoniae	-	-	-	+	-
32	T 2181	K.pneumoniae	-	-	-	+	-
33	T 2233	k.pneumoniae	+	-	+	-	+
34	T 2274	K.pneumoniae	-	-	-	+	-
35	T 2622	K.pneumoniae	+	-	+	-	+
36	T 2715	K.pneumoniae	-	-	+	+	-
37	T 2819	E.cloacae	+	-	-	-	-
38	T 2901	K.pneumoniae	-	-	-	+	-
39	T 2902	K.pneumoniae	-	-	-	+	-
40	T 2925	E.coli	-	+	+	-	-
41	T 2940	K.pneumoniae	-	-	-	-	-
42	T 2969	K.pneumoniae	+	-	+	+	-
43	T 2990	K.pneumoniae	+	-	+	+	-
44	T 2962	K.pneumoniae	+	-	+	-	+
45	T 3157	K.pneumoniae	-	-	-	+	-
46	T 3348	M.morganii	-	-	-	+	-
47	T 3367	K.pneumoniae	+	-	+	-	-
48	T 3427	E.coli	-	-	-	+	-
49	T 3358	E.coli	-	-	-	-	-
50	T 3477	K.pneumoniae	-	-	-	+	-

No.	Code	Organism	carbapenemase phenotypes			ESBLs phenotypes	AmpC β-lactamases phenotype
			Modified Hodge test	Boronic acid-based inhibition test	EDTA-meropenem combined-disk test	Combination disk test	Modified Hodge test
53	T 3525	E.cloacae	+	-	+	-	+
54	T 3625	E.aerogenes	-	-	-	+	-
55	T 3665	K.pneumoniae	-	-	+	+	-
56	T 3664	K.pneumoniae	-	-	-	+	-
57	T 3630	K.pneumoniae	-	-	+	+	-
58	T 3689	K.pneumoniae	+	-	+	-	-
59	T 3695	K.pneumoniae	+	-	+	+	+
60	T 3922	E.aerogenes	-	-	-	-	-
61	T 4009	E.cloacae	+	-	-	-	-
62	T 4036	K.pneumoniae	+	-	+	-	-
63	T 4060	K.pneumoniae	-	-	-	+	-
64	T 4080	E.coli	+	-	+	+	+
65	M 1	K.pneumoniae	-	-	-	+	-
66	M 2	E.cloacae	-	-	-	+	-
67	M 4	E.cloacae	+	-	-	+	+
68	M 7	K.pneumoniae	-	-	-	+	-
69	M 9	K.pneumoniae	-	-	-	+	-
70	M 11	E.cloacae	-	-	-	+	-
71	M 12	K.pneumoniae	-	+	-	-	-
72	M 13	E.cloacae	-	-	-	+	-
73	M 18	E.coli	-	-	-	+	-
74	M 20	K.pneumoniae	-	-	-	+	-
75	M 30	E.coli	-	-	-	+	-
76	M 33	E.coli	-	-	-	+	-
77	M 34	E.coli	+	-	-	+	-
78	M 35	E.coli	-	-	-	+	-
79	M 38	E.cloacae	+	-	+	+	+
80	M 42	K.pneumoniae	-	-	-	+	-
81	M 43	K.pneumoniae	-	-	-	+	-
82	M 48	K.pneumoniae	-	-	-	+	-
83	M 49	E.coli	-	-	-	+	-
84	M 50	E.coli	+	-	-	+	+
85	M 57	K.pneumoniae	+	-	+	+	-
86	M 64	E.cloacae	-	-	-	+	-
87	M 65	K.pneumoniae	+	+	-	+	+
88	M 68	K.pneumoniae	-	-	-	+	-
89	M 69	K.pneumoniae	-	-	-	+	-
90	M 73	E.coli	-	-	-	+	-
91	M 74	K.pneumoniae	-	-	-	+	-
92	M 76	E.cloacae	+	-	-	+	+
93	M 81	E.cloacae	-	-	-	+	-
94	M 84	K.pneumoniae	+	-	+	+	-
95	M 86	E.coli	-	-	-	+	-
96	M 89	E.cloacae	+	-	+	+	+
97	M 90	E.cloacae	-	-	-	+	-
98	M 93	E.cloacae	+	-	-	+	+
99	M 96	E.coli	+	-	-	+	-
100	M 113	K.pneumoniae	-	-	-	+	-
101	S 212	E.coli	-	-	+	+	-
102	S 804	K.pneumoniae	+	-	+	+	-
103	S 992	K.pneumoniae	+	-	+	+	-
104	S 1596	K.pneumoniae	+	-	+	+	-