PHENOTYPIC DETECTION OF CARBAPENEMS RESISTANT *ENTEROBACTERIACEAE* AND PREVALENCE OF COLISTIN RESISTANCE IN CARBAPENEMS RESISTANT *ENTEROBACTERIACEAE* IN LOWER NORTHERN REGION OF THAILAND

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Thesis entitled "Phenotypic detection of carbapenems resistant *Enterobacteriaceae* and prevalence of colistin resistance in carbapenems resistant *Enterobacteriaceae* in Lower Northern region of Thailand" submitted by Farida Noothong has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Sciences in Biomedical Sciences of Naresuan University.

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ABSTRACT

The emergence and global spread of carbapenem-resistance *Enterobacteriaceae* (CRE) has been reported and becoming a major concern in public health worldwide. The emerging of CRE is one of the most important emerging antibiotic resistances in Thailand. In this present study revealed the prevalence of CRE from 7 hospitals clinical microbiology laboratories located in the lower north of Thailand. The CRE prevalence was approximately 31.48% and all of CRE isolates were detected for carbapenemase production by phenotypic methods. 75 isolates (63.03%) of carbapenemase producing trains were investigated for the associated with the combination of colistin resistant. Within overall isolates, 7 isolates (5.88%) includes, 4 isolates of *Enterobacter cloacae* and 3 isolates of *Klebsiella pneumoniae* were resistant to colistin and showed MICs ranging from 32 to $\geq$ 128 μg/ml. However, the involvement of carbapenemase producing *Enterobacteriaceae* in colistin resistant isolates was not related to the plasmid-mediated colistin resistance gene, *mcr- 1*. Eventually, this found is preliminary prevalence, with the increasing of antibiotic combination resistance is becoming a major public health concern. The information documented in this study may offer

significant help in the control to reducing the spread of carbapenemase producing colistin-resistant *Enterobacteriaceae.*

LIST OF CONTENTS

LIST OF CONTENTS

LIST OF TABLES

LIST OF TABLES (CONT.)

LIST OF FIGURES

LIST OF FIGURES (CONT.)

Figures **Page**

LIST OF ABBREVIATIONS

Abbreviation or Symbol	Meaning
˚C	Degree Celsius
α	Alpha
β	Beta
Ω	Omega
µg/ml	Microgram per milliliter
µg/mg	Microgram per milligrams
µl	Microliter
× g	Relative Centrifugal Force
ACA	Aminocephalosporanic acid
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
Asn	Asparagin
ATCC	American Type Culture Collection
bp	Base pair
Carba NP	Carbapenemase Nordmann-Poirel
Ca^{2+}	Calcium ions
CFU/ml	Colony-forming units per milliliter
CFU/spot	Colony-forming units per spot
cis	Configurational isomerism
CLSI	Clinical and Laboratory Standards Institute
CMS	Colistin methanesulfonate
CRE	Carbapenem-resistant *Enterobacteriaceae*
Dab	Diaminobutyric acid

LIST OF ABBREVIATIONS (CONT.)

Abbreviation or Symbol	Meaning
DNA	Deoxyribonucleic acid
DW	Distilled water
EDTA	Ethylenediaminetetraacetic
ETP	Ertapenem
EUCAST	European Committee on Antimicrobial Susceptibility Testing
E-test	Epsilometer test
GES	Guiana extended spectrum
GIM	German imipenemase
Gly	Glycine
His	Histidine
IMI	imipenem-hydrolysing β-lactamases
IMP	Imipenem
KPC	*Klebsiella pneumoniae* carbapenemase
LPS	Lipopolysaccharide
Lys	Lysine
L-Ara4N	4-amino-4-deoxy-l-arabinose
MBLs	Metallo-beta-lactamases
mcr-1	Plasmid-borne colistin resistance gene
MDR	Multiple drug resistance
MEM	Meropenem
mg	Milligram
mg/dl	Milligram/deciliter
Mg^{2+}	Magnesium ion
MHA	Mueller-Hinton agar
MHT	Modified Hodge test

LIST OF ABBREVIATIONS (CONT.)

Abbreviation or Symbol	Meaning
MIC	Minimum inhibitory concentration
MIL	Motility-indole-lysine medium
ml	Milliliter
mm	Millimeter
mM	Millimolar
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaCl	Sodium chloride
NAG	N-acetylglucosamine
NAM	N-Acetylmuramic acid
NARST	The National antimicrobial resistance surveillance center
NCTC	National Collection of Type Cultures
NDM	New Delhi metallo-beta-lactamase
OXA	Oxacillinases
PBPs	Penicillin Binding Proteins
PCR	Polymerase chain reaction
PEtN	Phosphoethanolamine
pH	A logarithmic scale used to specify the acidity or basicity of an aqueous solution by hydrogen ions
Phe	Phenylalanine
RIFLE	Risk-Injury-Failure-Loss of Kidney Function-End-stage Kidney disease
RPM	Revolutions per minute

LIST OF ABBREVIATIONS (CONT.)

Abbreviation or Symbol	Meaning
Ser	Serine
s	Second
SIM	Seoul imipenemase
SPM	Sao Paulo metallo-beta-lactamase
Thr	Threonine
Tn	Transposon
TSB	Tryptic soy broth
TSI	Triple sugar iron agar
UK	The United Kingdom
USA	The United States of America
VIM	Verona intergron-encoded metallo-beta-lactamase
Zn	Zinc
Zn^2	Zinc ion

CHAPTER I

INTRODUCTION

Statement of purpose

β-lactam antibiotics are a class of broad-spectrum antibiotics active against various both gram negative and gram positive bacteria. The mechanism of β-lactam antibiotics are inhibition of bacterial cell wall synthesis (1). At present there are four major β-lactam subgroups that include penicillins, cephalosporin, monobactams and carbapenems. Unfortunately, the clinical use of β-lactam antibiotics influence on a selective pressure for the survival of resistant strains. Resistance to antibiotics is currently a major health concern in treating infectious diseases. There are four common defense mechanisms against β-lactam antibiotics include destruction of the antibiotic by β-lactamases, decreasing affinity of the PBP to the antibiotic, decreasing penetration through the porin of the antibiotic to reach the target site and increasing efflux pump activity for antibiotic extrusion. The most common mechanism of resistance to β-lactam antibiotics is the production of β-lactamases enzyme

Classification of β-lactamases has two major schemes in traditional use to classify the enzymes. The classification has been based on either molecular classification (Ambler classification) or functional classification (Bush-Jacoby-medeiros classification) (2). The molecular classification of β-lactamases is based on the nucleotide and amino acid sequences in these enzymes which divided into four molecular classes include classes A, B, C and D. The functional classification based on their ability to hydrolyze specific β–lactam classes and on the inactivation properties of the β–lactamases inhibitors by providing selective resistance to different classes of β-lactam antibiotics. The major grouping includes group 1, 2, 3 and 4 (2).

Carbapenemases are members of the molecular class A, B, and D β-lactamases, known as carbapenem-hydrolyzing enzyme. These enzymes have ability to hydrolyze almost all β-lactam antibiotics including penicillins, cephalosporins, monobactams, carbapenems and most are resistant to β-lactamase inhibitors (3). With the increasing numbers of serious infections in which the carbapenemase activity

rendered man β-lactams ineffective by carbapenemases-producing bacteria, particularly the emergence of carbapenemases-producing in *Enterobacteriaceae* family (4-5).

The emergence and global spread of carbapenemase producers among *Enterobacteriaceae* has been reported worldwide and possibly carried this resistance either on chromosome or acquired via plasmid mediated resistance that spread rapidly between bacterial isolate. For epidemiology of carbapenems-resistant *Enterobacteriaceae* (CRE) in Thailand, The surveillance of CRE among clinical isolates of *Enterobacteriaceae* from the hospitals in Thailand during 2012-2013, the CRE prevalence in each regions were 21.9% (52/237 isolates) in the north, 27.4% (65/237 isolates) in the Northeastern, 24.5% (58/237 isolates) in the Central, 9.3 % (22/237 isolates) in Bangkok, 6.3 % (15/237 isolates) in the East and 10.6% (25/237 isolates) in the south of Thailand (6). Notably, NDM-1 has been mostly identified from isolates. Nevertheless, the increasing of CRE and the use of other antibiotic include β-lactam is ineffective (6).

Therefore, colistin is increasingly considered as the final option of antibiotic therapy for multiple drug resistance (MDR) bacteria that are resistant to almost all other currently available antibiotics. However, re-introduced of colistin into clinical practice for treatment of multidrug resistant gram negative bacteria may lead to the development of resistance as well. Resistance to colistin has been reported throughout the world. Moreover, colistin has emerged as a therapeutic option against other MDR gram negative bacteria and CRE (7). With the increased usage of colistin to treat serious infection, there is beginning to be reported of resistance to colistin in isolates of CRE (8). From previous study, colistin resistance has evolved chromosomal-encoded mechanism, especially in certain members of *Enterobacteriaceae*. The mechanism is associated with two-component systems by the alteration of lipopolysaccharide (LPS) via lipid A modification. Very recently study has been reported the emergence of plasmid-mediated *mcr -1* colistin resistance via horizontal gene transfer, routine surveillance project on antimicrobial resistance in commensal *Escherichia coli* from food animal in China. The resistance has also been found in isolates of *E. coli* and *K. pneumoniae* in animal and human. The mechanism of resistance via *mcr-1* gene related to the family of phosphoethanolamine transferase

enzymes (9). The retrospective study showed that, the *mcr-1* gene has already been detected in no fewer than 16 countries, including 7 countries in Asia and Southeast Asia, includes China, Japan, Thailand, Laos, Vietnam , Cambodia and Malaysia (10).

Furthermore, the documented data from the research on the diversified *mcr-1* reservoirs confer resistance to colistin in human gut microbiota showed the resistance in at least 3 enterobacterial species from host reservoirs included at least three kinds of poultry and livestock chickens, pigs, and cattle. Moreover, it has been already being found animal-to-human transmission in China, Thailand, Laos, and Denmark. Therefore, the emergence of plasmid-mediated *mcr-1* colistin resistance has become a serious public health worldwide (10).

For the emergence of *mcr-1* gene in Thailand, the first detection of plasmid-mediated colistin resistance in human isolate in Thailand has been reported in *E. coli* isolates. The national antimicrobial resistance surveillance center Thailand (NARST) documented that the finding evolves a 63 years old man whose urine contained *E. coli* infected harboring the *mcr-1* colistin resistance gene with underlying intracerebral hemorrhage was admitted to Vichein Buri hospital, Petchaboon. Therefore, this was a first case of colistin resistance established in human (6). Moreover, from research study reported the emergence of colistin-resistant *K. pneumoniae* isolated from healthy individuals and patients in Thailand as well (11). Furthermore, the use of antibiotic in livestock animals in Thailand is one of the major contributors to antibiotic resistance. They are widely used in animal production including poultry and pig farms may lead to development of commensal bacteria on animal farms (12). Therefore, they are possible as the important reservoir for acquired antimicrobial resistance genes and have a possible exchange between animals to consumers. Recently, *mcr-1* colistin resistance gene was discovered in the plasmid mediated acquired resistance gene distributing in *E. coli* from human and livestock animals in Thailand (11,13-14). For these reasons, Plasmid carrying *mcr-1* was also mobilized to the recipient bacterial cell by conjugation rapidly and maintained in host cell. The emergence of *mcr-1* conduces to the breach of the last group of antibiotic, colistin by plasmid mediated resistance (12).

Therefore, the aim of this study is to determine the prevalence of CRE and determine the prevalence of colistin resistant in CRE of clinically isolated from

clinical microbiology laboratory, located in the lower north region of Thailand by phenotypic and genotypic methods.

Research Aim

The aims of this study are as following

1. To determine the prevalence of carbapenems resistant *Enterobacteriaceae* by phenotypic method using disk diffusion methods and the prevalence of carbapenemase producing in carbapenem resistant *Enterobacteriaceae* using modified hodge test (MHT) and carbapenemase Nordmann-Poirel (Carba NP) test.

2. To determine the prevalence of colistin resistant in carbapenem-resistant *Enterobacteriaceae* by using agar dilution method.

3. To detect the presence of *mcr-1* plasmid-mediated colistin resistance gene in colistin resistant strains by genotypic detection method.

CHAPTER II

LITERRATURE REVIEW

β-Lactam

β-lactam antibiotics are group of antibiotic that contain with four-membered nitrogen-containing β-lactam ring at the core of structure (A lactam is a cyclic amide). The antibacterial activity of these molecules resides in the ring itself. β-lactam antibiotics are generally bactericidal against organisms which are effective against a broad range of bacteria, both gram negative and gram positive bacteria. The mechanism of β-lactam antibiotics are inhibition of bacterial cell wall synthesis (1).

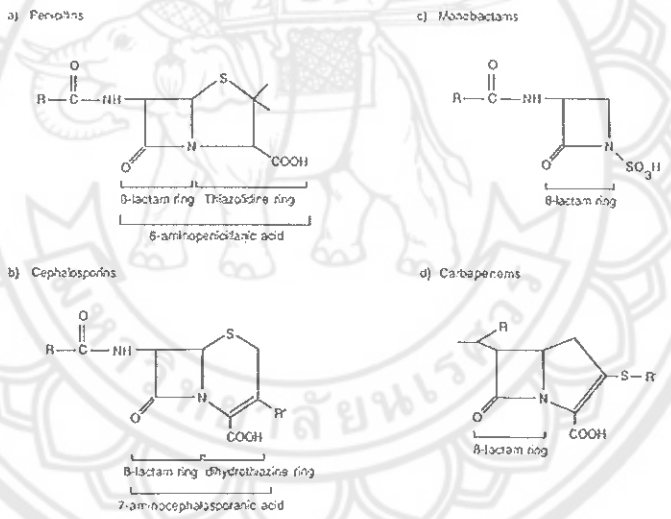


Figure 1 β-lactam antibiotics contains a β-lactam ring in molecular structures. (15)

Bacterial cell wall provides structural integrity to the cell by the presence of peptidoglycan lying outside the plasma membrane. Peptidoglycan is made up of a polysaccharide backbone consists of alternating N-Acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues in equal amounts and are connected through a β- (1,4)-glycosidic bond in transglycosylation reaction by transglycosylase. The pentapeptide side chains are connected to the carboxyl group of NAM. The most of pentapeptide structure in gram negative bacteria is usually composed of L-alanine, D-

glutamate, meso-Diaminopimelic acid, D-alanine and D-alanine, consecutively. In gram positive bacteria replace meso-Diaminopimelic acid with L-lysine in the third position of amino acid (15). To make a strong, peptidoglycan must be joined by pentaglycine cross-links between the peptides. The cross-links peptide is connected directly from the carboxyl group of the terminal D-alanine in the fourth amino acid position to the amino group in the third amino acid position on another pentapeptide side chains by the action of transpeptidase in transpeptidation reaction (1).

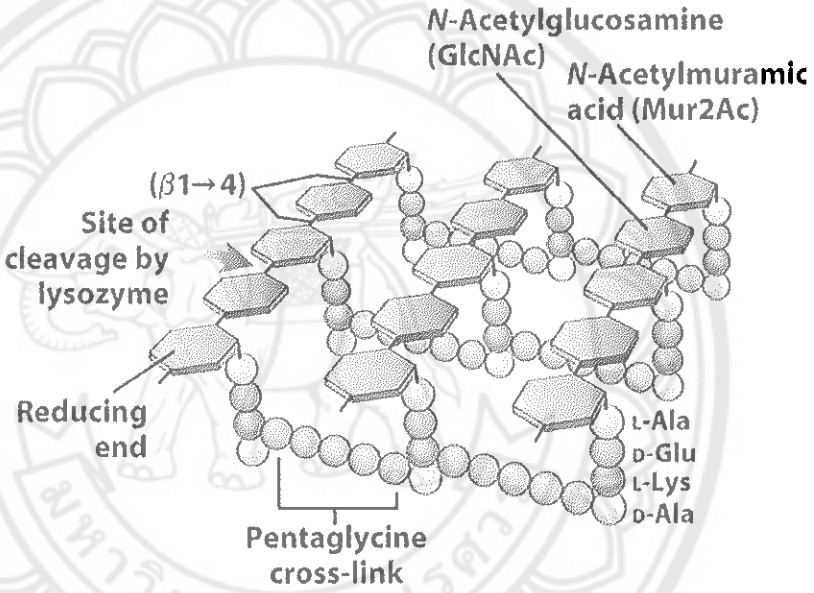


Figure 2 Bacterial cell wall structure is composed of chains of linked peptidoglycan subunits. (16)

All β-lactam antibiotics owe their activity to their ability to act as irreversible inhibitor of the transpeptidases because of the β-lactam ring structure gives these compounds a three dimensional shape to mimics the shape of the terminal D-Ala-D-Ala peptide sequence that serves as the substrate for cell wall transpeptidases that form covalent bonds between different peptidoglycan chains. The ring structure and associated side groups result in tight binding to the active site of transpeptidases and inhibits enzyme activity. For this reason, transpeptidases are collectively known as Penicillin Binding Proteins (PBPs) (19-20). Upon β-lactam binding to PBPs, rendered them unable to perform their role in cell wall synthesis. When bacterial cell wall

synthesis is inhibited, it cans auses cell death of the bacteria due to osmotic instability or autolysis (1).

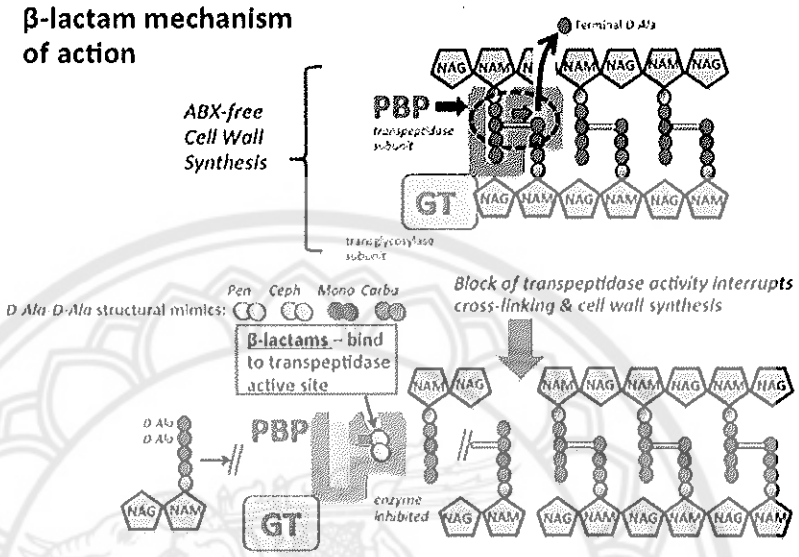


Figure 3 Mechanisms of action of β-lactam antibiotics (21).

β-lactam antibiotics are among the most commonly prescribed drugs, grouped together based upon a shared common structural feature of the β-lactam ring. The families of β-lactams antibiotics have distinctly different side rings and side chains with different antibacterial spectrums of activity and different pharmacokinetic properties. At present, there are four major β-lactam subgroups that includes penicillins, cephalosporin, monobactams and carbapenems (22).

1. **Penicillins**

The penicillins are a group of natural and semi synthetic antibiotics was discovered in 1928 by Scottish scientist Alexander Fleming at St. Mary's Hospital in London, England. The naturally occurring compounds are produced by a number of Penicillium notatum. Penicillins are containing the chemical nucleus 6-aminopenicillanic acid, which consists of a β-lactam ring fused to a thiazolidine ring (23). The bicyclic system (Penam) confers greater ring strain on the β-lactam ring, which is important for activity. An amide and a carboxylic acid group are also present. The carboxylic acid group is a possible site of modification to make prodrugs. Note the stereochemistry of the acyl amino side chain with respect to the 4-membered ring

and the cis stereochemistry for the hydrogen atoms. The penicillins differ from one another in the substitution at position 6, where changes in the side chain may modify the pharmacokinetic and antibacterial properties of the drug (22). Penicillin antibiotics were among the first medications to be effective against many bacterial infections and still widely used today.

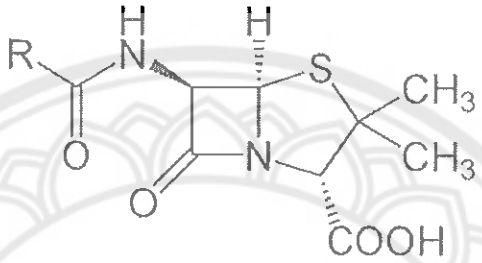


Figure 4 A general penicillin structure (24).

The penicillins can be classified according to their antibacterial activity into the following groups.

1.1 Natural penicillins

Natural penicillins, the first agents in the penicillin family, are obtained directly from the Penicillium mold and do not require further modification. The natural penicillins are active against non β-lactamase–producing gram positive cocci few gram negative cocci, gram-positive bacilli, anaerobes and spirochetes. Group of natural penicillins are consisted of many types of antibiotic according to their therapeutic indications such as penicillin G (Benzylpenicillin), procaine penicillin G, benzathine penicillin G, penicillin V (25). Penicillin G is the prototype of the class and the most potent of all penicillins against susceptible gram positive bacteria. It is sensitive to stomach acids and requires intravenous or intramuscular administration. Penicillin G is short acting, procaine and benzathine have extended duration of action because they can distribute into storage tissues to be released slowly (26).

1.2 Penicillinase-resistant penicillins

The penicillinase-resistant penicillins have bulky side chains that prevent their inactivation by the staphylococcal β-lactamases. These penicillins have a very narrow spectrum as they were developed solely for killing β-lactamase producing staphylococci which useful in treating infections caused by *Staphylococcus aureus* and *Staphylococcus epidermidis*. Due to the bulky side group, all of the penicillinase-resistant penicillins have difficulty penetrating the cell membrane and have a poor range of activity compared to other penicillins. Group of these penicillins are consisted of many types of antibiotic according to their therapeutic indications such as cloxacillin, dicloxacillin, oxacillin, nafcillin and methicillin. Methicillin was the first member of this group, followed by oxacillin, nafcillin, cloxacillin and dicloxacillin. Methicillin was the first penicillin developed through rational drug modification. Since then all bacteria which are resistant to any type of penicillin are designated as methicillin resistant e.g. methicillin-resistant *S. aureus* (MRSA) (26).

1.3 Aminopenicillins

The aminopenicillins have a wider range of activity than natural or antistaphylococcal penicillins. However, they lack the bulky side groups and are susceptible to inactivation by β-lactamases. Aminopenicillins have additional hydrophilic groups, allowing the drug to penetrate into gram-negative bacteria via the porins. The advantages of aminopenicillins include higher oral absorption, higher serum levels, and longer half-lives. Aminopenicillins are resistant to gastric acids so can be administered orally. Group of aminopenicillins are consisted of many types of antibiotic according to their therapeutic indications such as ampicillin, amoxicillin, bacampicillin, cyclacillin, hetacillin (1).

1.4 Extended spectrum penicillins

Extended-spectrum penicillins are also called antipseudomonal penicillins because the major advantage of these penicillins is their activity against *Pseudomonas aeruginosa* and certain indole-positive Proteus species that are resistant to aminopenicillins In general, they have greater activity than do other penicillins against gram negative bacteria due to enhanced penetration through the cell wall of these bacteria. Extended-spectrum penicillins are similar to the aminopenicillins in structure but have either a carboxyl groups or urea group instead

of the amine. Group of these penicillins are categorized into subgroup such as carboxypenicillins which include carbenicillin, ticarcillin and the ureidopenicillins include piperacillin, azlocillin, and mezlocillin. The extended-spectrum penicillins are not used in the treatment of infections caused by gram positive bacteria because penicillin G and aminopenicillins are more potent against these organisms. Nevertheless all antipseudomonals are destroyed by β-lactamases (27).

2. Cephalosporins

The cephalosporins are subgroup of β-lactam antibiotic, whose bicyclic system, called the cephem nucleus, consists of a four-member β-lactam ring fused through the nitrogen and adjacent tetrahedral carbon atom to a second heterocycle forming a six-member dihydrothiazine ring. Substance of cephalosporin C was first isolated from a fungus named as Cephalosporium acremonium by Dr.Abraham in 1948 (27). Most of the available cephalosporins are semi-synthetic derivatives of cephalosporin C, a compound with antibacterial activity produced by the fungus Cephalosporium. Cephalosporin C was limited by its generally weak antibiotic activity. The improvement of activity was developed by changing enzymatic cleavage of the aminoadipoyl side chain, 7-acylamino group. Cephalosporin C was developed by developing a chemical method to convert cephalosporin C efficiently into 7–aminocephalosporanic acid (7-ACA). The nucleus of 7-ACA has been modified with difference side chains. Modifications at position 7 of the β-lactam ring are associated with changes in antibacterial activity and stability of β-lactamases. Substitution at position 3 of the dihydrothiazine ring affects the metabolism and pharmacokinetic properties of the antibiotics to a greater extent than it affects antibacterial activity. Chemical reacylation of 7-ACA provided several classes of cephalosporins which became clinically available. Classification of cephalosporins by using any criteria such as microbiologic profiles, pharmacokinetic parameters and β-lactamase stability has been proposed. In general classification into four generations according to spectrum of activity is adopted (22).

2.1 First-generation cephalosporins

The compound have a relatively narrow spectrum of activity, being effective primarily against gram positive cocci and have moderate activity against community-acquired *Moraxella catarrhalis*, *E. coli*, *Proteus mirabilis*, *K. pneumoniae*

and being inactive against MRSA and *Enterococcus* spp.. The first generation cephalosporins are consisted of many types of antibiotic includes the parenteral administration such as cefazolin, cephalothin, cephapirin, cephradine and the oral administration such as cefadroxil, cephalexin (27).

2.2 Second-generation cephalosporins

The second-generation cephalosporins are more stable to β-lactamase and have greater activity across gram negative bacilli but less active against certain gram-positive cocci than the first generation agents (22). This generation of compounds can be considered in three groups: the cephalosporins, the cephamycins and the carbacephem. In comparison with the first-generation agent, the cephalosporins of this group include a range of parenteral and oral antibiotic that provide significantly improve activity against *Haemophilus influenzae*, *M. catarrhalis*, *Neisseria meningitidis* and *Neisseria gonorrhoeae* as well as enhanced activity against staphylococci and non enterrococci. Group of these cephalosporins are consisted of many types of antibiotic includes the parenteral administration such as cefamandole, cefonicid, ceforanide, cefuroxime and the oral administration such as cefaclor, cefprozil, axetilare The cephamycins are chemically related to cephalosporins, differing primarily by processing a 7α-methoxy group, which enhances their stability to certain β-lactamase. The cephamycins have inferior activity against staphylococci but active against *Bacteroides* spp., particularly *Bacteroides fragilis*. The cephamycins are consisted of cefmetazole, cefotetan and cefoxitin. The carbacephem is a member of the second-generation cephalosporins. Structurally, it is a 1-carbacephem that is identical to the cephalosporins, except that a methylene group replaces the sulfur atom at position 1 of the dihydrothiazine ring, resulting in a tetrahydropyridine ring. The carbacephemhas comparable activity to this group of cephalosporins against *S. aureus*, *Streptococcus pneumoniae* and *Streptococcus* spp but has a modestly increased activity against *H. influenzae* and *M catarrhalis*. The member of semi synthetic carbacephem antibiotic is loracarbef (27).

2.3 Third-generation cephalosporins

Third-generation cephalosporins are more active than the first and second-generation drugs against gram negative organisms. These drugs were introduced primarily to overcome the shortcomings of the earlier cephalosporins.

Thus, they have a broader antibacterial spectrum, increased resistance to β-lactamase inactivation, a better penetrability through the porins and are less toxic. They are highly active against most strain of facultative enteric gram-negative bacilli including *E. coli, P. aeruginosa, Klebsiella* spp., *P. mirabilis, Citrobacter* spp., *Providencia* spp. and *Serratia marcescens.* In addition, they have potent activity against *S. pneumoniae, Streptococcus pyogenes* and other streptococci including *H. influenzae, N. meningitidis, N. gonorrhoeae* and *M. catarrhalis.* Their additional advantages in clinical situation that are not satisfactorily addressed by the earlier cephalosporins. The member of third-generation cephalosporins antibiotics are consisted of the parenteral administration such as cefoperazone, cefotaxime, ceftazidime, ceftrizoxime, ceftriaxone and the oral administration such as cefdinir, cefixime, ceftibuten, cefpodoxime, proxetil. Particularly, cefoperazone and ceftazidime are the only two cephalosporins of this generation with significant antimicrobial activity against *P. aeruginosa* (27).

2.4 Fourth-generation cephalosporins

They have been classified as fourth generation cephalosporins because their potentics against members of the *Enterobacteriaceae* are higher than those of the earlier broad-spectrum cephalosporins. In addition, both most antibiotics remain effective against β-lactamase-overproducing gram negative strain resistant to other expanded-spectrum cephalosporins. There are only two antibiotics in these generation including cefepime and cefpirome that have been approved for clinical use. The fourth generation cephalosporins are characterized by a quaternary ammonium substituent at C-3' of the cephem nucleus. Cefepime has a N-methylpyrrolidine group and cefpirome has a cyclopentane-pyridinium group. The quaternary ammonium group provides one positive charge in the molecule, and since there is one carboxy group at position 4, these cephalosporins are in a Zwitterionic form (one positive and one negative charge in the same molecule). Permeability plays a key part in β-lactam antibiotic activity in gram-negative bacteria, since the β-lactam molecule have to penetrate the outer membrane before they reach their target at the surface of the bacterial inner membrane. Cefepime and cefpirome possess increased penetration through the porin channels, which is more rapid than that of the third-generation cephalosporins (27).

3. Monobactam

The first monobactams to be discovered were naturally occurring compounds isolated from bacteria (e.g. *Gluconobacter* spp., *Acenetobacter* spp. and *Chromobacterium* spp.) but they exhibited poor antimicrobial activity. These naturally occurring monobactams are characterized by the 2-oxoazetidine-1-sulfonic acid moiety with an acyl side chain at the 3 position with the β-orientation which most of monobactams is 3-α-methoxy group. Aztreonam is the only member of the monobactam subgroup of β-lactam antibiotic. It is a totally synthetic monabactam. It was rationally designed because of the successful structure-activity relationships developed for third-generation cephalosporins, such as the amino-thiazol-2-caboxy-2-oxypropaneimino group for ceftazidime. Adding a 4-α-methyl group as the substituent enhances the stability of the ring to β-lactamases catalytic hydrolysis. Aztreonam exhibits potent and specific activity against the wide range of β-lactamase-producing and non-producing aerobic gram negative bacteria, including *P. aeruginosa*. It causes minimal inhibition of gram positive bacteria and anaerobes. The exceptional activity of aztreonam appears to be its high and somewhat exclusive affinity for essential PBP3 of gram negative bacteria. As a sequence of this interaction, bacterial cell division is inhibited to cell death (27).

4. Carbapenem

The carbapenems are a subgroup of β-lactam antibiotics with a common carbapenem nucleus. Carbapenems are broad-spectrum antibiotics with activity against gram positive and gram negative bacteria. A member of a new group containing a 1-carbapenem ring system was reported in 1976. This compound was named thienamycin and was isolated from fermentation broths of the soil microorganism Streptomyces cattleya. Thienamycin has an aminoethylthio substituent at the C-2 and a 1-hydroxyethyl at C-6 of the carbapenem nucleus. It should be noted that it has a trans-substitued configuration at C-5 and C-6 of the carbapenem nucleus. Carbapenems are characterized by the presence of bicyclic ring system which processes a double bond at C-2 and C-3 in the five-member ring. It was a novel β-lactam antibiotic of particular interest because of its exceptional antibacterial potency and spectrum, including activity against Pseudomonas spp. and β-lactamase-producing species. Carbapenems are also the drugs of choice for extended-spectrum

beta-lactamase–producing organisms. It has been suggesting that this activity is due to the combined effects of good penetration into gram negative bacteria via a specific carbapenem uptake pathway involving the OprD porin channel, good stability to hydrolysis by most β-lactamases and strong binding to essential penicillin binding protein. The member of carbapenems antibiotics are consisted of imipenem, meropenem, ertapenem and doripenem (27).

4.1 Imipenem

This antibiotic is the N-formimidoyl derivative of thienamycin. It has excellent *in vitro* activity against aerobic gram positive species, including *Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp. (*Enterococcus faecalis* is usually susceptible but *Enterococcus faecium* isolates are usually resistant). Among gram negative bacteria, the imipenem spectrum includes most members of *Enterobacteriaceae* and also has excellent activity against *N. meningitidis, N. gonorrhoeae* and *H. influenzae*, including β-lactamase producing strains. Imipenem is very active against *Acinetobacter* spp. strains and its *in vitro* potency againsts *P. aeruginosa* (27).

4.2 Meropenem

Meropenem is a β-lactam antibiotic belonging to the carbapenem subgroup. It possesses a 1-β-methyl group on the carbapenem nucleus and a substituted 2' side chain. It exhibits potent antibacterial activity against a wide range of gram-positive and gram-negative bacteria. Meropenem is slightly more active against gram negative organism and also highly active *in vitro* against *Listeria monocytogenes*. It has moderate activity against β-lactamase-resistant in *Streptococcus pneumoniae* (27).

4.3 Ertapenem

Ertapenem is structurally very similar to meropenem in that it possesses a 1-β-methyl group on the carbapenem nucleus. It retains activity against most *Enterobacteriaceae* producing β-lactamases. It is marketed by Merck as a first-line treatment for community-acquired pneumonia infections (27).

4.4 Doripenem

Doripenem is the newest antibiotic agent in this group. It was recently approved by the Food and Drug Administration for the treatment of complicated intra-abdominal infections and complicated urinary tract infections. It has a broad spectrum againsts both gram positive and gram negative bacteria. It most closely resembles the profile of meropenem, except for its increased potency of *in vitro* activity against *P. aeruginosa* (27).

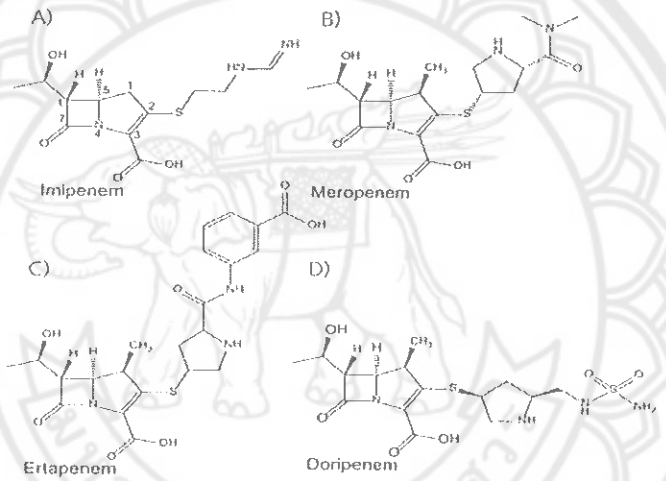


Figure 5 The structure of the carbapenems antibiotic (18)

β-lactamase inhibitors

B-lactamase inhibitors block the activity of β-lactamase enzymes. These compounds are very slowly released after attack of the ß-lactamase. The strategy for combating β-lactamase-mediated resistance is the use of agents designed to bind at the active site, which are frequently β–lactams ring. This strategy can take two forms that are create substrates that reversibly and irreversibly bind the enzyme with high affinity but form unfavorable steric interactions as the acyl-enzyme or develop mechanism-based or irreversible "suicide inhibitors". This protects the penicillins from degradation and expands their utility. Examples of these inactivators are the commercially available clavulanate, sulbactam, tazobactam, and avibactam. These are

β-lactamase inhibitors that have little intrinsic antibacterial activity but inhibit the activity of a number of plasmid-mediated β-lactamases (28). They generally do not inhibit chromosomally mediated β-lactamases (29). Combination of these agents with ampicillin, amoxicillin, ticarcillinand and piperacillin results in antibiotics with an enhanced spectrum of activity against many, but not all, organisms containing plasmid-mediated β-lactamases. In addition, these compounds inhibit the chromosomal β-lactamase of many *Bacteroides* spp., extending the spectrum of coverage for these organisms as well. There are β-lactamase inhibitors combinations using in clinical practice by following (25).

1. **Amoxicillin-clavulanate**

This compound has an activity inhibit most strains of oxacillin-sensitive *S. aureus* and β-lactamase producing *H. influenzae* (30).

2. **Ampicilin-sulbactam**

It is a parenteral formulation that expands the spectrum of ampicillin to include most strains of *S. aureus* and β–lactamase producing *H. influenzae* and some *Enterobacteriaceae* (31).

3. **Ticarcillin-clavulanate**

It is expand the spectrum of the respective penicillins to include β–lactamases producing *S. aureus, H. influenzae, N. gonorrhoeae* and some *Enterobacteriaceae* (32).

4. **Piperacillin-tazobactam**

Piperacillin-tazobactam is also expand the spectrum of the respective penicillins with an activity inhibit the strain of β–lactamase *S. aureus, H. influenzae, N. gonorrhoeae* some *Enterobacteriaceae* and anaerobe (32).

Antibiotic resistance

Antibiotic resistance is the ability of bacteria to resist the effects of antibiotic agent that was originally effective for treatment of infections. Resistance to antibiotic agent in bacteria is a natural phenomenon that has existed since before they were introduced into human or veterinary medicine, because most of the classes of compounds used clinically are produced by microorganism in the environment and bacteria use to against the other. Under these circumstance leading to a low-level of

natural selection for resistance to antibiotics, resulting in a selective pressure for the survival of resistant strains of bacteria. However, the current higher-levels of antibiotic-resistant bacteria are attributed to the increasing clinical use of antibiotic overuse and abuse of antibiotics that have been used so widely and for so long. Prolonged therapy with antibiotics can lead to the development of resistance in a microorganism that initially is sensitive to antibiotics, but later it can adapt gradually and develop resistance to antibiotics (33). The development of a phenotype resistant to antibiotic agents depends on various factors such as drug factors : the dosage regiment, the duration of therapy, the type of antibiotic mechanism include pharmacokinetic factor in patient, the mechanisms of resistance factors in microorganism and also rapidly changing populations which are related to human population and environmental factors (34).

Mechanism of resistance to antibacterial agent

Antimicrobial resistance in bacteria is a phenomenon resulting from biochemical processes that are encoded by bacterial genes. It arises by the general mechanism of resistance as follow.

1. Genetics of antibiotic resistance

Expression of bacterial resistance to antibiotics by the chemical processes, are general encoded by bacterial genetic loci associated with antibiotic resistance. For some types of resistance there is a large diversity of responsible genetic determinant. For this reason, it is importance to discuss the genetic underpinnings of resistance and its evolution. Bacterial resistance to antibiotics can be intrinsic, which intrinsic resistance is the innate ability of a bacterial species to resist activity of a particular antibacterial agent through its inherent structural or functional characteristics that allow tolerance of a particular drug or antimicrobial class. Intrinsic resistance can be due to the lack of affinity of the drug to the target of bacterial, loss accessibility of the drug into the bacterial cell, chromosomal encoded active transporters for drug extrusion and innate substance production to inactivate the drug (35). Genetics aspect of antibiotic resistance can also be acquired resistance, occurs when a particular microorganism obtains the ability to resist the activity of a particular antimicrobial agent to which it was previously susceptible. This can result from a mutation of

cellular genes, the acquisition of exogenous resistance genes or a combination of these two mechanisms. Therefore, there are two main reason of acquiring antibiotic resistance include (i) the mutation in difference chromosomal loci involved cellular genes and exogenous resistance genes (ii) through horizontal gene transfer (36).

1.1 Mutation

Spontaneous mutation is rarely found and occur randomly as error of replication or an incorrect repair of damaged DNA in actively dividing cell without of any selective pressure. This mutation also known as growth dependent mutation, which plays an important mode of generating antibiotic resistance. Furthermore, bacteria with an elevated mutation rate which during prolong non-lethal antibiotic selective pressure in a small bacteria population enters a transient state of a high mutation rate are currently accepted as hypermutable state. If a cell in this hypermutable state achieves a useful mutation, thus relieving the selective pressure from antibiotic, the cell begins to grow and reproduce at the same time exits the hypermutable state (33). Even though the most of mutation occur as the consequence of error during the DNA replication process or an incorrect repair of damaged DNA. However, the recent study has shown that mutation can also arise in non-dividing or slowly dividing cells and have some relation to the selective pressure used. These mutations called adaptive mutations occur only during non-lethal selective pressure. This is the main feature that distinguishes them from the growth dependent mutation. The adaptive mutation process is one of the main sources of the antibiotic resistant mutants to under originate under normal condition (33).

1.2 Horizontal gene transfer

Spreading of antibiotic resistance can occur by acquiring of foreign genetic material that transfer resistance genes from one bacterium to another called "horizontal gene transfer" (37). The principle mechanisms of resistance gene transfer in bacterium from a donor cell to a recipient cell are conjugation, transformation and transduction. Resistance genes can be incorporated into the recent chromosome by recombination (33). Recombination can bring about large change in the genetic material. These genes may contain single mutation or more severe sequence changes include antibiotic resistance gene. When new genotype arise by genetic material transform, they are likely to be expressed phenotypically (38). Conjugation is a type of

bacterial mating in which DNA is transferred from one bacterium to another by conjugative plasmid. Conjugative plasmid is dependent upon the *tra* genes that encode instruction for the bacterial cell to produce a sex pilus to allow cell-to-cell contact plasmid transfer from donor cell to a recipient (38). Most plasmid is double-stranded circular DNA, which more effective transfer resistance genes than chromosomal mutation. (36).

Some bacteria are naturally competent cell, which can be transformed by free DNA present in their environment in transformation process. They take up DNA fragment of related species from lysis of other organisms across their cell walls. Ones taken into the cell, chromosomal DNA must recombine with a homologous segment of the recipient chromosome to be stably maintained and inherited. Especially, plasmid DNA may be transformed into a cell and express without recombination. Thus, DNA fragment that encoded antibiotic resistance genes can be uptake by competent cell and express the feature of genes encoding. The other important mechanism is transduction; transduction involves the transfer of genetic material by infection with a bacteriophage. The replication process of bacteriophage, other DNA in the cell is packaged into the virus head that can transfer the DNA into a recipient cell with or without recombination into recipient genome as transformation (38).

As mentioned above, horizontal gene transfer of resistance genes is a mechanism for the dissemination of multiple drug resistance because resistance genes can be found in cluster and transfer together to the recipient (36).

2. Biochemistry of antibiotic resistance

The mechanism of antibiotic resistance from biochemical process has been a significant mechanism in bacteria. There are various biochemical mechanisms that employ the development of drug resistance. Although antibiotic resistance to a particular drug can be achieved by more than one mechanism and vary among bacteria species, resistance is created by a major mechanism in one microorganism (39). The main types of biochemical mechanisms that bacteria use for defense are as follow.

2.1 Decreased permeability

Most gram negative bacteria are employed the resistance by this mechanism because, gram negative bacteria possess an outer membrane consisting of an inner layer containing phospholipids and an outer layer containing the lipid A moiety of lipopolysacharides. Drug molecule can penetrate though outer membrane by several modes, which depends on its chemical composition. In consequence of this main composition of outer membrane, drug will be slowed down penetrate into the cell and inhibited the transport across outer membrane by reduce outer membrane permeability (33).

2.2 Modification of the target molecule

The interaction of antibiotic with target molecule is generally quite specific so even small changes in a target molecule can influence antibiotic binding to a target or cannot bind properly (36). However, in the presence of modification in the target, it is possible for mutational changes or other changes in the cell to compensate and retaining the cellular function. (33).

2.3 Metabolic bypass of antibiotic inhibition

Microorganisms develop an altered metabolic pathway that alteration target is resistant to inhibition of antibiotic. At the same time, bacteria as well produce a native target, which is sensitive to antibiotics. An alteration target allows bacteria to survive by adopting the role of native protein, for that reason the cell is not affected by drug against (40).

2.4 Efflux pump

The efflux pump is membrane protein that export antibiotic from the cell and maintain their low intracellular concentration in bacterial cell (33). Several classes of pumps in bacteria may be quite selective or they may have broad substrate specificity. The majorities of these pumps are located in the cytoplasmic membrane and use proton motive force to drive drug efflux. Efflux pump can be a single component or multicomponent efflux systems, they both transfers their substrates across the cytoplasmic membrane (41). They can be specific to antibiotic and most of them are multidrug transporters that are capable to pump a wide range of unrelated antibiotics. For that reason, it is significantly contributed to multiple drug resistance (33).

2.5 Antibiotic inactivation or modification

The bacterial defense mechanisms within the category of antibiotic inactivation by destroying the active component of the antimicrobial agent can occur via several mechanisms. Many antibiotic inactivations includes the production of enzymes that degrade or modify a chemical structure of drug. Biochemical strategies are group transfer, redox mechanism and hydrolysis. Antibiotic inactivation by group transfer is an enzyme-mediated resistance, which the most diverse family of resistant enzymes is the group of transferases and inactivate antibiotic by chemical substitution. Therefore, the inactivated antibiotics are affected in their binding to a target (33). Antibiotic inactivation by redox process is a type of oxidation or reduction of antibiotic. However, there are a few of studies of this strategy (41). The last mechanism is Antibiotic inactivation by hydrolysis, which many antibiotics have hydrolytically susceptible chemical bonds. Several enzymes can destroy antibiotic activity by targeting and cleaving these bonds. Bacteria can excrete these enzymes to inactivate the drug before they reach their target in the cell (42). There are three main enzymes that inactivate antibiotic included β-lactamases, aminoglycoside-modifying enzymes and chloramphenicol acetyltransferases (43).

Resistance to β-lactam antibiotic

Bacteria have four common defense mechanism against β-lactam antibiotics includes destruction of the antibiotic by β-lactamases, decreased affinity of the PBP to the antibiotic, decreased penetration through the porin of the antibiotic to reach the target site and increased efflux pump activity for antibiotic extrusion.

1. Production of β-lactamase enzymes

The production of β-lactamases is a major mechanism of β-lactam resistance, particularly amongst gram-negative bacteria. β-lactamases are members of a superfamily of active-site serine proteases or D,D-peptidases, hydrolytic enzymes comprised of short chains of amino acids that disrupt the amide bond of the characteristic four-membered β-lactam ring cause the antimicrobial effective (44). These enzymes produced by bacteria of both gram-positive and gram negative bacteria. Their productions are variably chromosomally or plasmid encoded, often associated with mobile genetic element such as transposons and integrons (45).

2. Alteration of Penicillin-Binding Protein (PBPs)

The target sites for the β-lactams are the PBPs in the cytoplasmic membrane, the transpeptidases that manufacture peptidoglycan. The interaction of β-lactam molecules know as autolysins, which rupture the cell, leading to cell death. Therefore, the alterations in PBPs may influence their binding affinity for β-lactam antibiotics and the sensitivity of the altered bacterial cell to inhibition by these antibiotics. PBP-mediated resistance for bacteria takes several forms, including overproduction of a PBP, acquisition of a foreign PBP with low affinity, recombination of a susceptible PBP with more resistance verities, and point mutations with PBPs that lower affinity for the β-lactam antibiotic. This mechanism is predominantly found in gram-positive bacteria. Such a mechanism is responsible for MRSA that provided resistant to β-lactam antibiotic includes cephalosporins, monobactams and carbapenems (23).

3. Decreased expression of outer membrane protein (OMPS)

The entry of hydrophilic antimicrobials such as β-lactams into gram negative bacteria occurs via channels in the outer membrane formed by porins (46). Reduction the outer membrane permeability to β-lactams by porin loss of or changes in porin structure can promote resistance to these agents. Porin deficiency is an important determinant of carbapenem resistance or reduced susceptibility too, particularly in Pseudomonas aeruginosa where loss or mutation of the OprD porin is common in carbapenem, especially imipenem-resistant strains (47).

4. Efflux pumps

Efflux is a mechanism of antimicrobial resistance that extrudes one or more antibiotic classes from bacterial cells. Several classes of pumps have been described for gram positive and gram-negative bacteria. They may be quite selective or may have broad substrate specificity. The majority family of efflux pump that found in gram negative bacteria is Resistance Nodulation Division (RND), including AcrAB-TolC of *Enterobacteriaceae* and MexAB-OprM of *P. aeruginosa* (23).

β-lactamases

β-lactamases are enzymes produced by both gram positive and gram negative bacteria. These enzymes are members of a superfamily of active-site serine proteases

or D,D-peptidases that inactivate antibiotic by hydrolytic cleavage the β-lactam ring of β-lactam antibiotics. β-lactam antibiotics are a class of broad-spectrum antibiotics that use in bacterial infection treatment active against gram positive bacteria and various gram negative bacteria. For this reason, Resistance to β-lactam antibiotics by production of β-lactamase enzyme has become a major public health problem.

1. Classification of β-lactamases

Classification of β-lactamases has two major schemes are traditional use to classify the enzymes. The classification has been based on either the functional characteristics of the enzymes (5) or their primary structure (48).

1.1 Molecular classification (Ambler classification)

The molecular classification of β-lactamases is based on the nucleotide and amino acid sequences in these enzymes and divided into four molecular classes are recognized classes A, B, C and D based on conserved and distinguishing amino acid motifs correlating with the functional classification. Classes A, C, and D utilized serine for β-lactam hydrolysis and Class B metalloenzymes which require divalent zinc ions for substrate hydrolysis (3).

1.1.1 Class A β-lactamases

Class A β-lactamases possess four important structural motifs that create a complex hydrogen-bonding network to fix the β-lactam in the substrate-binding pocket. Residues Ser70-Xaa-Xaa-Lys73, Ser130-Asp131-Asn132 and Lys/Arg234-Thr/Ser235-Gly236 define the conserved residues critical for β-lactam binding and hydrolysis. The Ω loop (amino acids Arg164 to Asn179) is unique in class A β-lactamases. A highly conserved Glu166 that functions as a general base (electron donor) in the catalytic process is located in the Ω loop. The two commonly encountered class A β-lactamases found in *Enterobacteriaceae* are designated TEM-1 and SHV-1, TEM-1 and SHV-1 β-lactamases are primarily penicillinases with diminished activity against cephalosporin substrates (23).

1.1.2 Class B β-lactamases

Class B β-lactamases are metalloenzymes that contain αββα motif with a central β-sandwich and two helices on each side. Consequently, class B β-lactamases require zinc or another heavy metal for catalysis and their activities are inhibited by chelating agents for example ethylenediaminetetraacetic (EDTA) and

dipicolinic acid. This zinc atom is held in place by three histidines and a water molecule. Some metallo-β-lactamases contain a second Zn^{2+} binding site. These two sites function separately, with the primary Zn^{2+} binding site assisted by the secondary site. A coordinated water molecule also plays a critical role in catalysis (23). Because of metallo-β-lactamases began to appear on transferable elements, so they became more promiscuous and were subject to evolutionary pressures in a variety of hosts, resulting in enzyme families with several dozen unique variants. Class B β-lactamases can be grouped into three different subclasses depending on their requirements for zinc as follows.

Subclass B1

Subclass B1 enzymes contain the largest number of metallo-β–lactamases and share more than 23% identity. For this subclass, catalysis is proposed to be associated only with the di-zinc enzyme. Enzyme catalysis initiates with the binding of the β-lactam at the metal center with the carbonyl oxygen interacting with Zn1 and the carboxyl group on the 5-or 6-membered fused ring bound to Zn2 (49). The hydroxide ion is stabilized by Zn1 and Zn2 and resides between the metal ions in a position to attack the carbonyl carbon. These enzymes include the clinically important and transferable IMP, VIM and NDM type enzymes (50).

Subclass B2

The subclass B2 β-lactamases differ from B1 and B3 enzymes in that they contain one zinc in the active site and they display a narrow substrate spectrum focused almost exclusively on carbapenem hydrolysis (51). Enzyme reaction mechanisms of subclass B2 for biapenem have been proposed based on the structures. The mechanisms have common features, are an interaction between Zn2 and the conserved Lys224 residue with the carbapenem β-lactam C-3 carboxyl group. In addition, a water molecule is held by interactions with His and Asp residues and one of these could act as a general base to activate water for attack on the C-7 carbonyl carbon of the substrate (50).

Subclass B3

These subclass enzymes are similar to subclass B1 and the mechanisms have common features in that they have activity against cephalosporins.

They contain two zinc in the active site. Subclass B3 is usually plasmid mediated, those found in *Stenotrophomonas maltophilia* (50).

1.1.3 Class C β–Lactamases

Class C β-Lactamases are also known as Class C serine cephalosporinases, commonly found in extended-spectrum cephalosporin - resistant gram negative. AmpC type β-Lactamases usually encoded by genes located on the bacterial chromosome of many gram negative bacteria including *Citrobacter* spp., *Serratia* spp. and *Enterobacter* spp., where its expression is usually inducible, although AmpC enzymes are more prevalent by plasmid-borne. Production of chromosomal AmpCs in gram negative bacteria is repressed at a low level but can be derepressed by induction with β–lactams explosure (23).

1.1.4 Class D β–Lactamases

Class D β–lactamases or Class D serine oxacillinases were initially categorized as oxacillinases because of their ability to hydrolyze oxacillin at a rate of at least 50% of that of benzylpenicillin, in contrast to the relatively slow hydrolysis of oxacillin by classes A and C (8). In gram negative bacteria OXA enzymes confer resistance to a wide variety of penicillins, cephalosporins, extended-spectrum cephalosporins (OXA-type ESBLs) and carbapenems (OXA-type carbapenemases) (23). In additional study, sodium chloride at concentrations of greater than 50 to 75 mM inhibits some carbapenem hydrolyzing oxacillinases such as OXA-25 and OXA-26 (52).

1.2 Functional classification

The functional classification provides the opportunity to relate these varied enzymes to their clinical role, based on their ability to hydrolyze specific β-lactam classes and on the inactivation properties of the β–lactamases inhibitors by providing selective resistance to different classes of β-lactam antibiotics (53). It scheme proposed initially by Bush in 1989 and updated herein is based on the 1995 proposal by Bush and George A. Jacoby, Antone A. Medeiros (54). Enzyme grouping has been taken into account substrate and inhibitor profiles that can be correlated with their phenotype in clinical isolates. The functional classification generally correlates with the more broadly based molecular classification. The major grouping updated system includes group 1 cephalosporinases, group 2 broad-spectrum, inhibitor-

resistant, and extended-spectrum β-lactamases, serine carbapenemases and group 3 metallo-β–lactamases. (53).

1.2.1 Group 1 cephalosporinases

These enzymes are belonging to molecular class C that generally encoded on the chromosomes of many *Enterobacteriaceae* family and a few other organisms. Group 1 cephalosporinases are more active on cephalosporins than benzylpenicillin, they are usually resistant to inhibition by clavulanic acid and active on cephamycins (53).

1.2.2 Group 2 serine β-lactamases

This group is the largest group of β–lactamases including molecular classes A and D. Group 2 serine β-lactamases are divided into each subgroup as follows.

Subgroup 2a

This subgroup is known as penicillinases, that represent a small group of β–lactamases. These enzymes preferentially hydrolyze benzylpenicillin and many penicillin derivatives, with poor hydrolysis of cephalosporins, carbapenems, or monobactams. They are also inhibited by clavulanic acid and tazobactam. (53).

Subgroup 2b

This subgroup hydrolyzes penicillins and narrow-spectrum cephalosporins but could be strongly inhibited by clavulanic acid and tazobactam. The majority of these enzymes are plasmid-mediated, that cause the widespread increase of β–lactamases producing bacteria , for example TEM and SHV genes, group of TEM are commonly found in *Enterobacteriaceae* family and SHV in *K. pneumoniae* (53).

Subgroup 2be

These enzymes retain the activity against penicillins and extended-spectrum cephalosporins, that similar to subgroup 2b β-lactamases and also hydrolyze one or more oxyimino-β-lactams, such as cefotaxime, ceftazidime, and aztreonam. Because of hydrolytic activity, this subgroup 2be is represented extended-spectrum β-lactamase (ESBLs) (53).

Subgroup 2br

These broad-spectrum β-lactamases generally have acquired resistance to clavulanic acid and related inhibitors or known as inhibitor-resistant β-lactamase while retaining a subgroup 2b spectrum of activity. Include some ESBLs with relative resistance to clavulanic acid inhibition (53).

Subgroup 2c

This subgroup is also penicillinases that have an ability to hydrolyze carbenicillin or ticarcillin as rapidly as benzylpenicillin. These enzymes are generally easily inhibited by clavulanic acid or tazobactam. Because the major ability to hydrolyze carbenicillin, so that subgroup 2c is represented as carbenicillinase (CARB). The recent study has described extended-spectrum carbenicillinase or CARB-10 with expanded activity against cefepime and cefpirome therefore, this new enzyme has formed into subgroup 2ce (55).

Subgroup 2d

The enzymes of this subgroup were distinguished by their ability to hydrolyze cloxacillin or oxacillin at a rate of greater than 50% that for benzylpenicillin thus, they are known as OXA enzymes. In addition, many β-lactamases in this subgroup are inhibited by NaCl. They have various spectrum of activity to hydrolyze β-lactam, now OXA-related enzymes comprise the second largest family of β-lactamases. In the new subgroup 2de are cloxacillin or oxacillin-hydrolyzing enzymes with an extended spectrum that includes oxyimino-β-lactams but not carbapenems. The majority of 2de enzymes are derived from OXA-10 by between 1 and 9 amino acid substitutions and include enzymes such as OXA-11 and OXA-15. Another new subgroup is 2df β-lactamases are OXA enzymes with carbapenem-hydrolyzing activities (53). They appear most frequently in *Acinetobacter baumannii* and usually produced by genes that are located on the chromosome (56), although plasmid-borne OXA-23 and OXA-48 enzymes have been identified in the *Enterobacteriaceae* (2).

Subgroup 2e

This subgroup is also cephalosporinases that have an ability to hydrolyze extended-spectrum cephalosporins and to be inhibited by clavulanic acid or tazobactam. Their activity similar to ESBLs in subgroup 2be enzymes, but can be

differentiated from that subgroup by their poor affinity for penicillins and monobactams (53).

Subgroup 2f

Subgroup 2f is serine carbapenemases from molecular class A β-lactamases. They have ability to hydrolyze carbapenem, which can be inhibited better by tazobactam than by clavulanic acid. Furthermore, these enzymes are plasmid-mediated β-lactamases including KPC and some GES in particular have recently been associated with major outbreaks of multidrug-resistant gram negative infections, particularly in *Enterobacteriaceae* with their spread now becoming worldwide (57).

1.2.3 Group 3 (Metallo-β-lactamases)

Metallo-β-lactamases differ structurally from the other β-lactamases by their requirement for a zinc ion or another heavy metal for catalysis at the active site. These enzymes can be distinguished by their ability to hydrolyze carbapenems, but some serine β-lactamases now have also acquired that ability (53). On the contrary, serine β-lactamases, the MBLs have poor affinity or hydrolytic capability for monobactams and are not inhibited by clavulanic acid or tazobactam. Their activities are inhibited by chelating agents for example EDTA and dipicolinic acid or 1,10-o-phenanthroline (58). These metalloenzymes have been subdivided based on function as subgroups 3a, 3b, and 3c. The increasing numbers of Metallo-β-lactamases be described by the major plasmid-encoded MBL families, particularly in subgroup 3a such as the IMP, VIM and NDM enzymes that have spread now worldwide and most frequently in *Enterobacteriaceae* (53)

1.2.4 Group 4 β-lactamases

These enzymes are penicillinases that are not inhibited by β-Lactamase inhibitor. They have as yet been incompletely characterized and most likely would be included in one of the existing enzyme groups if more information about them were available (53).

Table 1 Classification schemes for bacterial β-lactamases, based on functional classification and molecular classification (53).

Molecular class	Functional class	Representative enzyme	Main attribute
A	2a 2b 2c	TEM-1, TEM-2 SHV-1	Hydrolyzes penicillins, cephalosporins, Penicillinase, inhibit by β-lactamase inhibitors.
	2be	ESBLs e.g. TEM, SHV Except TEM-1,TEM-2, SHV-1	Extended spectrum β-lactamase inhibit by β-lactamase inhibitors
	2br	TEM-30 TEM-31	Similar hydrolysis of ESBLs and resistance to inhibition by β-lactamase inhibitors
	2e	CTX-M	Cephalosporinase inhibition by clavulanate and similar hydrolysis of ESBLs
	2f	NmcA, IMI, SME GES, KPC	Increased hydrolysis of cephamycins, carbapenems combined with resistance to clavulanate
B	3a 3b 3c	MBLs e.g. IMP, VIM NDM, CcrA, L1	Zinc-dependent β-lactamase ,similar hydrolysis of subgroup 2f with resistance to β-lactamase inhibitors
C	1	AmpC	Similar hydrolysis of ESBLs with increased hydrolysis of cephamycins and β-lactamase inhibitors
D	2d	Oxacillinase	Oxacillin-hydrolysing and increased hydrolysis of β-lactamase inhibitors
		OXA-23, OXA-24/40, OXA-48, OXA-58	Similar hydrolysis of subgroup 2f
-	4	Miscellaneous enzyme	Incompletely characterized

Carbapenemase

Carbapenemase represent broad-spectrum of activity and the most versatile family of β-Lactamase. Although carbapenemase are known as carbapenem-hydrolyzing enzyme, these enzymes have ability to hydrolyzed almost all β-lactam antibiotics including penicillins, cephalosporins, monobactams, carbapenems and most are resistant to β-lactamase inhibitors (4). Carbapenemases are members of the molecular class A, B, and D β-lactamases. There are increasing numbers of serious infections in which the carbapenemase activity renders many β-lactams ineffective by carbapenemases-producing bacteria, particularly the emergence of this group of β-lactamases in the *Enterobacteriaceae* (51). Carbapenemases are broadly divided into two types based on molecular class include serine β-lactamase and metallo-β-lactamases as follow.

1. Serine β-lactamase

Serine carbapenemase are divided into 3 classes include class A, C and D as follows.

1.1 Class A carbapenemase

This class is serine carbapenemase related to group 2f of functional classification. These β-lactamases class have been detected in *Enterobacter cloacae, S. marcescens,* and *Klebsiella* spp. (60). The major of class A serine carbapenemases are divided into chromosomally encoded enzyme including SME, NMC and IMI enzyme and plasmid-encoded enzyme including KPC and GES (51).

1.2 Class C carbapenemase

Class C carbapenemase related to group 1 of functional classification. The first class C carbapenemase was isolated from a virulent strain of *Enterobacter aerogenes*. Normolly, they have poor affinity or hydrolytic capability for carbapenems. However, the mutations of these enzymes improve their activity against imipenem. These enzyme include CMY-10 and BER, that differ from AmpC β-lactamase with their extend spectrum of activity (51).

1.3 Class D carbapenemase

The OXA β-lactamase with carbapenemase activity is commonly found in *P. aeruginosa* and *Enterobacteriaceae* family. These enzymes have ability to hydrolyzed oxacillins and cloxacillins. They are inhibited by NaCl. The group of

OXA-type carbapenemase found in *A. baumannii* have an extend spectrum of activity to against imipenem (52).

2. Metalloβ-lactamase

This class of β-lactamases is characterized by the ability to hydrolyze carbapenems and by its resistance to the β-lactamase inhibitors but susceptibility to inhibition by metal ion chelators. The first metallo-β-lactamase detected were chromosomal enzyme present in *Bacillus cereus*, *Aeromonas* spp. and *S. maltophilia* (49).

Epidemiology of carbapenemase-producing *Enterobacteriaceae*

The emergence and global spared of carbapenemase producers in *Enterobacteriaceae* has been reported worldwide. Carbapenemase was first description in *Enterobacteriaceae* in 1993 (60). Since then, these enzymes have been emerged to different parts of the world. They are mostly identified belonging to main molecular classes includes class A, B and D β-lactamase (51). Carbapenemase are carried either on chromosome or acquired via plasmid mediated resistance (61).

1. **Class A carbapenemases**

The first carbapenemase was identified over 20 years ago in an *E. cloacae* isolate (63). This class has also been described both chromosomal encoded such as NmcA, Sme, IMI-1 and SFC-1 and plasmid encoded such as KPC, IMI-2, GES (60). KPC are frequently encountered enzymes and currently the most clinically significant enzymes in this group (63). The first report has been identified from a clinical *K. pneumoniae* strain in 1996 on the eastern coast of the USA. They confer high levels of resistance not only to carbapenems but also to most β-lactams antibiotic including broad-spectrum cephalosporins. The first KPC producer was identified (64). Several KPC clones are disseminating harboring different multilocus sequence type, *β*-lactamase content, and plasmids. All the same the KPC genes are flanked by the same transposon Tn4401 located on conjugative plasmids and are horizontally transferred (65). This lead to the extraordinary spreading capacity of these enzymes (66). The first outbreak of KPC in Southeast Asia is not well known. Although, China is considered to be a country where some areas are facing endemic situations (67). Moreover, there

are the most commonly identified carbapenemases being NDM and OXA-48-like enzymes in India and also show some KPC producers are occurring as well (68,69).

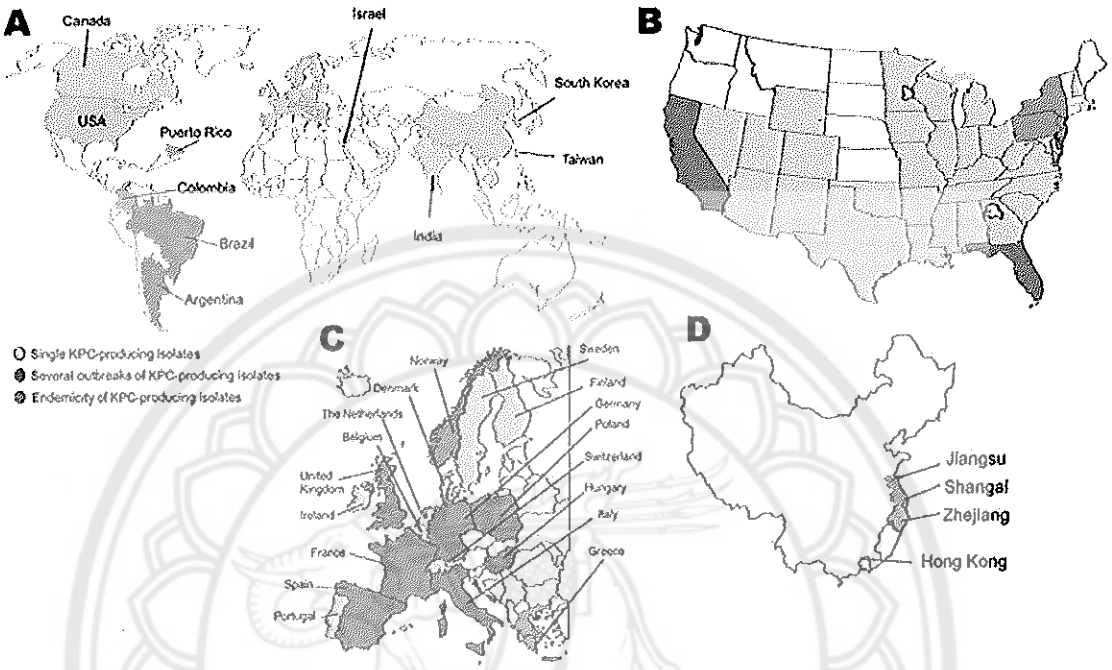


Figure 6 Geographical distribution of KPC producers (63).

2. Class B carbapenemases

Class B metallo-β-lactamases have been identified as acquired enzymes since the early 1990s, either in *Pseudomonas* spp. or in *Enterobacteriaceae* (70). They are mostly of the VIM and IMP types and, more recently, of the NDM-1 type (51). Even though, they have been reported worldwide, the VIM producing *Enterobacteriaceae* are highly prevalent in the southern part of Europe and around the Mediterranean Sea, whereas the IMP producers remain mostly located in Asia (70). VIM family includes 41 variants, which have been found in *P. aeruginosa* and *Enterobacteriaceae*. However, VIM-2 is frequently the most commonly reported MBL worldwide (71). For IMP-type β -lactamases were the first acquired MBLs, reported in *S. marcescens* from Japan in 1991. IMP family has been detected in a series of clinically important gram negative pathogens, such as *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp. (72). Nevertheless, the frequency of IMP-

producing isolates worldwide is much less than that of KPC, VIM, NDM or OXA-48 producers. The wide spread of IMP-type enzymes has been demonstrated mainly in Japan, Taiwan, and eastern China, although there are single reports from many other countries, and isolates producing these enzymes have sometimes caused nosocomial outbreaks.(73). Another types of MBL which one of the most clinically significant carbapenemase is NDM-1. This enzyme was first described in *E. coli* and *K. pneumoniae* in Swedish patient returning from India in 2008 (74). The important reservoir of NDM-producing *Enterobacteriaceae* is the Indian subcontinents. Because, the main spread of NDM producers has been extensively identified among patients from the Indian subcontinent. The prevalence of carriage is estimated to be 5–15% in that part of the world (75). In addition, spreading of NDM producing strain has been identified in the UK and correlated with India and Pakistan (76). Afterward, NDM producing *Enterobacteriaceae* have been reported in almost all of world, including many countries in Asia, Africa, Australia, the Americas, and Europe (77).

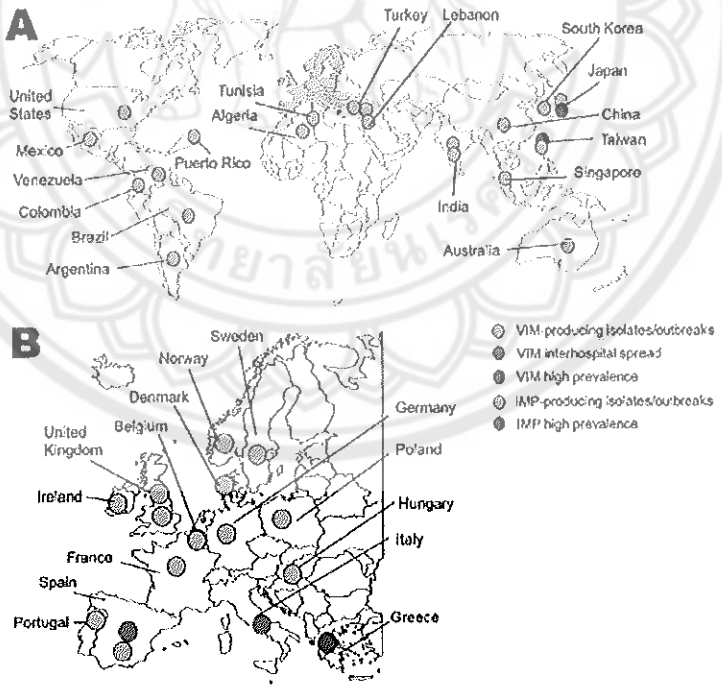


Figure 7 Geographical distribution of Class B Carbapenemases includes, VIM, IMP enterobacterial producer (63).

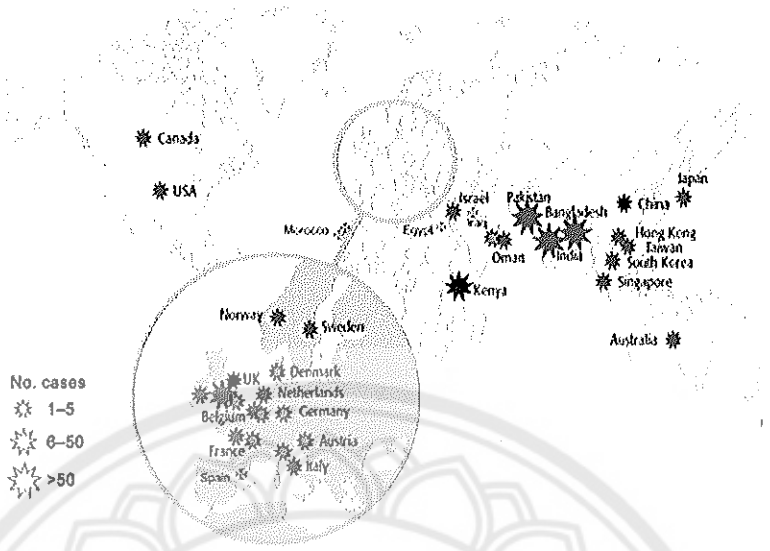


Figure 8 Geographic distribution of New Delhi metallo-β-lactamase-1 producers (July 15, 2011. Star size indicates number of cases reported. Red stars indicate infections traced back to India, Pakistan, or Bangladesh, green stars indicated infections traced back to the Balkan states or the Middle East; and black stars indicate contaminations of unknown origin) (63)

3. Class D carbapenemases

Class D β-lactamases, which are also known as OXAs for oxacillinases with more than 400 variant types (78). Firstly, OXA β-lactamases were reported from *P. aeruginosa* but until now, these enzymes have been detected in many other gram negative bacteria, including *Enterobacteriaceae* (79). OXA-48 and its deriverties is the main enzymes have been identified in *Enterobacteriaceae* and usually isolated worldwide (80). This enzyme was initially found in *K. pneumoniae* isolates from Turkey in 2001 (81). Afterward, OXA-48 producing strains have been extensively reported as sources of nosocomial outbreaks in many countries, notably in Mediterranean countries. A point mutation analog of OXA-48, namely OXA-181 with sharing the similar hydrolytic activity, has been identified in enterobacterial isolate from patient involve the Indian subcontinent. (82).

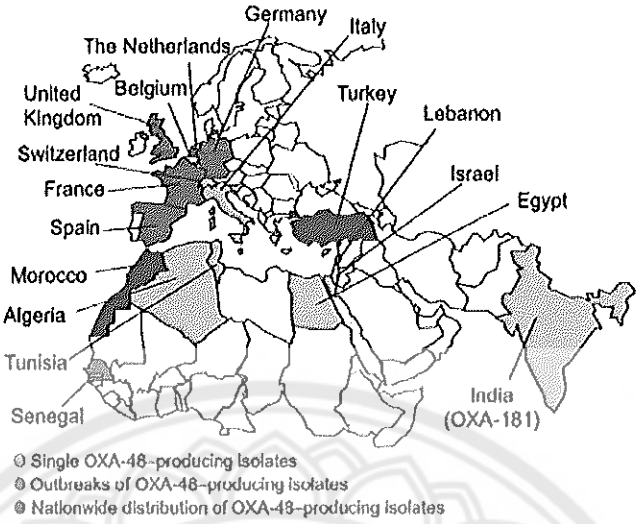


Figure 9 Geographic distribution of OXA-48 type producers (63).

Epidemiology carbapenem-resistant *Enterobacteriaceae* in Thailand

A retrospective study on CRE in Thailand in 2016, documented the prevalence of CRE among *Enterobacteriaceae* clinical isolates at Siriraj Hospital has been increasingly from 0.5% to 2.5 % in range of five years, during 2012 to 2016. The resistance most found in *K. pneumoniae, E. coli* and *Enterobacter* spp., respectively (83). Moreover, the Update of CRE from NARST in 2012 shown the prevalence carbapenem resistance in *P. aeruginosa* and *A. baumannii* were 28.7%, 76.3% respectively. In addition, the prevalence and characteristic of CRE had been increasing 3.5% to 30.7% for a year, during 2012-2013. The surveillance of CRE among clinical isolates of *Enterobacteriaceae* from the hospitals in Thailand during 2012-2013, the CRE prevalence in each region were 21.9% (52/237 isolates) in the north, 27.4% (65/237 isolates) in the Northeastern, 24.5% (58/237 isolates) in the Central, 9.3 % (22/237 isolates) in Bangkok, 6.3 % (15/237 isolates) in the East and 10.6% (25/237 isolates) in the south of Thailand. Notably, NDM-1 has been mostly identified from isolates (9). For class A carbapenemases, they have been found KPC-13 producing strain, which mainly identified in *E. cloacae* at Siriraj Hospital during 2012-2014. Furthermore, the prevalence of KPC-13 among *Enterobacteriaceae* clinical isolates were increasing to 1.4% of 12,741 isolates from the largest university hospital in Thailand (84). The spreading of class B metallo-β-lactamases, they have been

identified either in *P. aeruginosa* and *Enterobacteriaceae.* IMP-14 and IMP-015 were first identified in *P. aeruginosa* in 2004. Since then in 2010, IMP-type enzyme has been found in two clinical isolates of *P. aeruginosa* at Buddhachinaraj hospital, Phitsanulok. The study of molecular epidemiology of carbapenem resistant in *A.baumannii* found two isolates of OXA-23 producing strains among clinical isolates at Buddhachinaraj hospital during 2003-2008 and also found OXA-23 production from *A. baumannii* isolated from 37 clinical specimens at Siriraj hospital. All of the above-mentioned, Thailand is one of a population mixing explaining the importance of the dissemination of carbapenemase producer (85).

Colistin

Colistin also known as polymyxin E is a member of polymyxin group of polypeptide antibiotics. Polymyxin E (colistin) and polymyxin B are the main antibiotics of this group and the only polymyxins used clinically. These antibiotics were discovered in the 1940s by nonribosomal peptide synthetase in *Bacillus polymyxa* subspecies *colistinus Koyama* (86) and has been marketed as its inactive prodrug colistin methanesulfonate (CMS) in previously. Colistin was the first antibiotic with significant activity against gram negative bacteria, including *P. aeruginosa, A. baumannii* (87). In 1970s, they were abandoned and replaced by other antibiotics because of concern about nephrotoxicity and neurotoxicity (10). Nevertheless, the increasing of MDR bacteria become public health problem and the developing of new drugs for treatment have not been breakthrough nowadays, and the use of other antibiotic include β-lactam is ineffective. Therefore, colistin is increasingly considered as the final option of antibiotic therapy for MDR bacteria that are resistant to almost all other currently available antibiotics (88).

1. Colistin structure

Polymyxin antibiotic structure is usually composed of a cyclic heptapeptide with a tripeptide side chain acylated by a fatty acid at amino terminus. Polymyxin B and polymyxin E (colistin) share almost identical primary sequence with major difference present at position 6 where D-Phe (D-phenylalanine) in polymyxin B is replaced by D-Leu (D-leucine) in colistin. They have cyclic heptapeptide loop to link between amino group of side chain on diaminobutyric acid (Dab) residue at

position 4 and carboxyl group of C-terminal L-Thr (L-threonine) residue at position 10. Their molecule were combined between lipophilic and hydrophilic groups make polymyxin structure amphipathic with polycationic domains, that conferring for its antibacterial activity (86).

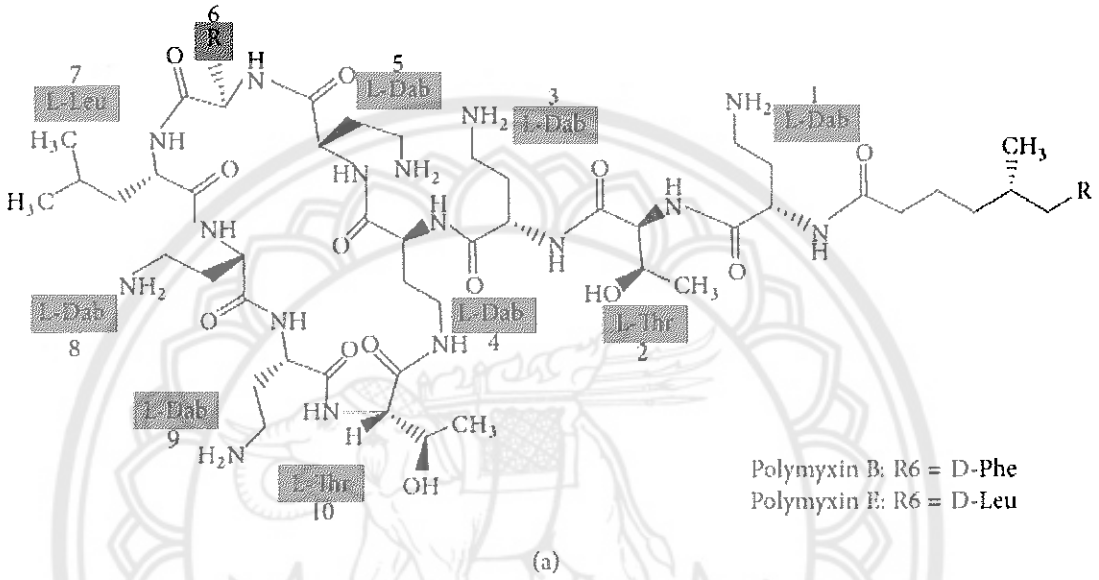


Figure 10 Chemical structure of polymyxin (89).

2. Spectrum of activity

The *in vitro* activity of colistin includes most gram negative aerobic bacilli and cause the rapid against bacterial in a concentration-dependent. It has an activity against some multidrug resistance gram negative pathogen, including *Acinetobacter* spp., *P. aeruginosa, Klebsiella* spp., *Enterobacter* spp., *E. coli, Salmonella* spp., *Shigella* spp., *Citrobacter* spp., *M. moganii, H. influenza,* and *S. maltophilia.* Colistin has also been reported to be potentially active against several mycobacterium spp. (90). However, *Proteus* spp, *Burkholderia mallei, Burkholderia cepacia, Providencia* spp., *Serratia* spp., *Edwardsiella* spp., and *Brucella* spp are all resistant to colistin. In addition, colistin has no activity against gram negative and gram positive aerobic cocci, gram positive aerobic bacilli, all anaerobes, fungi and parasites (91-92).

3. Mechanism of action

Colistin has a similar structure to polymyxin regularly most investigations have been conducted with polymyxin B as a model of polymyxin antibiotics. Previous studies have demonstrated the initial target of the antimicrobial activity of polymyxin is commonly the lipopolysaccharide (LPS) component of the outer membrane (93). However, some previous studies have shown three possible pathways of antimicrobial mechanism of polymyxin in gram negative bacteria (89).

3.1 Membrane lysis death pathway

Normally, the initial target of polymyxin is LPS of the outer membrane that acts as the first and foremost a permeability barrier in gram negative bacteria (89). LPS is composed of three domains: innermost lipid A, central core oligasacharide region and outermost O-antigen chain (94). Especially, lipid A, the most important domain which serve as a hydrophobic anchor with tight packing of fatty acyl chains to stabilized overall outer membrane structure. To make the stability of the membrane layer, there are some divalent cation such as Ca^{2+} and Mg^{2+} generally serve as a bridge between the adjacent LPS molecule (95). The polymyxins normally have a strong positive charge and a hydrophobic acyl chain that give them a high binding affinity between the cationic polypeptide molecule of the polymyxins and anionic LPS molecule of the outer membrane. The electrostatic interactions competitively derangement divalent cations on them, leading to a local disturbance of the outer membrane. Finally, the increasing of the permeability in cell membrane will destroy the physical integrity of phospholipids bilayer, leading to inner membrane lysis, leakage of cell contents and cell death respectively (96-98).

3.2 Vesicle-vesicle contact pathway

This pathway is an alternative mechanism, has been studied. The vesicle-vesicle contact is proposed that polymyxins can mediated the contacts between periplasmic leaflets of inner membrane and outer membrane which composed of an inner phospholipid leaflet and an outer leaflets that predominantly contains LPS, protein and lipoprotein. The cationic polypeptide molecule of polymyxin can bind to both anionic phospholipid vesicle of outer membrane and inner membrane then promote the exchange of phospholipid between vesicles. When the polymyxins bind to LPS with the electrostatic interaction, the molecule of polymyxin will induce the lipid

exchange between leaflets of outer membrane and inner membrane, then triggering the loss of specificity of phospholipids composition and can potentially cause an osmotic imbalance, leading to cell lysis (99).

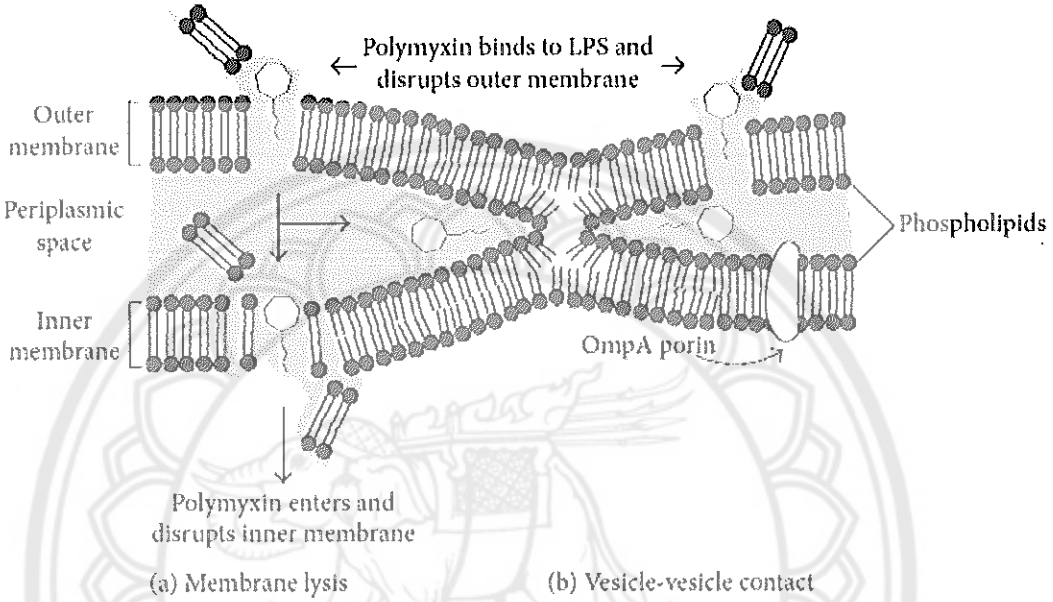


Figure 11 The antibacterial mechanisms of polymyxin: (a) classic mechanism of membrane lysis (b) alternative mechanism of vesicle-vesicle contact (99-100)

3.3 Hydroxyl radical death pathway

This mechanism has been reported, the antibacterial mechanism of polymyxin can possibly induce rapid cell death the accumulation of hydroxyl radical. This pathway is based on the hypothesis of oxidative stress due to polymyxin induced foramation of reactive oxygen species, inducing superoxide, hydrogen peroxide and hydroxyl radical in gram-negative bacteria cells (101). When polymyxin molecules enter into and cross outer membrane and inner membrane, the superoxide molecule will be induced and converted to hydrogen peroxide thought superoxide dismutases in cells (102). Consequently, the present hydrogen peroxide will oxidize ferrous iron to ferric iron, along with the formation of hydroxyl radical, which called Fenton reaction. After reaction, the concentration of hydroxyl radical is overwhelmingly established,

there will be the oxidative stress in result that can trigger the damage of DNA, the variety of lipid and protein, eventually the severe oxidative stress can cause cell death (103).

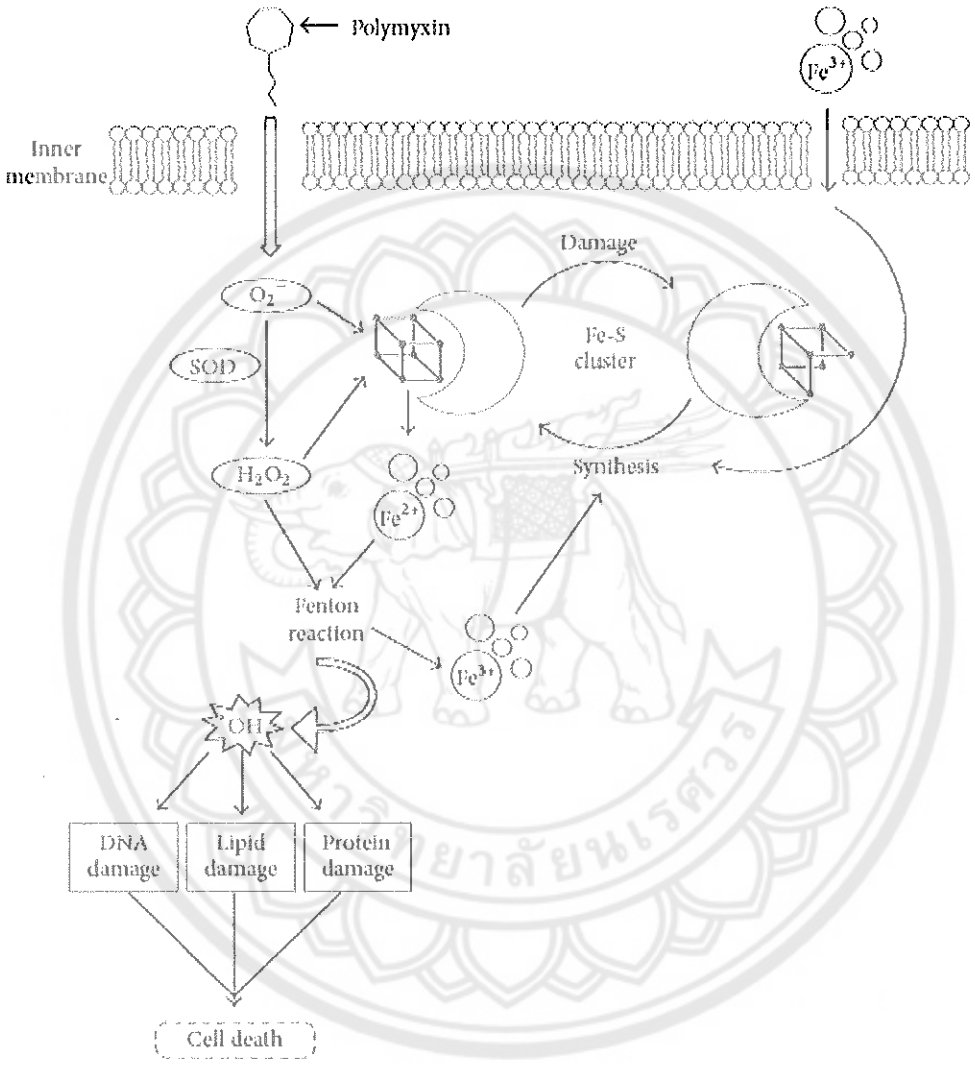


Figure 12 Hydroxyl radical death mechanisms in bacteria induced by polymyxin (89).

4. Mechanisms of resistance to polymyxins

Some bacteria have an intrinsic resistance to these drugs such as *Proteus* spp., *Serratia* spp. and *Burkhoderia* spp., whereas other gram negative bacteria can develop resistance to protect themselves from exposure to cationic

antimicrobial peptides in a process referred to as acquired resistance through mutation or adaptation mechanism (86,104). The previous study suggested that there might be possible mechanism of resistance related to the alteration of initial targets.

4.1 PhoP-PhoQ two-component system

It has been approved about the mechanism of resistance to polymyxin in gram negative bacteria that involves the multitier up regulation of a number of regulatory systems (105). Polymyxin antibiotic generally have a bactericidal activity by between positively charged on polymyxin molecule and negatively charged phosphate group on lipid A of LPS (97). Gram negative bacteria employ the mechanism to protect themselves from polymyxin attraction by the alteration of LPS via lipid A modification. The bacterial cell has a reduction in LPS by reduce the level of specific outer membrane proteins and net negative charge that related to the development of resistance (106). The alteration can be achieved by covalent modifications of the lpid A moiety of LPS to prevent from the attraction of polymyxin molecules. Therefore, The most common polymyxin resistance mechanism in bacteria is cationic substitution, that attributed to the shielding of phosphate on lipid A with positively charged group by the addition of phosphoethanolamine (PEtN) and L-4-aminoarabinose (L-Ara4N) (107-108). This mechanism is mediated by PhoP-PhoQ regulatory system encoded by PhoP locus (89). The activation of PhoP-PhoQ is the major regulatory to mediate the LPS modification in gram negative bacteria, which the PmrA-PmrB encoded by pmrCAB operon (109). PmrA-dependent modification can occur on each three distinct LPS domains include lipid A, core polysaccharide and O-antigen chain, respectively. The innermost of lipid A which has positively charged will be neutralized by either PEtN or L-Ara4N to protect the interaction with negatively charged of phosphates group and confer resistance to polymyxin B (94). Moreover, PmrA will activate the ugd gene encoding UDP-glucose dehydrogenase and pbg gene encoding L-Ara4N transferase that necessary for biosynthesis and incorporation of L-Ara4N (110). In addition, the inner membrane PmrC protein encoded by PmrA-activted pmrC gene is needed for pEtN incorporation into lipid A as well (89). The following part is the central core polysaccharide, this region will be modified via the decoration of heptose phosphate with pEtN can induce to polymyxin B resistance (111). This modification needs specific phosphotransferase by PmrA-activated cptA

gene encoding. In the outermost O-antigen chain, the increasing of the length and amount of O-antigen in LPS will result in the heightened resistance to polymyxin (112-113).

4.2 Specific-Specific resistance mechanism

There have been reports other mechanism besides the activation of two-component systems. There are often specific-specific mechanisms in polymyxin resistance. Multidrug efflux pump is one of antimicrobial resistance mechanism and play an importance role of polymyxin resistance in gram negative and gram-positive bacteria. The role of efflux pump in polymyxin resistance have been approved for example, the MexAB-OprM efflux pump in *P. aeruginosa*, the increasing expression of MexAB-OprM when exposure to the drug has been proposed to confer tolerance towards to polymyxin E (114-115). The AcrAB efflux pump encoded by acrAB operon confer resistance to polymyxin B in *K. pneumoniae* and *E. coli* (116). Moreover, A multidrug efflux pump NorM in *Burkholderia vietnamien* has also been proposed to contribute to polymyxin resistance (117). These are some examples of efflux pumps have been approved to transport and pump out polymyxin present in cells. In addition, there are another mechanisms of polymyxin resistance in gram-negative bacteria, for example in *P. aeruginosa* can promote resistance to polymyxin B via increasing expression of the outer membrane proteins OprH and membrane stabilization proteins (118). The outer membrane protein OmpA in *K. pneumoniae* confers resistance to antimicrobial peptide. OmpA generally regulate the expression of capsule polysaccharide which has a negatively charge whereas polymyxin has positively charge. For this reason, the capsule polysaccharide can bind to the polymyxin by electrostatic interaction to reduce the amount of peptide reaching the target site. This mechanism will be neutralized the bactericidal activity and associated increase resistance to polymyxin (119-120). Recently, it was found that the complete loss of LPS will decreased the ability to reach the target and lead to high-level polymyxin E resistance in *A. baumannii* (121).

5. Clinical use indications

Normally, there are two common forms of colistin available includes colistin sulphate and the commercially available parenteral formulation colistimethate sodium, which a prodrug (122). It has excellent bacterialcidal activity and should be

considered for the treatment of infection caused by multidrug resistance gram negative bacteria. Colistimethate sodium is administered parenterally, intravenously or intramuscularly (123). Colistin sulphate is administered either orally or topically (86). Both colistimethate sodium and colistin can also be given via inhalation. Nevertheless, colistimethate sodium is hydrolysed in aqueous solutions to colistin in a concentration-dependent manner, so it should be administered shortly after reconstitution to avoid the toxicity associated with colistin (124). There are some *in vitro* studies and clinical studies have indicated the synergistic activity effect of colistin with other antimicrobial agents against multidrug resistance gram negative bacteria. Synergy definition in these trials was based on standard methodology, which means a 2 log10 decrease in CFU/mL between the combination and the most active single agent at the different time-points. The synergistic effect was observed with many antimicrobial agents for example, the combination between colistin and antipseudomonal agents includes azlocillin, piperacillin, aztreonam, ceftazidime, imipenem, or ciprofloxacin which, the result was more effective than colistin monotherapy (10). Other study, imipenem–colistin synergy was reported in 50% of *K. pneumoniae* strains with the blaVIM-1 genotype (125). And also showed the significant synergy when colistin was combined with a glycopeptide against *A. baumannii* (126). An *in vitro* pharmacokinetics/pharmacodynamic interaction between colistin and doripenem combination and reduction demonstrated substantial reductions in regrowth and delay in the emergence of colistin-resistant subpopulations of *P. aeruginosa* (127). Possible mechanisms for synergy with colistin and carbapenems or rifampin are subpopulation synergy and mechanistic synergy (128). In subpopulation synergy, one drug kills the subpopulations that are resistant to the other drug and the mechanistic synergy means that, because each drug acts on a different cellular pathway, one drug increases the rate or extent of killing caused by the other drug. Moreover, there were several *in vitro* studies demonstrated the regrowth with colistin monotherapy so that the concerning about colistin monotherapy include heteroresistance among gram negative bacterial populations exposed to colistin alone have been raised (129-130). Because of the resistance, use of two antibiotics might prevent the emergence of resistance to either and the combination of antibiotic might allow lower doses of colistin to reduce toxicity.

6. Toxicity and adverse effects

From previous studies, the most common adverse effect of colistin therapy is nephrotoxicity and neurotoxicity. Early reported, with colistin revealed a high incidence of toxicity related renal toxicity. The indication includes acute tubular necrosis manifested as decreased creatinine clearance and increased serum urea and creatinine levels (86). Some studies used any of the RIFLE criteria (131), some used the threshold of failure or above, and others defined renal failure as creatinine >2 mg/dl. Many risk factors for nephrotoxicity found in different studies included older age, pre-existing renal insufficiency, hypoalbuminaemia and concomitant use of non-steroidal anti-inflammatory drugs (93). Neurotoxicity is less common than nephrotoxicity. The toxicity is associated with dizziness, weakness, facial and peripheral paresthesia, vertigo, visual disturbances, confusion, ataxia, and neuromuscular blockade, which can lead to respiratory failure or apnea. However, neurotoxicity effects are usually mild and the development related to the frequently of colistin treatment in patients. Most of all, both nephrotoxicity and neurotoxicity are considered to be dose-dependent. So that, the manifested will be resolved after early discontinuation of therapy with the antibiotic (86).

7. Epidemiology of colistin-resistant

Colistin has been re-introduced into clinical practice for treatment of multidrug resistant gram negative bacteria after the reports on nephrotoxicity and neurotoxicity, it was abandoned in the 1970s and the parenteral use was almost completely abandoned in the 1980s (89,93). The revival of polymyxin has been coming in therapeutic use since the mid of 1990s, because the paucity of novel antibiotics against prevent multidrug resistant infections (132). While polymyxin remains a last resort agent to treat multi-drug resistant, especially those caused by gram negative pathogen. Bacteria are usually able to evolve the strategies to protect them self by resistance mechanism to antibiotic agents including colistin. However, there are reports the emergence of heteroresistance and resistance after colistin treatment. Resistance to colistin has been reported throughout the world. Normally, colistin resistance has high rates among *A. baumannii* and *P. aeruginosa*. Because, these pathogens are resistance to all most all available antibiotic including β–lactams, fluoroquinolones, tetracyclines, aminoglycosides and carbapenems (133). It leads to

the emerging of pandrug-resistant and extremely drug-resistant isolates (134). Since colistin is frequently the only antibiotic remaining to treat multidrug resistant *A. baumannii* and *P. aeruginosa* infections. Therefore, heteroresistance and resistance against colistin of these pathogens have been increasingly reported in clinical setting (135). The highest rates colistin resistance has been reported from Asia followed by Europe and others parts of the globe, respectively. The first case was reported from Czech Republic in 1999 (136). The surveillance study of USA hospitals revealed the resistance rate of colistin approximately 5.3% in Acinetobacter isolates (137). So from then on, the emergence of colistin resistant in *A. baumannii* has been increasingly reported every year and observed in several countries around the world. The clinical importance of colistin-resistant strains may be obrserved in the context of heteroresistance in *A. baumannii* strains and the emergence of colistin-resistant pathogens following treatment of MDR isolates with colistin (138). In any case, the Clinical and Laboratory Standards Institute (CLSI) has selected an MIC of ≤ 2 μg/ml as susceptible and an MIC of ≥ 4 μg/ml as resistant for colistin (139). This type of resistance can described as an adaptive resistance with the rapid development of resistance in the presence of antibiotics and reversal to susceptibility in the absence of the same (140).

Meanwhile, colistin has emerged as a therapeutic option against other multidrug resistant gram negative bacteria and CRE. With the increased usage of colistin to treat serious infection, there are beginning to be reports of resistance to colistin in isolates of CRE. Several studies have been reported a higher prevalence of mortality rates of these pathogens form the increasing usage of colistin (11). Previous studies documented the in vivo evolution of a KPC-producing isolate to a stable colistin-resistant phenotype following low-dosage colistin use (141), and colistin heteroresistance among *Enterobacteriaceae* isolates following colistin therapy has also been reported (142). Furthermore, several focused studies have documented case reports and the possible hospital transmission of carbapenemase-producing, colistin resistant *Enterobacteriaceae* (143). These reports provide growing evidence of an increasing association of CRE and resistance to colistin.

From previous study, colistin resistance has evolved chromosomal-encoded mechanism, especially in certain members of *Enterobacteriaceae*. The

mechanism is associated with two-component systems by the alteration of LPS via lipid A modification. Very recently study, the emergence of plasmid-mediated *mcr - 1* colistin resistance via horizontal gene transfer, from a routine surveillance project on antimicrobial resistance in commensal *E. coli* from food animal in China has been reported. The resistance has also been found in isolates of *E. coli and K. pneumoniae* in animal and human. The mechanism of resistance via mcr-1 gene related to the family of phosphoethanolamine transferase enzymes (9). The retrospective study showed that the mcr-1 genes have already been detected in no fewer than 16 countries, including 7 countries in Southeast Asia China (9), Thailand, Laos (13), Japan (144), Vietnam (145), Cambodia (146) and Malaysia (13) and 9 European countries Denmark (147), United Kingdom (England and Wales), Netherlands, France , Portugal, Switzerland, Germany, Belgium and Algeria (13). Moreover, the document from the research on the diversified mcr-1 reservoirs confer resistance to colistin in human gut microbiota showed the resistance in at least 3 enterobacterial (10) species (*E. coli*, *Salmonella enterica* and *K. pneumoniae*) from host reservoirs included at least three kinds of poultry and livestock chickens, pigs, and cattle. Furthermore, it has been already being found animal-to-human transmission in China, Thailand, Laos, and Denmark. Therefore, the emergence of plasmid-mediated *mcr-1* colistin resistance has become a serious public health worldwide (10).

8. **The emergence of *mcr-1* gene in Thailand**

The first detection of plasmid-mediated colistin resistance in human isolate in Thailand has been reported in *E. coli* isolates. The National antimicrobial resistance surveillance center, Thailand (NARST) documented that the finding evolves a 63 years old man whose urine contained *E. coli* harboring the mcr-1 colistin resistance gene with underlying intracerebral hemorrhage was admitted to Vichein Buri hospital, Petchaboon during 23 January to 25 February 2016. Sincethen, during the hospitalization with developed to bronchitis. Susceptibility testing revealed the pathogen to be resistant to ertapenem, imipenem, and meropenem. The resistance has been developed during hospitalization. Therefore, this was a first case of colistin resistance established in human (148). Moreover, from research study reported the emergence of colistin-resistant *K. pneumoniae* isolated from healthy individuals and patients in Thailand (11). Furthermore, the use of antibiotic in livestock animals in

Thailand is one of the major contributors to antibiotic resistance. They are widely used in animal production including poultry and pig farms, to prevent infections and treat disease and to improve growth performance (149). Nearly all antimicrobial classes important for human medicine are also used in animal production that lead to development of commensal bacteria on animal farms (150). Therefore, they are possible as the important reservoir for acquired antimicrobial resistance genes and have a possible exchange between animals to consumers. Recently, *mcr-1* colistin resistance gene was discovered in the plasmid mediated acquired resistance gene distributing in *E. coli* from human and livestock animals in Thailand (14). Unfortunately, the use of antibiotics has been quite liberal and not necessarily under veterinary control in Thailand, few data are available from semi-intensive systems prevalent in developing countries, where farmers typically have little access to veterinary support (12). For these reasons, Plasmid carrying *mcr-1* was mobilized to the recipient bacterial cell by conjugation rapidly and maintained in host cell as well. The emergence of *mcr-1* conduces to the breach of the last group of antibiotic, colistin by plasmid mediated resistance (10).

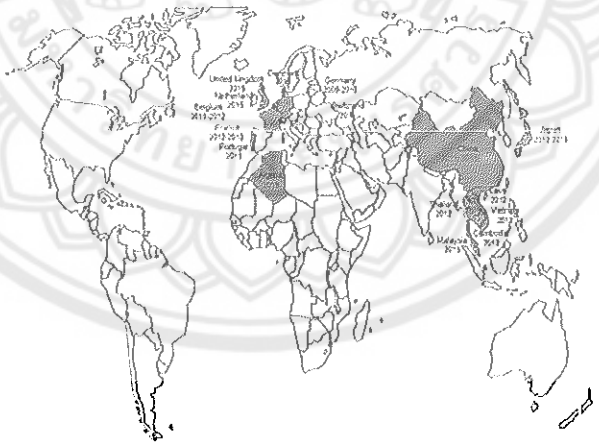
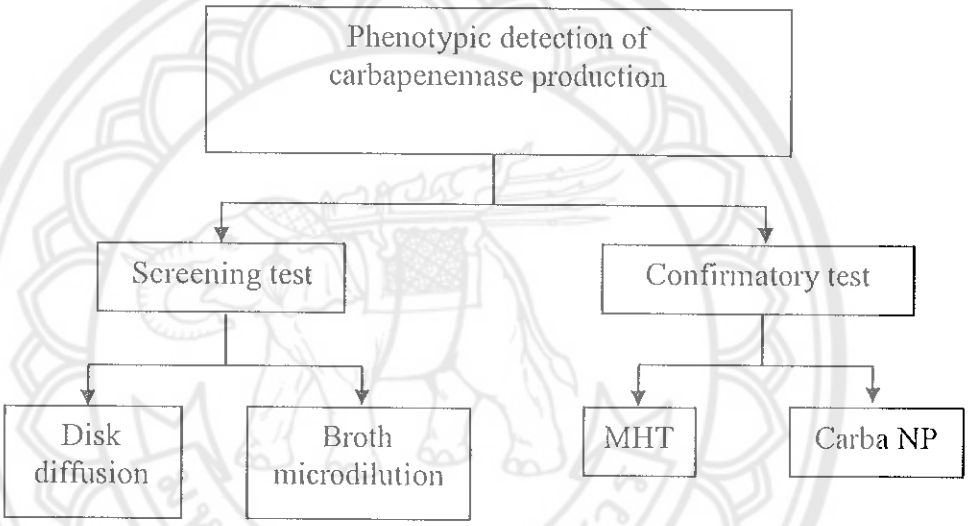


Figure 13 Global distribution of the *mcr-1* colistin resistance gene. The countries where the mcr-1 gene was discovered are highlighted in blue (13).

Detection of carbapenemase production

Detection of carbapenemase production is one of the most important methods to identify patients colonized with carbapenems resistant or carbapenamase-producing bacteria. CLSI has published guidelines for phenotypic detections includes, screening and confirmatory tests. Moreover, the genotypic detection is one of the methods to approach the molecular test for carbapenemase gene.

1. Phenotypic detection of carbapenemase production according to the CLSI guidelines.



The diagram represents a phenotypic detection of carbapenemase production according to the CLSI guidelines

1.1 Screening test of carbapenemase production

The detection of carbapenemase producer in clinical specimen is based first on the analysis of susceptibility testing. Therefore, a greater attention has been paid to antimicrobial activity screening method. According to the CLSI guidelines (M100-S24), the breakpoint for carbapenem is a device for detecting carbapenemase producers at first (151).

1.1.1 Disk diffusion method

Agar disk diffusion testing is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing and use for carbapenemase production basic screening. The antimicrobial susceptibility will be tested using disk diffusion methods in accordance with the M02-A11 CLSI

guideline. All *Enterobacteriaceae* isolates will be prepared the inoculums for Antimicrobial susceptibility testing. The bacterial inoculums from previously prepared tryptic soy broth (TSB) will be adjusted the turbidity of the suspension to achieve a turbidity equivalent to 0.5 McFarland standard. The result in a suspension containing approximately 1-2 x 10^8 CFU/ml. After adjusting the turbidity of the inoculums suspension, A sterile cotton swab will be dropped into the adjusted suspension and rotated several time to remove excess fluid from the swab. The inoculums will be inoculated on Mueller-Hinton agar (MHA) plate by streaking the swab over the entire agar surface. The same procedure will be repeated two more times with rotated the plate approximately 60° each time and swabbed the rim of the agar at a final step. A MHA plate will be left for 3-5 minutes. In this study will be evaluated susceptibility testing of carbapenems using the carbapenems disk including imipenem (IPM), meropenem (MEM), ertapenem (ETP) in each concentration at 10 µg. Plate will be incubated at 35±2 °C for 16-18 hours. After of the incubation period, the inhibitory zone will be measured in a diameter (mm.) each plate as shown in table 2. For quality control *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 will be used as quality control organisms. The screening result interpret as positive when the diameter of inhibition zone is 16-21 mm for imipenem or 14-21 mm. for meropenem (152).

1.1.2 Broth microdilution method

Dilution method is one the most appropriate for detect carbapenemase production by MIC values to estimate the concentration of the tested antibiotic agent. Prepare the bacterial inoculums by adjusted the turbidity of the suspension to achieve a turbidity equivalent to 0.5 McFarland standard. This result in a suspension containing approximately 1-2 x 10^8 CFU/ml. Inoculate the bacterial inoculums into cation-adjusted Mueller-hinton broth. The procedure involves preparing each dilution of carbapenem antibiotic in liquid medium. For, ertapenem, meropenem and imipenem, prerare the concentration at 1 µg/ml. Then, inoculated the inoculums at 35±2 °C for 16-20 hours. The growth of tested isolated after incubation indicate that the MIC value is less than 2 µg/ml. Afterward, The MIC of either carbapenems antibiotic will be tested to detect the carbapenemase production. The positive result of screening test will be interpreted when MIC of ertapenem is 2-4

μg/ml and 2-8 μg/ml for meropenem and imipenem to detect the production of carbapenamase (151).

1.1.3 Antimicrobial gradient method (E-test)

This method combines the principle of dilution methods with that of diffusion methods in order to determine the MIC value. The principle is based on the possibility of creating a concentration gradient of the antimicrobial agent tested in the agar medium. MIC value is determined at the intersection of the strip and the growth inhibition ellipse (153) .The detection will be interpret as positive result when MIC of carbapenem includes imipenem, meropenem and doripenem are in range of 2-4 μg/ml and ertapenem (ETP) is 2 μg/ml.

1.2 Confirmatory test of carbapenemase production

After screening test of carbapenemase production, tested isolate that represent positive result with resistance to at least extended spectrum cephalosporin required the confirmatory test for affirm carbapenemase production of each tested isolate by following methods.

1.2.1 Modified Hodge test (MHT)

This test is based on the inactivation of carbapenem by carbapenemase producing strains that enable a carbapenem susceptible indicator strain (*E.coli* ATCC 25922) to extend growth towards a carbapenem-containing disc along the streak of inoculum of the test strain. Positive test result is a characteristic cloverleaf-like indentation as shown in figure 14 (151).

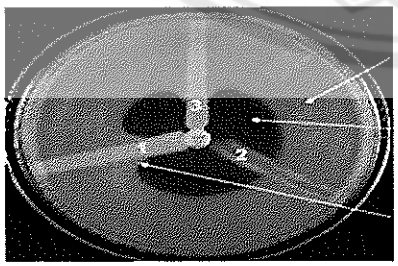


Figure 14 The MHT performed on a MHA plate with (1) MHT positive control *K. pneumoniae* ATCC BAA-1705 (2) MHT negative control *K. pneumoniae* ATCC BAA-1706 and (3) a clinical isolate with positive result (85)

1.2.2 Carba NP test

The Carba NP test is a novel phenotypic method developed for carbapenemase detection. It is based on *in vitro* hydrolysis of carbapenems by a bacterial lysate, which is detected by changes in pH values using the indicator phenol red (red to yellow/orange). It was reported to be 100% sensitive and specific for *Enterobacteriaceae* and 100% specific and 94.4% sensitive for *Pseudomonas* spp. harboring carbapenemases. Carba NP test will be performed for an identification of carbapenemases production as shown in figure 15 (154).

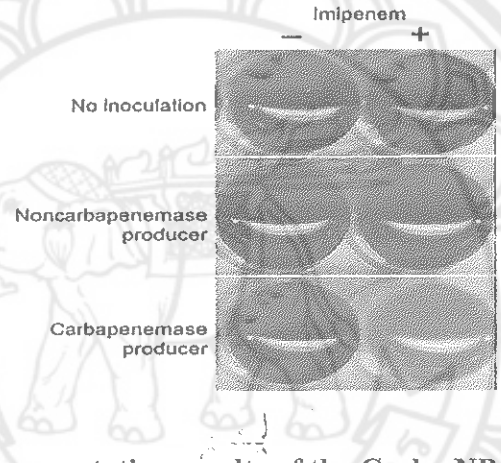


Figure 15 Representative results of the Carba NP test (154)

2. Genotypic detection of carbapenemase gene

Genotypic detection is one of molecular confirmation of carbapenemase-producing. Additionally, the increasing number of new carbapenemases makes molecular tests unsuitable for the initial detection of carbapenemase production. To screen for the presence of carbapenem resistance genes includes bla_{KPC}, bla_{GES}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{OXA-48} and bla_{OXA-58}, many molecular technique have been use for the detection carbapenemase genes such as conventional polymerase chain reaction (conventional PCR), multiplex PCR and real-time PCR. (155).

2.1 Conventional PCR

PCR is a powerful method for amplifying particular segments of DNA, enables the synthesis of specific DNA fragments using a DNA-polymerase enzyme, which takes part in the replication of the cellular genetic material. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling

of the reaction for DNA melting and enzymatic replication of the DNA. DNA-polymerase synthesizes a complementary sequence of DNA, as a small fragment (primer) is connected to one of the DNA strands in the specific site chosen to start the synthesis. Primers limit the sequence to be replicated and the result is the amplification of a particular DNA sequence (156). Additionally, to amplify several different DNA sequences simultaneously, multiplex PCR was developing. This process amplifies DNA in samples using multiple primers sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences (157).

2.2 Real-time PCR

Another technique known as quantitative polymerase chain reaction is a real-time polymerase chain reaction (RT-PCR). This PCR technique monitors the amplification of a targeted DNA molecule during the reaction. There are two common methods for the detection of PCR products in real-time PCR includes, the first is non-specific fluorescent dyes that intercalate with any double-stranded DNA, the second is sequence-specific DNA probes consisting of oligonucleotide that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence (158).

Detection of colistin resistance

1. Susceptibility testing methods for polymyxin

There are various methods to determine the resistance to polymyxin in pathogen. The current guideline recommends routine colistin susceptibility testing by estimate of MIC because the detection by disc diffusion test dose not reliability to the revel of resistance as well as the E-test method which base on the same principle of diffusion method. In addition, many published studies have employed Agar dilution method as a standard method, but this has been used infrequency by the CLSI in recent years as a reference. However, the complicated by the lack of a reliable reference standard methods which still debatable. This leaves broth microdilution as a primary reference method to perform polymyxins MIC testing. The susceptibility testing by broth microdilution requires fastidious attention to prevent a varying degree of adherence to organic and inorganic material due to its polycationic nature (159). As

recommended by CLSI guidelines provided MIC breakpoint for *Acinebacter* spp. and *Pseudomonas* spp. with colistin or polymyxin B MICs ≤ 2 μg/ml will categorized as susceptible and those with MICs ≥ 4 μg/ml will categorized as resistant (160). Formerly, there is no breakpoint of polymyxin available for *Enterobacteriaceae* according to CLSI guidelines. Therefore, MIC of polymyxin for *Enterobacteriaceae* usually interprets by the breakpoints of European Committee on Antimicrobial Susceptibility Testing (EUCAST) for reference. Enterobacterial isolates with colistin or polymyxin B MICs ≥ 2 μg/ml were categorized as resistant (161).

1.1 Broth microdilution

Broth microdilution is one the most appropriate reference method to perform polymyxins MIC testing. Prepare the bacterial inoculums by adjusted the turbidity of the suspension to achieve a turbidity equivalent to 0.5 McFarland standard. This result in a suspension containing approximately 1-2 x 10^8 CFU/ml. Inoculate the bacterial inoculums into cation-adjusted Mueller-hinton broth. CLSI recommend to add polysorbate-80 (P-80) when testing with cationic antimicrobial agent. To prepare microdilution trays, make intermediate twofold dilutions of polymyxin agent volumetrically in sterile DW. For the intermediate (10×) polymyxin solutions, dilute the concentrated antimicrobial stock solution by making serial twofold dilutions. Use one pipette for measuring all diluents and then for adding the stock antimicrobial solution to the first tube. For each subsequent dilution step, use a new pipette. Dispense the antimicrobial/broth solutions into the plastic microdilution trays. Prepare the polymyxin solutions at twice the desired final concentration, and fill the wells wit 0.1 mL by pipette. Each tray should include a growth control well and a sterility (uninoculated) well. After that, in a process of testing, each well contains approximately 5×10^5 CFU/mL therefore, if the volume of broth in the well is 0.1 mL and the inoculum volume is 0.01 mL, then the 0.5 McFarland suspension (1×10^8 CFU/mL) should be diluted 1:20 to yield 5×10^6 CFU/mL. When 0.01 mL of this suspension is inoculated into the broth, the final test concentration of bacteria is approximately 5×10^5 CFU/mL. Then, inoculated the inoculums at 35±2 °C for 16-20 hours. After an incubation period, the MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the microdilution wells as detected by the unaided eye. Compare the amount of growth in the wells containing

the antimicrobial agent with the amount of growth in the growth-control wells or tubes (no antimicrobial agent) used in each set of tests when determining the growth end points (122). For a test to be considered valid, acceptable growth (≥ 2 mm button or definite turbidity) must occur in the growth-control well. The MIC levels of *E. coli* ATCC 25922 (colistin-susceptible strain) and *E. coli* NCTC 13846 (colistin-resistant strain) were used as quality control organisms for testing methods. The results were interpreted in accordance with The European Committee on Antimicrobial Susceptibility Testing (EUCAST)

1.2 Agar dilution method

The agar dilution method is a well-established technique for detect of MIC. The antimicrobial agent is coprporated into the agar medium, with each plate containing a different concentration of the agent. Preparing agar dilution plates, polymyxin solution should prepare in an intermediated concentration (10×) by making successive 1:2. 1:4 and 1:8 dilutions or making a serial twofold dilution. Then, add one part of the 10× colistin solution to nine parts of molten agar. After preparing an appropriate dilutions of polymyxin solution, add each concentration of agent solution to molten test agar (molten MHA have been allowed to equilibrate in water bath to 45 to 50 °C). Mix the agar and polymyxin solution thoroughly and pour into petri dishes on a level surface to result in an agar depth of 3 to 4 mm.Pour the plates quickly after mixing to prevent cooling and partial solidification in the mixing conatainer. Avoid generating bubble when mixing. Allow the agar to solidify at room temperature, and either use the plates immediately or store them in sealed plastic bags at 2 to 8°C for up to five days for reference work (162).

In a section of the inoculum preparation, to prepare the inoculum for antimicrobial susceptibility testing by adjusting turbidity of the suspension to achieve a turbidity equivalent to 0.5 McFarland standard. This result in a suspension containing approximately 1-2 x 10^8 CFU/ml. After adjusting the turbidity of the inoculums suspension, the final inoculum required was approximately 10^4 CFU/spot), use the adjusted suspension for final inoculation within 15 min of preparation.Then, apply an aliquot of each inoculum to the agar surface, a growth control plate (no colistin contaninig) was the first inoculated. Then, starting inoculates with the lowest to higher concentration. The inoculated plates were left to dry at room temperature,

but no more than 30 min. Invert the plates and incubated at 35±2 °C for 16-20 hours. After an incubation period, Record the MIC as the lowest concentration of colistin that completely inhibit growth. The MIC levels of *E. coli* ATCC 25922 (colistin-susceptible strain) and *E. coli* NCTC 13846 (colistin-resistant strain) were used as quality control organisms for testing methods (123). The results were interpreted in accordance with The European Committee on Antimicrobial Susceptibility Testing (EUCAST) as shown in table 3

2. Molecular detection of colistin resistance gene

For molecular detection of colistin resistance gene, the mechanism of resistance has been through both chromosomal-encoded mechanism and plasmid-mediated *mcr-1* colistin resistance gene. To detect the chromosomal DNA that possibly involved in colistin resistance includes the *pmrA, pmrB, phoP, phoQ,* and *mgrB* genes by using PCR Amplification and sequencing technique. In addition, for detect plasmid-mediated *mcr-1* colistin resistance gene, the mcr-1 gene specific primers and PCR condition will performed (14).

CHAPTER III

MATERIAL AND METHODS

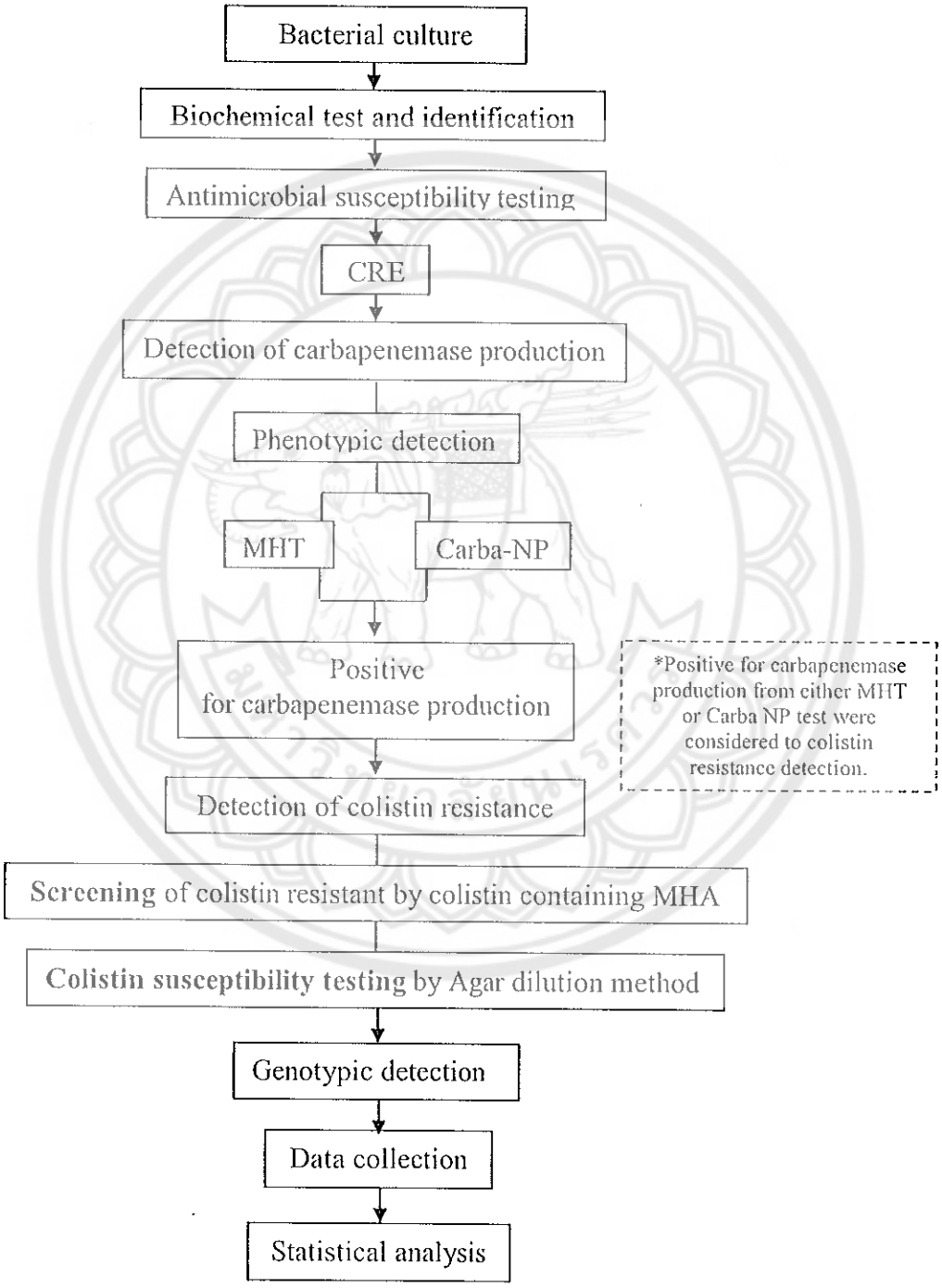
Bacterial strains

Clinical isolates of CRE were obtained from hospital clinical microbiology laboratory, entirely located in the lower north region of Thailand. All isolates were preliminary determined for their susceptibility to carbapenems by standard disk diffusion method using Kirby-Bauer disk, according to clinical breakpoints recommended by the Clinical Laboratory Standards Institute (CLSI). The resistant iolates that non-susceptible to at least a carbapenem agent were used as samples in this study. Bacterial strains were collected, isolated in pure culture and stored in nutrient agar slant, then put in a suitable position in transport containers to laboratory. The samples were obtained from following hospitals during January 2016- June 2017.

1. Naresuan university hospital, Phitsanulok
2. Budhachinnaraj Hospital, Phitsanulok
3. Srisangworn hospital, Sukhothai
4. Phetchabun hospital, Phetchabun
5. Wichian Buri Hospital, Phetchabun
6. Uthaithani Hospital, Uthaithani
7. Sawanpracharak Hospital, Nakhon Sawan

Experimental design

Diagram of experimental design



Bacterial culture

Bacterial samples were picked from nutrient agar by using a sterile inoculation loop or needle and inoculated on MacConkey agar plate as isolation streak plate technique. The bacterial cultures were incubated at 35±2 °C for 18-24 hours.

Biochemical test and identification

Bacterial isolates were grown for identification process by using a sterile inoculation loop, picked 3-5 colonies of test and inoculate in tryptic soy broth (TSB), incubated at 35±2 °C for 2 hours to identify organisms. The biochemical methods includes Triple sugar iron agar (TSI), Motility-indole-lysine medium (MIL), Citrate, Malonate and Urease test were used for identified isolated and then incubated at 35±2 °C for 18-24 hours. The results of biochemical tests were interpreted as genera and species of *Enterobacteriaceae*.

Antimicrobial susceptibility testing

The antimicrobial susceptibility was tested using disk diffusion methods in accordance with the M02-A11 Clinical and Laboratory Standards Institute (CLSI) guideline. All isolates were prepared the inoculums for antimicrobial susceptibility testing. The bacterial inoculums of *Enterobacteriaceae* were inoculated in TSB and adjusted the turbidity of the suspension to achieve a turbidity equivalent to 0.5 McFarland standards with a result in suspensions containing approximately 1-2 x 10^8 CFU/ml. After adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dropped into the adjusted suspension and rotated several time to remove excess fluid from the swab. The inoculums were inoculated on Mueller-Hinton agar (MHA) plate by streaking the swab over the entire agar surface. The same procedures were repeated two more times with rotated the plate approximately 60° each time and swabbed the rim of the agar at a final step. A MHA plate was left for 3-5 minutes. In this study was evaluated susceptibility testing of carbapenems using the carbapenems disk including imipenem (IPM), meropenem (MEM), ertapenem (ETP) and doripenem (DOR) in each concentration at 10 µg. The plate was incubated at 35±2 °C for 16-18 hours. After of the incubation period, the inhibitory zone was measured in a diameter (mm) each plate as shown in table 2. For quality control, *S. aureus* ATCC 25923,

E. coli ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control organisms.

Table 2 Zone diameter interpretative standard for *Enterobacteriaceae* (151) (According to the M100-S24 CLSI guideline)

Carbapenems	Susceptible (S) mm.	Intermediate (I) mm.	Resistance (R) mm.
Doripenem (DOR)	≥ 23	20-22	≤ 19
Imipenem (IPM)	≥ 23	20-22	≤ 19
Meropenem (MEM)	≥ 23	20-22	≤ 19
Ertapenem (ETP)	≥ 22	19-21	≤ 18

Detection of carbapenemase production

The detection of carbapenemase production was tested using two approaches, phenotypic method and genotypic method.

1. Phenotypic Detection Method

1.1 Modified Hodge test (MHT)

The Modified Hodge test (MHT) was detected carbapenemase production in isolates of *Enterobacteriaceae*. All isolates were tested intermediate or resistant to one or more carbapenems using the current interpretive criteria as listed in table 2. A culture of *E. coli* ATCC 25922, the indicator organism as recommended by CLSI was prepared a 0.5 McFarland standard suspension containing approximately 1-2 x 10^8 CFU/ml. After adjusting the inoculums, 1:10 dilution of inoculums suspension were diluted in sterilized normal saline solution or broth. The inoculums were inoculated on MHA plate as for the routine disk diffusion procedure. A MHA plate was allowed to dry for 3-10 minutes. A 10 µg of ertapenem or meropenem disk was placed in the center of the test area. The test or QC organism was picked 3-5 colonies using inoculating loop and inoculated in a straight line out from the edge of the disk. The streak was at least 20-25 mm. in length. The QC organism of testing was performed with each run, *K. pneumoniae* ATCC 1705 as positive control and

K. pneumoniae ATCC 1706 as negative control. The plate was incubated at 35±2 °C in ambient air for 16-20 hours.

For the interpretation, positive MHT was showed a clover leaf-like indentation (cloverleaf shape) of the *E. coli* ATCC 25922 strain growing along the test organism growth streak within the disk diffusion zone indicating production of carbapenemase and a negative test was showed no growth of the *E. coli* ATCC 25922 along the test organism growth streak within the disk diffusion.

1.2 Carbapenemase Nordmann-Poirel (Carba NP) test

Carba NP test was performed for an identification of carbapenemases production as follows. In this study, the Carba NP test protocol was modified from CLSI guideline M100-S26 and Nordmann method. First step, the Carba NP test solution was prepared using two separate solutions include solution A and solution B. Carba NP solution A was prepared by mixing 2 mL of phenol red solution solution 0.5% (wt/vol) with 16.6 mL of distilled water. Then, the pH value was adjusted to 7.8 by adding drops of 0.1 N NaOH and 10% HCl. For solution B, the same procedure was repeated and 6 mg of imipenem reference standard powder was added in the solution. After Carba NP test solutions was prepared, the bacterial isolates were grown overnight on blood agar plate to be tested. Extraction of bacterial protein, 1 µL loopful of the tested strain was extracted for isolation and purification of proteins from the cell using 100 µL of bacterial protein extraction reagent containing Tris-HCl buffer, pH 7.4 in Microcentrifuge tube. Each tube was vortexed for 5 seconds and this bacterial suspension was centrifuged at 10,000 × g at room temperature for 5 minutes. The enzymatic bacterial suspension in supernatant was used for detect carbapenemases production. 96-well microtiter plate was used according to Nordmann method, 50 µl of solution A and solution B were added in each well separately. After that, 50 µl of the enzymatic bacterial suspension was added in each solution respectively. 96-well microtiterplate was incubated at 35±2 °C for up 2 hours. QC organism of testing was performed with each run, by using *K. pneumoniae* ATCC 1705 as positive control and *K. pneumoniae* ATCC 1706 as negative control.

An interpretive criterion for detection of carbapenemase production by phenotypic methods has some limitation of each test. For the MHT, false-positive results can occur in isolates that produce ESBL or AmpC enzymes coupled with porin

loss. Moreover, false-negative results are occasionally noted from some NDM carbapenemase producing isolates. In addition, the limitation of Carba NP test can occur with some isolates of carbapenemase types, for example OXA-type and chromosomal encoded enzyme are not consistently detected. Therefore, positive isolates for carbapenemase production from either MHT or Carba NP test was considered to colistin resistance detection.

1.3 Modified carbapenem inactivation method (mCIM)

A new specific phenotypic method was performed for an identification of carbapenemases production. This test is based on the principle that when a meropenem disk is incubated for 4 hours in an aqueous suspension of a carbapenemase-producing microorganism, the carbapenem in the disk is degraded by carbapenemase. In contrast, if the test microorganism does not produce carbapenemase, MEM retains its antimicrobial activity after incubation in the bacterial suspension. This new method was established by CLSI guideline M100-S27, as the following protocol. Firstly, 1-µl loopful of the tested isolate from an overnight blood agar plate was emulsifying in 2 ml TSB and vortexed for 10-15 s. Then, 10 µg meropenem disk was added and emmersed in the suspension to each tube using sterile forceps. Each tube was incubated at 35±2 °C in ambient air for 4 hours ± 15 minutes. After that, just before or immediately following completion of the meropenem disk suspension incubation, the inoculum of *E. coli* ATCC 25922 was prepared in 0.5 McFarland standard suspensions containing approximately 1-2 x 10^8 CFU/ml. After incubating preriods, the suspension of *E. coli* ATCC 25922 was inoculated on MHA plate as the routein disk diffusion procedure. The inoculum suspention preparation and MHA plate inoculation steps were each completed within 15 minutes then allowed plate to dry for 3-10 minutes before adding the meropenem disk. The meropenem disk was removed from each TSB-meropenem disk suspension using 10-µl loop by placing the flat side of the loop against the flat edge of the disk and using surface tension to pull the disk out of the liquid. MEM disk was dragged and pressed by loop along the inside edge of the tube, expelling excess liquid from the disk. Then, MEM disk was placed on the MHA plate previously inoculated with the meropenem-susceptible *E. coli* ATCC 25922 an indicator strain. After that, MHA plate was inverted and

incubated at 35±2 °C in ambient air for 18-24 hours. After the following incubation, the zone of inhibition was measure as the routine disk diffusion method.

For the interpretation, if the test isolate produces a carbapenemase, the MEM disk will be hydrolyzed and there will be no inhibition or limited growth inhibition of the meropenem-susceptible *E. coli* ATCC 25922, will be interpreted as "Carbapenemase positive" in zone diameter of 6-15 mm. or presence of pinpoint colonies within a 16-18 mm.

Otherwise, if the test isolate dose not produces a carbapenemase, the MEM disk will not be hydrolyzed and will inhibit growth of the meropenem-susceptible *E. coli* ATCC 25922, will be interpreted as "Carbapenemase negative" in zone diameter of ≥19 mm. clear zone.

In addition, there might be interpreted as "Carbapenemase intermediated", if the presence or absence of a carbapenemase cannot be confirmed in zone diameter of 16-18 mm. or the zone diameter of ≥19 mm within the pinpoint colonies.

In this present study, the mCIM was added in the final of experimental study. This method was performed for suspected carbapenemases production of carbapenemase-producing, colistin resistant *Enterobacteriaceae* as a following protocol recently describes in CLSI guideline M100-S27.

2. Genotypic Detection Method

Genotypic detection of carbapenemase gene was an additionally test to confirm the presence of carbapenems resistance genes. In this study, all the isolates of the carbapenemase-producing, colistin resistant *Enterobacteriaceae* were sent to the clinical microbiology labolatory department of pathology, Faculty of medicine Ramathibodi hospital Mahidol University to identify the precise carbapenemase gene. The molecular method was performed using AMR direct flow chip kit (Master Diagnóstica). DNA Flow Technology for the detection of antibiotic-resistant markers by multiplex PCR and reverse hybridization to detect two genes associated with β-lactam antibiotic(broad-spectrum bla_{SHV} and bla_{CTX-M}) and fifteen carbapenem resistance genes includes, bla_{KPC}, bla_{sme}, $bla_{nmc/imi}$, bla_{GIM}, bla_{GES}, bla_{SPM}, bla_{SIM} bla_{IMP}, bla_{VIM}, bla_{NDM}, $bla_{OXA-23-like}$, $bla_{OXA-24-like}$, $bla_{OXA-48-like}$, $bla_{OXA-51-like}$, $bla_{OXA-58-like}$

Detection of colistin resistant

1. The screening of colistin resistant by colistin containing MHA

The colistin containing MHA was used for screening colistin resistant. The optimal colistin concentration was prepared at 2 µg/ml by weighing colistin powder (colistin sulfate salt) 10 mg, making 100 µg/ml of a stock solutions. To prepare 100 ml of a stock solutions containing 100 µg/ml of colistin sulfate with a potency of 1000.413 µg/mg, 10 mg of colistin powder was dissolved in 100 ml of sterile distilled water. To achieve desired concentration, stock solution was diluted with 250 ml of DW to make a final concentration at 2 µg/ml. The diluted of MHA was autoclave at 121°C for 15 min. After that, melted MHA was cooled down to 56 °C and then 2 µg/ml of colistin working solution was added. Poured plates were stored at 4 °C, protected from direct light exposure for up to one week (161). For the screening test, the inoculum was adjusted turbidity of the suspension to achieve a turbidity equivalent to 0.5 McFarland standards. This result in a suspension was containing approximately 1-2 x 10^8 CFU/ml. After adjusting the turbidity of the inoculums suspension, the final inoculum required was 10^6 CFU/spot of 10 µl (The final inoculum on the agar was approximately 10^4 CFU/spot). A MHA plate was left to dry and incubated at 35±2 °C for 16-18 hours. *E. coli* ATCC 25922 (colistin-susceptible strain) and *E. coli* NCTC 13846 (colistin-resistant strain) were used as quality control organisms for testing methods. The interpretation of colistin resistant screening was interpreted using reference breakpoint in accordance with The European Committee on Antimicrobial Susceptibility Testing (susceptible $\leq$ 2 µg/ml, resistant $\geq$ 2 µg/ml). If two or more colonies growth on plate the interpretation considered as colistin resistant.

2. Colistin susceptibility testing

All of colistin resistant isolates from screening test was detected for MIC of colistin by agar dilution method ranging 0.125-128 µg/ml. Cultures were prepared the inoculums for antimicrobial susceptibility testing by adjusting turbidity of the suspension to achieve a turbidity equivalent to 0.5 McFarland standards. A result in suspension containing approximately 1-2 x 10^8 CFU/ml. After adjusting the turbidity of the inoculums suspension, the final inoculum required was 10^6 CFU/spot of 10 µl (The final inoculum on the agar was approximately 10^4 CFU/spot), the adjusted suspension for final inoculation was used within 15 min of preparation. An aliquot of

each inoculum was applied to the agar surface, a growth control plate (no colistin contaninig) was the first inoculated. Then, the tested isolated were inoculated from the the lowest to higher concentration later. The inoculated plates were left to dry at room temperature, but no more than 30 min. the plates were inverted and incubated at 35±2 °C for 16-20 hours. After an incubation period, MIC of each isolate was recorded as the lowest concentration of colistin that completely inhibits growth. The MIC levels of *E. coli* ATCC 25922 (colistin-susceptible strain) and *E. coli* NCTC 13846 (colistin-resistant strain) were used as quality control organisms for testing methods. The results were interpreted in accordance with The European Committee on Antimicrobial Susceptibility Testing (EUCAST) as shown in table 3 (161).

Table 3 EUCAST clinical breakpoint for *Enterobacteriaceae* (161)

Agent	MIC breakpoint (mg/L)	
	Susceptible (S)	Resistance (R)
Colistin	≤2	>2

3. Genotypic detection method

The plasmid-mediated colistin resistance gene, *mcr-1*, will be detected in colistin resistant strains by conventional PCR technique with the following conditions (12).

Genomic DNA extraction

Genomic DNA was obtained by a pre-screening of susceptibility testing colistin resistant isolates. Bacterial cultures were grown in TSB broth overnight. 1-5 mL of each culture was taken and suspended in a centrifuge tube, centrifuging for 1 min at 10,000 rpm (~11,500 × g), the supernatant was discarded as possible. Then, the pellet lelf over from suspension was resuspended in 200 µL Buffer GA and 20 µL Proteinase K, mixed thoroughly by vortex. Afterwards, 220 µl Buffer GB was added to the sample, vortexed for 15 s, and incubated at 70°C for 10 min to yield a homogeneous solution to completely cell lysed. To adjust binding condition, 220 µl ethanol (96-100%) was added to the sample and mixed thoroughly by vortex for 15 s.

In a step of DNA isolation, the mixture was pipetted into the Spin Column CB3 (in a 2 ml collection tube) and centrifuged at 12,000 rpm (~13,400 × g) for 30 s. The spin column was discarded flow-through and placed into the collection tube. 500 µL Buffer GD (Ensure ethanol 96-100% has been added) was added to Spin Column CB3 for washing silica membrane. The Spin Column was centrifuged at 12,000 rpm (~13,400 × g) for 30 s, subsequently. Next, the spin column was discarded flow-through and placed into the collection tube again. 600 µL Buffer PW (Ensure ethanol 96-100% has been added) was added to Spin Column CB3 and centrifuged at 12,000 rpm (~13,400 × g) for 30 s. The spin column was discarded flow-through and placed into the collection tube and centrifuge at 12,000 rpm (~13,400 × g) for 2 min to dry the membrane completely. DNA elution step was performed by membrane washing as follow, the Spin Column was placed in a new clean 1.5 ml centrifuge tube and pipetted 50-200 µL Buffer TE or distilled water directly to the center of the membrane. Then, the Spin Column was incubated at room temperature (15-25°C) for 2-5 minute and centrifuged for 2 minute at 12,000 rpm (~13,400 × g). Afterwards, the eluting DNA that containing chromosomal as well as plasmid DNA fractions were directly used as sample in PCR technique . For further experiments the samples were stored at -20°C.

Primers

Colistin resistant strains were screened by PCR-based diagnostics with *mcr-1*-specific primers to probe the presence of the *mcr-1* gene. A pair of primer was designed for specific part of PCR product, 309 base-pair of *mcr-1* gene as following (9).

Target gene	Primer sequences
mcr-1	CLR F 5'-CGGTCAGTCCGTTTGTTC-3'
	CLR R 5'-CTTGGTCGGTCTGTAGGG-3'

Reaction mix

Reaction mix was prepared the following mix in a microcentrifuge tube (for a 25 µL reaction). Additionally, DNA free water was used as negative control and the colistin resistant *E. coli* NCTC 13846 (*mcr-1* positive) was used as positive control. The protocol was optimized for the Qiagen PCR Kit (Qiagen), in which might

need some optimization to adjust for the particular conditions that contains mastermix 25 µL, forward primer 0.2 µL, reverse primer 0.2 µL, DNA template 2 µL and water up to 50 µL.

Conditions for the PCR

PCR with the following conditions: an initial denaturation step at 94°C for 15 min followed by 25 cycles - denaturation at 94°C for 30 s then annealing at 58°C for 90 s and elongation at 72°C for 60 s Before finishing the run a final elongation step at 72°C for 10 min was attached.

Detection and analysis of PCR products

The product of PCR was detected by gel electrophoresis. For assembling the results, stained agarose gel was interpreted the size of the specific product band (309 bp) and compared with the predicted result or molecular maker, including positive and negative control run on the same gel.

Statistical analysis

The experiment data will be statistically analyzed as following

1. The results of carbapenems susceptibility testing data by disk diffusion method were statistically analyzed into resistant and susceptible percentage, divided into the following hospitals in each species of isolate.

2. The results of carbapenemase production by modified Hodge test and Carba-NP test were statistically analyzed into percentage.

3. The results of the screening of colistin resistant by colistin containing MHA were statistically analyzed into resistant and susceptible percentage, divided into the following hospitals in each species of isolate.

4. The results the presence of *mcr-1* plasmid-mediated colistin resistance gene in colistin resistant strains by of genotypic detection method were statistically analyzed into percentage.

CHAPTER IV

RESULTS

The clinical isolates of CRE from clinical microbiology laboratory, located in the lower north region of Thailand during January 2016-June 2017 were determined the prevalence of resistance to carbapenems and the prevalence of carbapenemase producing in CRE by phenotypic method. All CRE strains were determined the prevalence of colistin resistance and detected the presence of plasmid-mediated colistin resistance gene. The results of experimental data were statistically analyzed as follows.

Bacterial strains

The clinical isolates were obtained from 7 hospitals clinical microbiology laboratory, located in the lower north region of Thailand. Bacterial strains were collected, isolated in pure culture and stored in nutrient agar slant. A total of different 418 obtained clinical isolates as shown in table 4.

Table 4 A total obtained clinical isolates from 7 clinical microbiology laboratories located in the lower north region of Thailand

Hospitals	Province	Isolates
Naresuan university hospital	Phitsanulok	18
Budhachinnaraj hospital	Phitsanulok	34
Srisangworn hospital	Sukhothai	51
Phetchabun hospital	Phetchabun	149
Wichian Buri hospital	Phetchabun	52
Uthaithani hospital	Uthaithani	93
Sawanpracharak hospital	Nakhon Sawan	21
Total		**418**

Bacterial culture

Bacterial samples were picked from nutrient agar and inoculated on MacConkey agar plate as isolation streak plate technique. The growth isolates were identified as following method as shown below in table 5.

Table 5 A total of bacterial culture from clinical isolates from 7 clinical microbiology laboratories located in the lower north region of Thailand

Hospitals	Province	Obtained isolates	Culture isolates
Naresuan university hospital	Phitsanulok	18	18
Budhachinnaraj hospital	Phitsanulok	34	34
Srisangworn hospital	Sukhothai	51	51
Phetchabun hospital	Phetchabun	149	145
Wichian Buri hospital	Phetchabun	52	52
Uthaithani hospital	Uthaithani	93	65
Sawanpracharak hospital	Nakhon Sawan	21	19
Total		**418**	**384**

Bacterial identification

In total of 384 non-duplicated clinical isolates from 7 separate hospitals clinical microbiology laboratories were cultured and identified by biochemical methods. This identification included 378 isolates (98.43%) of *Enterobacteriaceae* and 6 isolates (1.56%) of non-*Enterobacteriaceae*. All of various species of *Enterobacteriaceae* family included 199 isolates of *E. coli* (51.82%), 121 isolates of *K. pneumoniae* (31.51%), 22 isolates of *E. cloacae* (5.73%), 7 isolates of *C. freundii* (1.82%), 2 isolates of *C. diversus* (0.52%), 7 isolates of *Enterobacter* spp. (1.82%), 9 isolates of *P. mirabilis* (2.34%), 3 isolates of *S. marcescens* (0.78%), 3 isolates of *M. morganii* (0.78%), 5 isolates of *Salmonella* spp. (1.30%) . A total of identified isolated were separately reported below in each hospital and presented in table 6 as follows.

Naresuan University hospital

All of 18 clinical isolates from Naresuan University hospital clinical microbiology laboratory were culture and identified as *Enterobacteriaceae* family, included 3 isolates of *E. coli*, 12 isolates of *K. pneumoniae*, 2 isolates of *C. freundii* and 1 isolate of *Enterobacter* spp.

Budhachinnaraj hospital

All of 34 clinical isolates from Budhachinnaraj hospital clinical microbiology laboratory were culture and identified as *Enterobacteriaceae* family, included 7 isolates of *E. coli*, 25 isolates of *K. pneumoniae*, 2 isolates of *E. cloacae*.

Srisangworn hospital

All of 51 clinical isolates from Srisangworn hospital clinical microbiology laboratory were culture and identified as various species of *Enterobacteriaceae* family, included 31 isolates of *E. coli*, 10 isolates of *K. pneumoniae*, 3 isolates of *E. cloacae*, 2 isolates of *P. mirabilis*, 2 isolates of *M. morganii*, 1 isolate of *Salmonella* spp., 1 isolate of *C. diversus*, 1 isolate of *Enterobacter* spp.

Phetchabun hospital

All of 145 clinical isolates from Phetchabun hospital clinical microbiology laboratory were culture and identified as 142 isolates of *Enterobacteriaceae* family, included 85 isolates of *E. coli*, 35 isolates of *K. pneumoniae*, 13 isolates of *E. cloacae* and 3 isolates of *P. mirabilis*, 3 isolates of *C. freundii*, 2 isolates of *S. marcesens*, 1 isolate of *Salmonella* spp., and other 3 isolates of non-*Enterobacteriaceae* included 2 isolates of *Aeromonas hydrophila* and 1 isolate of *Aeromonas veronii*.

Wichian Buri hospital

All of 52 clinical isolates from Wichian Buri hospital clinical microbiology laboratory were culture and identified as 50 isolates of *Enterobacteriaceae* family, included 27 isolates of *E. coli*, 15 isolates of *K. pneumoniae*, 4 isolates of *Enterobacter* spp. and 2 isolates of *P. mirabilis*, 1 isolate of *Citrobacter diversus*, 1 isolate of *S. marcesens*. Also 2 isolates of *A. baumannii* as non-*Enterobacteriaceae* family.

Uthaithani hospital

All of 65 clinical isolates from Uthaithani hospital clinical microbiology laboratory were culture and identified as 64 isolates of *Enterobacteriaceae* family, included 39 isolates of *E. coli*, 15 isolates of *K. pneumoniae*, 2 isolates of *C. freundii*, 1 isolate of *M. morganii*, 2 isolates of *P. mirabilis*, 1 isolate of *E. cloacae*, 1 isolate of *Enterobacter* spp., 2 isolates of *Salmonella* group B, 1 isolate of *Salmonella* group D. Also 1 isolate of *A. baumannii* as non-*Enterobacteriaceae* family.

Sawanpracharak hospital

All of 19 clinical isolates from Sawanpracharak hospital clinical microbiology laboratory were culture and identified as *Enterobacteriaceae* family, included 7 isolates of *E. coli*, 9 isolates of *K. pneumoniae* and 3 isolates of *E. cloacae*.

Table 6 Total of bacterial identification in clinical isolates from 7 clinical microbiology laboratories located in the lower north region of Thailand

Organisms	Hospitals							Total (384)	%
	Naresuan university hospital (18)	Budhachinnaraj hospital (34)	Srisangworn hospital (51)	Phetchabun hospital (145)	Wichian Buri hospital (52)	Uthaithani hospital (65)	Sawanpracharak hospital (19)		
E. coli	3	7	31	85	27	39	7	199	51.82
K. pneumoniae	12	25	10	35	15	15	9	121	31.51
E. cloacae	-	2	3	13	-	1	3	22	5.72
Enterobacter spp.	1	-	1	-	4	1	-	7	1.82
C. freundii	2	-	-	3	-	2	-	7	1.82
C. diversus	-	-	1	-	1	-	-	2	0.52
P. mirabilis	-	-	2	3	2	2	-	9	2.34
S. marcescens	-	-	-	2	1	-	-	3	0.78
M. morganii	-	-	2	-	-	1	-	3	0.78
Salmonella spp.	-	-	1	1	-	3	-	5	1.30
Total of *Enterobacteriaceae* (384)	18	34	51	142	50	64	19	378	

Antimicrobial susceptibility testing

The overall identified of 378 *Enterobacteriaceae* isolates from 7 hospitals were determined antimicrobial susceptibility testing using disk diffusion method. Within the overall identified of isolate, 259 isolates (68.52%) were susceptible to all carbapenems with inhibition zone diameters as 23 to 41 mm, 22 to 38 mm, 23 to 39 mm and 21 to 39 mm in meropenem, ertapenem, doripenem and imipenem, respectively. For the other 119 non-susceptible isolates (31.48%), of 70 isolates were resistant to all carbapenems with inhibition zone diameters as 6 to 18 mm in ertapenem, 6 to 19 mm in meropenem, doripenem and imipenem. Meanwhile, other 59 of non-susceptible isolates showed intermediated or resistance inhibition zone diameters to only an antibiotic or more than one antibiotic in carbapenems group. The results of carbapenems susceptibility testing were statistically analyzed into resistant and susceptible percentage, divided into the following hospitals in each species of isolate.

Naresuan University hospital

A total of 18 *Enterobacteriaceae* isolates from Naresuan University hospital were determined for carbapenems susceptibility. Within the overall *Enterobacteriaceae* isolate, 13 isolates (72.22%) were susceptible to meropenem, ertapenem, doripenem and imipenem with inhibition zone diameters as 21 to 31 mm in each carbapenems. In total, 5 isolates (27.77%) of *K. pneumoniae* were resistant to all carbapenems with inhibition zone diameters as 10 to 14 mm in meropenems, 8 to 10 mm in ertapenem, 11 to 14 mm in doripenem, 13 to 16 mm in imipenem.

Budhachinnaraj hospital

Within the overall 34 isolates of *Enterobacteriaceae* from Budhachinnaraj hospital, only single isolate (2.94%) was susceptible to meropenem, ertapenam and doripenem with inhibition zone diameters as 26 to 27 mm in each drug. Nevertheless 2 isolates (5.88%) were susceptible to just only imipenem with inhibition zone diameters as 23 to 24 mm. In total, 33 isolates (97.06%) were resistant to meropenem, ertapenam and doripenem but 32 isolates were just resistant to imipenem. With the inhibition zone diameters as 6 to 17 mm in meropenem, 6 to 18 mm in doripenem and 6 to 14 mm in ertapenem and imipenem. Of the 33 individuals isolates were 26

K. pneumoniae isolates (78.79%), 5 *E. coli* isolates (15.15%) and 2 *E. cloacae* isolates (6.06%).

Srisangworn hospital

In total of 51 isolates of *Enterobacteriaceae* from Srisangworn hospital were determined as various susceptibilities in each carbapenems. The susceptibility of meropenem, 44 isolates (86.27%) were susceptible with inhibition zone diameters as 23 to 33 mm. Also, 5 isolates (9.8%) showed intermediated inhibition zone diameters as 21 to 22 mm and 2 isolates of *E. coli* (3.92%) were resistant to meropenem with inhibition zone diameters as 13 to 18 mm. For ertapenem susceptibility, 49 isolates (96.08%) were susceptible but 2 isolates of *E. coli* (3.92%) were resistant to ertapenem with inhibition zone diameters as 22 to 35 mm and 10 to 14 mm, respectively. In a part of doripenem susceptibility, 38 isolates (74.51%) were susceptible with inhibition zone diameters as 23 to 33 mm. Also, 11 isolates (21.57%) showed intermediated inhibition zone diameters as 20 to 21 mm and 2 isolates of *E. coli* and *K. pneumoniae* were resistant to doripenem with inhibition zone diameters as 18 to 19 mm. Last of all the susceptibility of imipenem were determined, 34 isolates (66.66%) were susceptible with inhibition zone diameters as 23 to 29 mm and showed intermediated inhibition zone of 14 isolates (27.45%) as 20 to 22 mm but 3 isolates (5.88%) of 2 *E. coli* and 1 *M. morganii* were resistant to imipenem with inhibition zone diameters as 14 to 18 mm.

Phetchabun hospital

A total of 142 *Enterobacteriaceae* isolates from Phetchabun hospital were determined for carbapenems susceptibility. Within the overall *Enterobacteriaceae* isolate, 113 isolates were (79.57%) susceptible to all carbapenems. They were susceptible to meropenem, ertapenem, doripenem and imipenem with inhibition zone diameters as 23 to 35 mm, 22 to 33 mm, 23 to 35 mm, 23 to 35 mm, respectively. For the other 29 non-susceptible isolates (20.42%), of 17 isolates (11.97%) were resistant to all carbapenems with inhibition zone diameters as 6 to 19 mm in meropenem, 6 to 18 mm in imipenem, ertapenem and doripenem. Meanwhile, 1 isolate of *E. coli*, 4 isolates of *E. cloacae*, 1 isolate of *Salmonella* spp., 1 isolate of *K. pneumoniae* were intermediated to ertapenem. Moreover, some of non-susceptible isolates were showed intermediated or resistance inhibition zone diameters to more than one antibiotics in

carbapenems. 1 isolate of *E. cloacae* was showed an intermediate inhibition zone diameters to ertapenem and imipenem, 2 isolates of *E. coli* were intermediate to meropenem and resistant to ertapenem, 1 isolate of K. pneumoniae was intermediate to doripenem and resistant to meropenem and ertapenem, 1 isolate of *C. freundii* was intermediate to imipenem and resistant to meropenem, ertapenem, doripenem as well.

Wichian Buri hospital

In total of 50 isolates of *Enterobacteriaceae* from Wichian Buri hospital were determined as various susceptibility in each carbapenems. Within the overall *Enterobacteriaceae* isolate, 40 isolates were (80%) susceptible to all carbapenems with inhibition zone diameters as 22 to 32 mm in each drugs. For the other 10 non-susceptible isolates (20%), 1 *Enterobacter* spp. and 1 *P. mirabilis* isolates were intermediate to ertapenem and imipenem, respectively. In addition, 3 isolates of *E. coli* and 2 isolates of *E. cloacae* were resistant to ertapenem. Moreover, some of non-susceptible isolates were showed intermediated or resistance inhibition zone diameters to more than one antibiotics in carbapenems. One isolate of *E. coli* was intermediate to meropenem and resistant to ertapenem, 1 isolate of *Enterobacter* spp. was intermediate to ertapenem and resistant to imipenem, 1 isolate of *K. pneumoniae* was intermediate to meropenem and resistant to and ertapenem and imipenem. Lastly, 1 isolate of *K. pneumoniae* was intermediate to meropenem and doripenem including ertapenem resistance.

Uthaithani hospital

In total of 64 isolates of *Enterobacteriaceae* from Uthaithani hospital were determined for carbapenems susceptibility. Within the overall *Enterobacteriaceae* isolate, 57 isolates (89.06%) were susceptible to all carbapenems. They were susceptible to meropenem with inhibition zone diameters as 28 to 37 mm, 25 to 38 mm in ertapenem, 26 to 39 mm in doripenem and imipenem. For the other 7 non-susceptible isolates, 6 isolates (9.38%) of 1 *E. coli* and 5 *K. pneumoniae* isolates were resistant to all carbapenems with inhibition zone diameters as 6 to 16 mm in meropenem, 6 to 15 mm in ertapenem, 6 to 18 mm in doripenem and imipenem, respectively. However, 1 isolate of *Enterobacter* spp. (1.56%) was just intermediate to only ertapenem with inhibition zone diameters as 15 mm.

Sawanpracharak hospital

A total of 19 *Enterobacteriaceae* isolates from Sawanpracharak hospital were determined for carbapenems susceptibility. Within the overall *Enterobacteriaceae* isolate, 7 isolates were (36.84%) susceptible to all carbapenems. They were susceptible to meropenem, ertapenem, doripenem and imipenem with inhibition zone diameters 31 to 41 mm, 25 to 35 mm, 31 to 37 mm, 27 to 36 mm, respectively. In total, 10 isolates (52.63%) of 2 *E. coli*, 6 *K. pneumoniae* and 2 *E. cloacae* isolates were resistant to all carbapenems with inhibition zone diameters as 6 to 19 mm in meropenems, 6 to 16 mm in ertapenem, 9 to 17 mm in doripenem, 13 to 19 mm in imipenem. Meanwhile, 1 isolate of *K. pneumoniae* (5.26%) was susceptible to imipenem with inhibition zone diameters as 23 mm but showed an intermediate to meropenem and doripenem with the same inhibition zone diameters as 22 mm and resistant to ertapenem with inhibition zone diameters as 16 mm. Also 1 isolate of *E.cloacae* (5.26%) was just intermediate to only imipenem with inhibition zone diameter as 20 mm, but resistant to meropenem, ertapenem, doripenem with inhibition zone diameters 17 mm, 9 mm, 6 mm, respectively.

The results of carbapenems susceptibility testing were statistically analyzed into resistant and susceptible percentage, divided into the following hospitals in each species of isolate. The susceptibility of *Enterobacteriaceae* isolates were presented in table 7 as follows.

Table 7 The susceptibility testing of carbapenems in clinical isolates from 7 clinical microbiology laboratories located in the lower north region of Thailand by disk diffusion method

Organisms	Hospitals														Total (378)			
	Naresuan University hospital (18)		Budhachinnaraj hospital (34)		Srisangworn hospital (51)		Phetchabun hospital (142)		Wichian Buri hospital (50)		Uthaithani hospital (64)		Sawanpracharak hospital (19)		Susceptible (S)		Non-Susceptible (NS)	
	S	NS	S	NS	S	NS	S	NS	S	NS	S	NS	S	NS	isolate	%	isolate	%
E. coli	3	-	-	7	20	11	73	12	23	4	38	1	5	2	162	42.86	37	9.78
K. pneumoniae	7	5	1	24	5	5	30	5	13	2	10	5	2	7	68	17.98	53	14.02
E. cloacae	-	-	-	2	1	2	4	9	-	-	1	-	-	3	6	1.59	16	4.23
C. freundii	2	-	-	-	-	-	1	2	-	-	2	-	-	-	5	1.32	2	0.53
C. diversus	-	-	-	-	1	-	-	-	1	-	-	-	-	-	2	0.53	-	-
Enterobacter spp.	1	-	-	-	-	1	-	-	-	4	-	1	-	-	1	0.26	6	1.59
P. mirabilis	-	-	-	-	1	1	3	-	1	1	2	-	-	-	7	1.85	2	0.53
S. marcescens	-	-	-	-	-	-	2	-	1	-	-	-	-	-	3	0.79	-	-
M. morganii	-	-	-	-	-	2	-	-	-	-	1	-	-	-	1	0.26	2	0.53
Salmonella spp.	-	-	-	-	1	-	-	1	-	-	3	-	-	-	4	1.06	1	0.26
Total (378)	13	5	1	33	29	22	113	29	39	11	57	7	7	12	259	68.52	119	31.48

Detection of carbapenemase production

A total of 119 non-susceptible isolates (shown an intermediated or resistant to at least a carbapenem agent) were used as samples for testing the production of carbapenemase by two approaches phenotypic method. Of 5 isolates (4.2%) shown positive results for only MHT. Meanwhile, 17 isolates (14.29%) shown positive results for only Carba-NP. The other isolates, 53 isolates (44.54%) shown positive result for both MHT and Carba-NP as well as 44 isolates (36.97%) shown negative result for both tests. Therefore, 75 isolates (63.03%) that positive for carbapenemase production from either MHT or Carba-NP test were considered to colistin resistance detection in further experiment. The results of the detection for carbapenemase producing strains were interpreted and statistically analyzed into percentage, divided into the following hospitals in each species of isolate as follow.

1. **Phenotypic Detection Method**

1.1 Modified Hodge test (MHT)

The Modified Hodge test (MHT) was detected carbapenemase production in carbapenems non-susceptible isolates. Within the overall of 119 non-susceptible isolates, 5 isolates (4.2%) shown positive results for only MHT and 53 isolates (44.54%) also shown positive result for both MHT and Carba-NP test. The results of the detection for carbapenemase producing strains were interpreted and statistically analyzed into percentage, divided into the following hospitals in each species of isolate. The detection of carbapenemase production by MHT was presented in table 8 as follows.

Naresuan University hospital

A total of 5 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. Within the overall, they were totally *K. pneumoniae* and shown positive result for MHT. For the interpretation, all of non-susceptible isolates were carbapenemase producing strains.

Budhachinnaraj hospital

A total of 33 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. All of non-susceptible isolate, 32 isolates were shown positive results for MHT. The carbapenemase producing

strains includes, 26 isolates of *K. pneumoniae*, 5 isolates of *E. coli* and 1 isolate of *E. cloacae.*

Srisangworn hospital

A total of 22 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. Within the overall of non-susceptible isolate, only 1 isolate of *E. cloacae* were carbapenemase producer.

Phetchabun hospital

A total of 29 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. All of non-susceptible isolate, 10 isolates were shown positive results for MHT. The carbapenemase producing strains includes 4 isolates of *E. coli,* 2 isolates of *K. pneumoniae*, 2 isolates of *E. cloacae* and 2 isolates of *C. freundii.*

Wichian Buri hospital

A total of 11 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. Within the overall of non-susceptible isolate, only 1 isolate of *E. cloacae* were carbapenemase producer.

Uthaithani hospital

A total of 7 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. All of non-susceptible isolates, only 1 isolate of *K. pneumoniae* was shown positive result indicating production of carbapenemase.

Sawanpracharak hospital

A total of 12 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. All of non-susceptible isolate, 8 isolates were shown positive results for MHT. The carbapenemase producing strains includes, 1 isolates of *E. coli,* 4 isolate of *K. pneumoniae* and 3 isolates of *E. cloacae.*

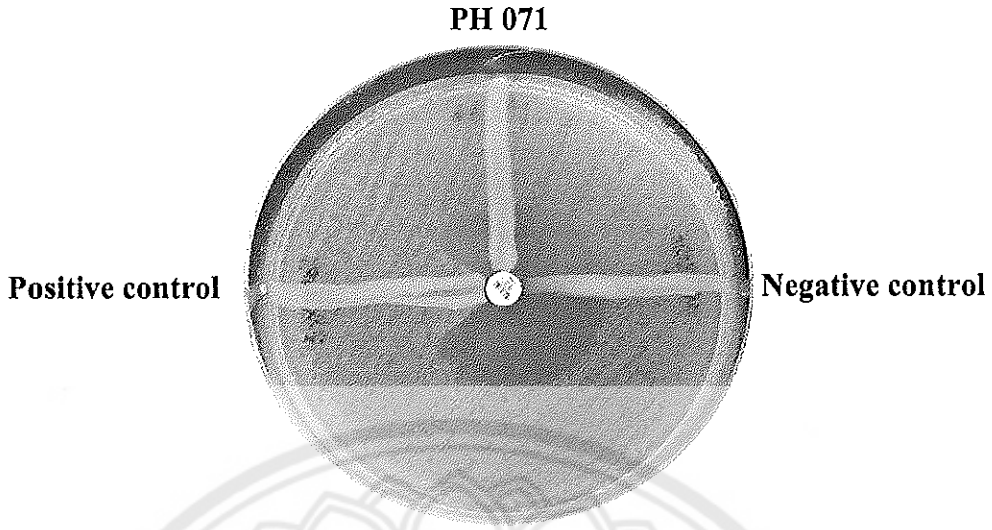


Figure 16 Representative a result of MHT using ETP disk (MHT positive control *K. pneumoniae* ATCC BAA 1705, MHT negative control *K. pneumoniae* ATCC BAA 1706 and test isolate of PH 071)

The positive control showed positive result with a clover leaf-like indentation of the *E. coli* ATCC 25922 strain growing along the test organism growth streak within the disk diffusion zone indicating production of carbapenemase whereas, the negative control and the isolate of PH 071 showed negative result with no growth of the *E. coli* ATCC 25922 along the test organism growth streak within the disk diffusion.

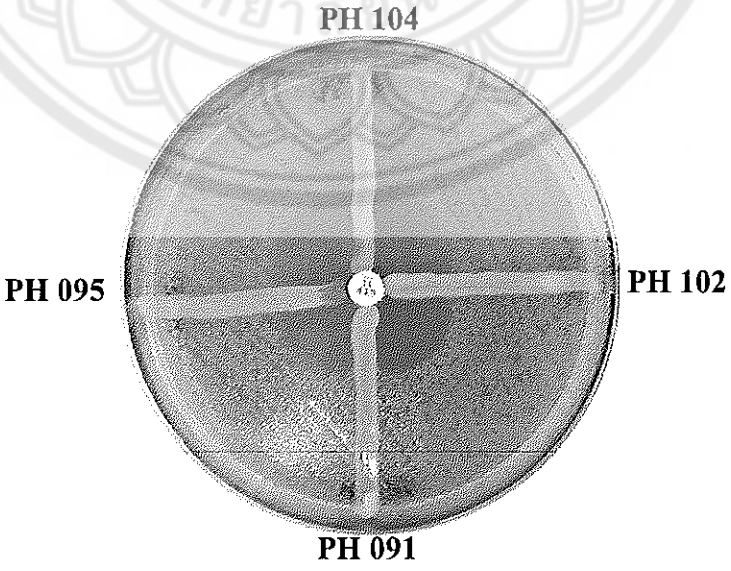


Figure 17 The isolates of *E. cloacae* PH091 and PH102 showed positive results, PH095 and PH104 showed negative results, respectively

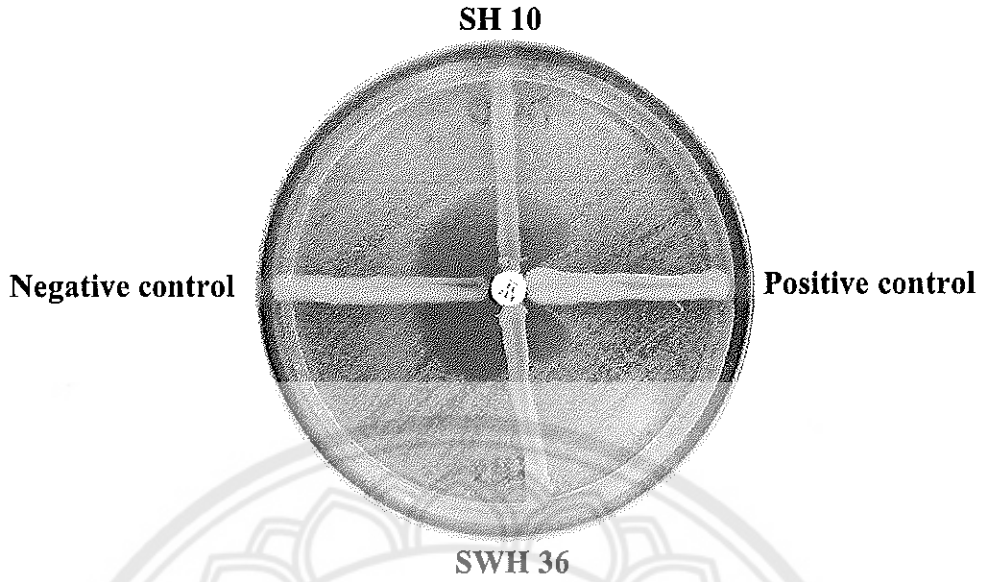


Figure 18 Representative a result of MHT with the negative result of *K. pneumoniae* SH10 and the positive result of *E. cloacae* SWH36 (MHT positive control *K. pneumoniae* ATCC BAA 1705, MHT negative control *K. pneumoniae* ATCC BAA 1706)

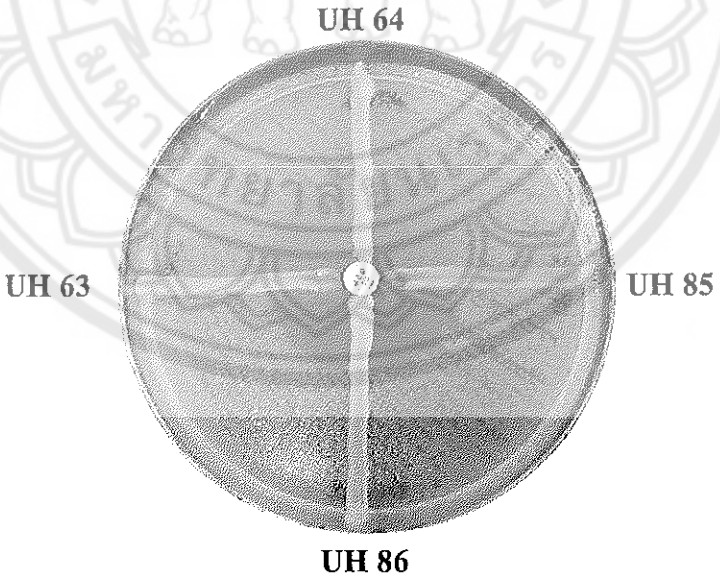


Figure 19 Representative a negative result of MHT with all clinical isolates of *K. pneumoniae* UH63, *K. pneumoniae* UH64, *E. coli* UH85 *and* *K. pneumoniae* UH86, respectively

1.2 Carba-NP test

The Carba-NP test was detected carbapenemase production in carbapenems non-susceptible isolates. Within the overall of 119 non-susceptible isolates, 17 isolates (14.29%) shown positive result for only Carba-NP and 53 isolates (44.54%) also shown positive result for both MHT and Carba-NP test. The results of the detection for carbapenemase producing strains were interpreted and statistically analyzed into percentage, divided into the following hospitals in each species of isolate. The detection of carbapenemase production by Carba-NP test was presented in table 9 as follows.

Naresuan University hospital

In total of 5 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. Within the overall, they were totally *K. pneumoniae* and shown positive result for Carba-NP test. For the interpretation, all of non-susceptible isolates were carbapenemase producing strains.

Budhachinnaraj hospital

A total of 33 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. All of non-susceptible isolate, 32 isolates were shown positive results for Carba-NP test. The carbapenemase producing strains includes, 26 isolates of *K. pneumoniae*, 5 isolates of *E. coli* and 1 isolate of *E. cloacae.*

Srisangworn hospital

A total of 22 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. Within the overall of non-susceptible isolate, 2 isolates were shown positive results for Carba-NP test. The carbapenemase producing strains includes, 1 isolate of *E. coli* and 1 isolate of *E. cloacae.*

Phetchabun hospital

A total of 29 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. All of non-susceptible isolate, 16 isolates were shown positive results for Carba-NP test. The carbapenemase producing strains includes, 8 isolates of *E. coli*, 1 isolate of *K. pneumoniae*, 5 isolates of *E. cloacae* and 2 isolates of *C.freundii.*

Wichian Buri hospital

A total of 11 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. Within the overall of non-susceptible isolate, there are none carbapenemase producer detected by the Carba-NP test

Uthaithani hospital

A total of 7 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. All of non-susceptible isolates, 6 were shown positive results for Carba-NP test includes, 1 isolate of *E. coli*, 1 isolate of *Enterobacter* spp. and 4 isolates of *K. pneumoniae*.

Sawanpracharak hospital

A total of 12 carbapenems non-susceptible *Enterobacteriaceae* isolates were detected for carbapenemase production. All of non-susceptible isolate, 9 isolates were shown positive results for Carba-NP test. The carbapenemase producing strains includes 2 isolates of *E. coli*, 4 isolates of *K. pneumoniae* and 3 isolates of *E. cloacae*.

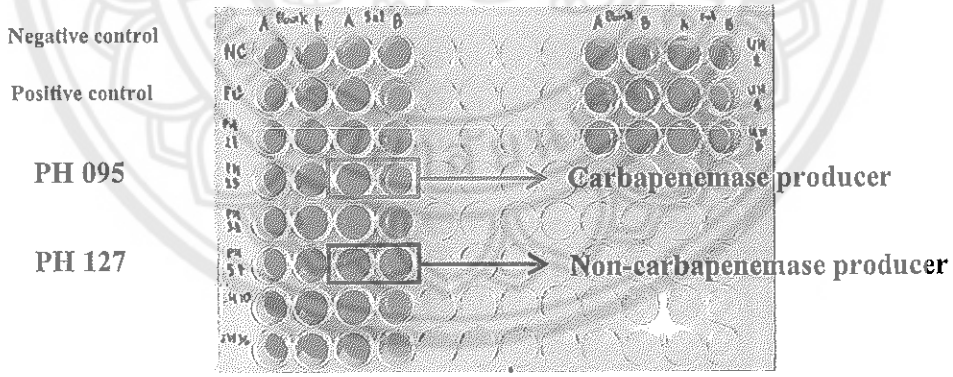


Figure 20 Representative a result of Carba-NP test based on acidification of phenol red when imipenem is hydrolyzed (evidenced by the color change of the test solution from red to yellow. The isolates of *E. cloacae* PH095 showed positive results and *K. pneumoniae* PH127 showed negative results, respectively, with the positive control of *K. pneumoniae* ATCC BAA 1705 and the negative control *K. pneumoniae* ATCC BAA 1706.)

Table 8 Detection of carbapenemase production in CRE by MHT and Carba NP test

Hospitals	CRE	Results							
		MHT				Carba-NP test			
		Negative		Positive		Negative		Positive	
		Isolates	%	Isolates	%	Isolates	%	Isolates	%
Naresuan university hospital (18)	5	-	-	5	4.2	-	-	5	4.2
Budhachinnaraj hospital (34)	33	1	0.84	32	26.89	1	0.84	32	26.89
Srisangworn hospital (51)	22	21	17.65	1	0.84	20	16.81	2	1.68
Phetchabun hospital (142)	29	19	15.97	10	8.4	13	10.92	16	13.45
Wichian Buri hospital (50)	11	10	8.4	1	0.84	11	9.24	-	-
Uthaithani hospital (64)	7	6	5.04	1	0.84	1	0.84	6	5.04
Sawanpracharak hospital (19)	12	4	3.36	8	6.72	3	2.52	9	7.56
Total (378)	119	61	51.26	58	48.74	49	41.18	70	58.82

Table 9 Detection of carbapenemase production in CRE by MHT and Carba NP test, divided into the following hospitals in each result of isolate

Detection of carbapenemase production		Hospitals							Total	%
MHT	Carba NP	NU (18)	BU (34)	SWH (51)	PH (142)	VH (50)	UH (64)	SH (19)	(378)	
Positive	Negative	-	-	-	2	1	-	2	5	4.2
Negative	Positive	-	-	1	8	-	5	3	17	14.29
Positive	Positive	5	32	1	8	-	1	6	53	44.54
Positive result for carbapenemase detection									75	63.03
Negative	Negative	-	1	20	11	10	1	1	44	36.97
Total of CRE (119)		5	33	22	29	11	7	12	119	
%		4.2	27.73	18.49	24.37	9.24	5.88	10.08		

Detection of colistin resistant

1. The screening of colistin resistant by colistin containing MHA

All of 75 carbapenemase producing strains (63.03%) were screened of colistin resistant by 2 μg/ml colistin containing MHA. The interpretation of colistin resistant screening was interpreted using reference breakpoint in accordance with The EUCAST (Susceptible ≤ 2 μg/ml, resistant ≥ 2 μg/ml). Within the overall strains, 7 isolates (5.88 %) includes, 4 isolates of *E. cloacae* and 3 isolates of *K. pneumoniae* were considered as colistin resistant. The results of the screening of colistin resistant were interpreted and statistically analyzed into percentage, divided into the following hospitals in each species of isolate as follow.

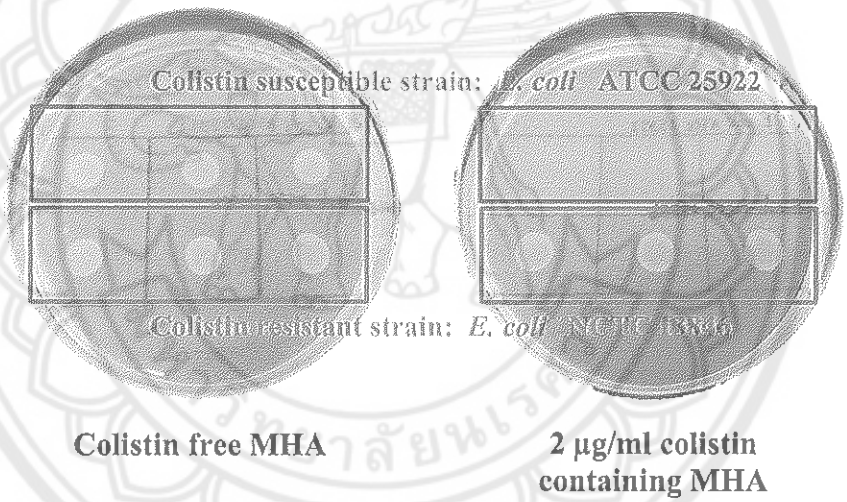


Figure 21 Representative the screening of colistin resistant by colistin containing MHA, the inoculum of QC organisms were spot on MHA to qualified test method.

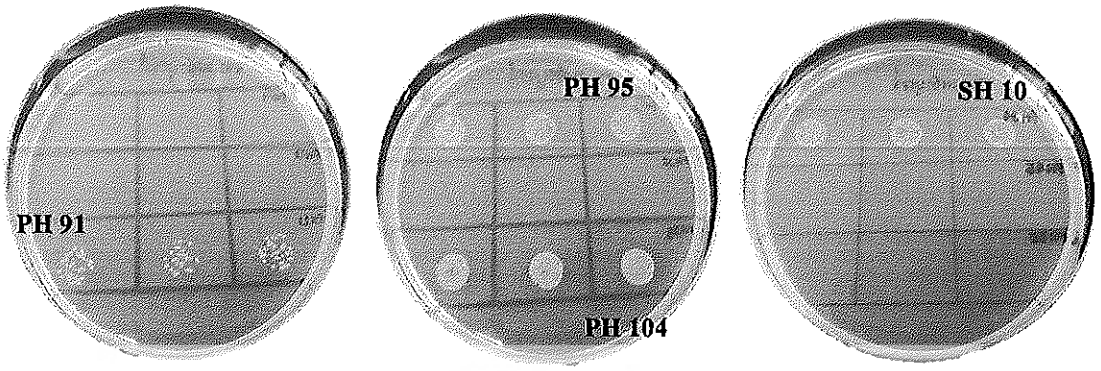


Figure 22 Representative the colistin resistant screening of the CRE clinical isolates. (If two or more colonies growth on plate, will be considered as colistin resistant using reference breakpoint in accordance with the EUCAST, Susceptible ≤ 2 μg/ml, resistant ≥ 2 μg/ml).

2. Colistin susceptibility testing

All of 7 colistin resistant isolates from screening test were detected for minimum inhibitory concentration (MIC) of colistin by agar dilution method ranging 0.125-128 µg/ml. Within the overall isolates shown colistin MICs ranging from 32 to ≥ 128µg/ml. Among 7 isolates, 1 isolate of *K. pneumoniae* UH063 shown resistant to colistin with MIC at 32 µg/ml, 2 isolates of *E. cloacae* PH091 and *K. pneumoniae* UH064 shown resistant to colistin with MIC at 64 µg/ml and 4 isolates of *E. cloacae* PH95, PH104, SWH036 and *K. pneumoniae* SH010 shown resistant to colistin with MIC at ≥ 128 µg/ml. The results of colistin susceptibility testing were interpreted and statistically analyzed into percentage, divided into the following hospitals in each species of isolate as follow.

Table 10 Determination of minimum inhibitory concentrations of colistin by Agar dilution (According to the EUCAST breakpoint for *Enterobacteriaceae*)

Code	Organism	Colistin concentration in agar dilution (µg/ml)											MIC
		0.125	0.25	0.5	1	2	4	8	16	32	64	128	
UH 063	*K. pneumoniae*	+	+	+	+	+	+	+	+	NG	NG	NG	32
PH 091	*E. cloacae*	+	+	+	+	+	+	+	+	+	NG	NG	64
UH 064	*K. pneumoniae*	+	+	+	+	+	+	+	+	+	NG	NG	64
PH 095	*E. cloacae*	+	+	+	+	+	+	+	+	+	+	+	>128
PH 104	*E. cloacae*	+	+	+	+	+	+	+	+	+	+	+	>128
SWH 036	*E. cloacae*	+	+	+	+	+	+	+	+	+	+	+	>128
SH 010	*K. pneumoniae*	+	+	+	+	+	+	+	+	+	+	+	>128

+ : in the table representative growth isolates of colistin resistant strains in each concentration of colistin MIC

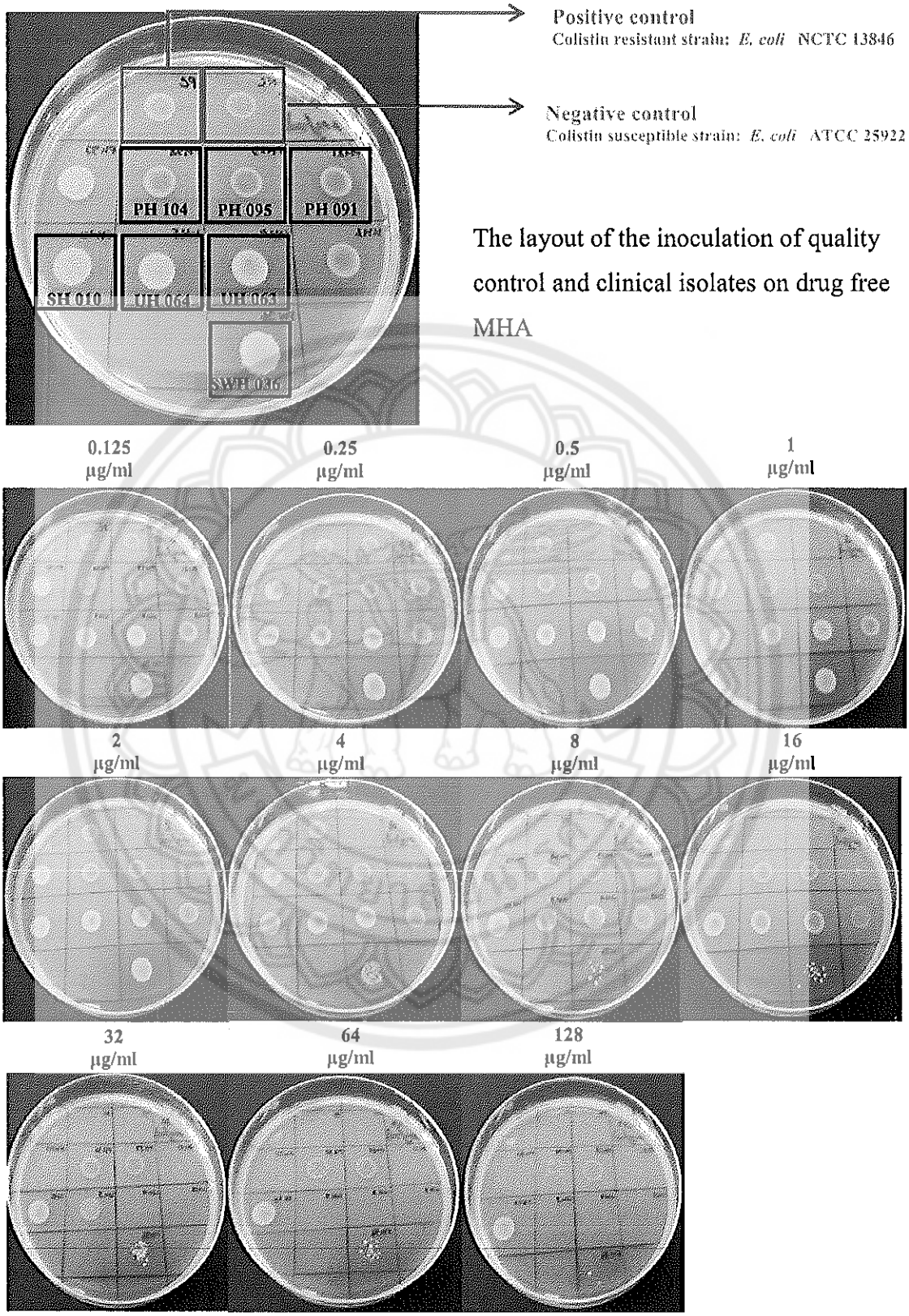


Figure 23 Representative the determination of MIC of colistin by agar dilution in ranging 0.125-128 µg/ml.

3. Genotypic detection method

The plasmid-mediated colistin resistance gene, *mcr-1*, were detected in colistin resistant strains by conventional PCR technique and the PCR products were detected by gel electrophoresis. The interpretation of specific product band (309 bp) was compared with the predicted result or molecular maker, including positive and negative control run on the same gel. A total of 7 colistin resistant isolates were negative to *mcr- 1* detection.

Figure 24 Representative a result of specific PCR product for mcr- 1 detection, (tracks 1 and 11 showed 100 bp DNA maker, track 2 showed positive control with the specific size PCR product band (309 bp), track 3 showed negative control and tracks 4-10 showed the results of, PH 091, PH 095, PH 104, SWH 036, UH 063, UH 064, SH 010, respectively.)

The additional data of carbapenemase producing, colistin resistant *Enterobacteriaceae*

1. Modified carbapenem inactivation method (mCIM)

In this present study, the mCIM was added in the final of experimental study. This method was performed for suspected carbapenemases production of carbapenemase-producing, colistin resistant *Enterobacteriaceae*, as a following protocol recently describes in CLSI guideline M100-S27

In this instance, in total of 7 isolates of carbapenemase-producing, colistin resistant *Enterobacteriaceae*, 6 isolates includes, *E. cloacae* PH91, *E. cloacae* PH95, *E. cloacae* PH104, *E. cloacae* SWH36, *K. pneumoniae* UH063 and *K. pneumoniae* SH10 were positive for mCIM. Otherwise, 1 isolate of *K. pneumoniae* UH064 was negative for mCIM

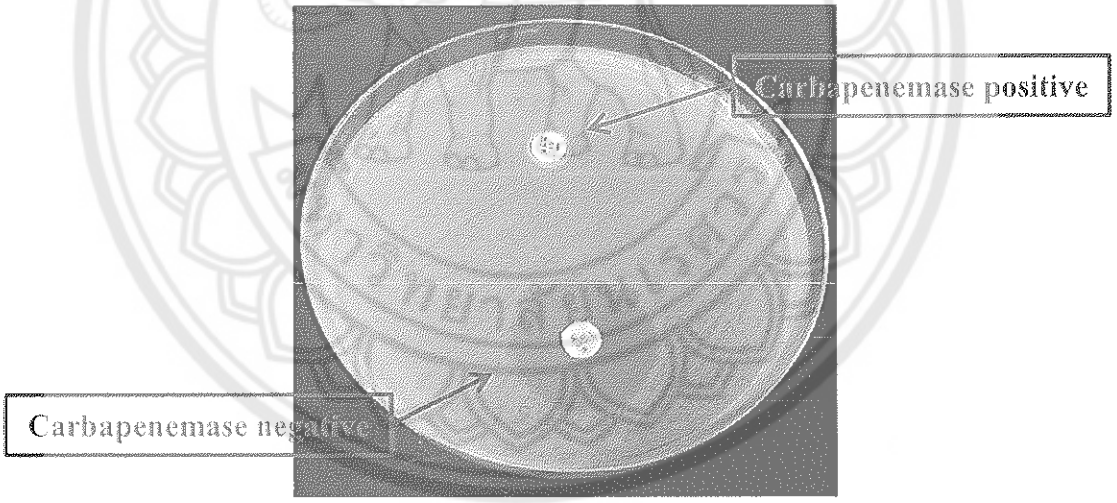


Figure 25 Representative a result of mCIM with carbapenamase positive *K. pneumoniae* ATCC BAA 1705 and carbapenamase negative *K. pneumoniae* ATCC BAA 1706

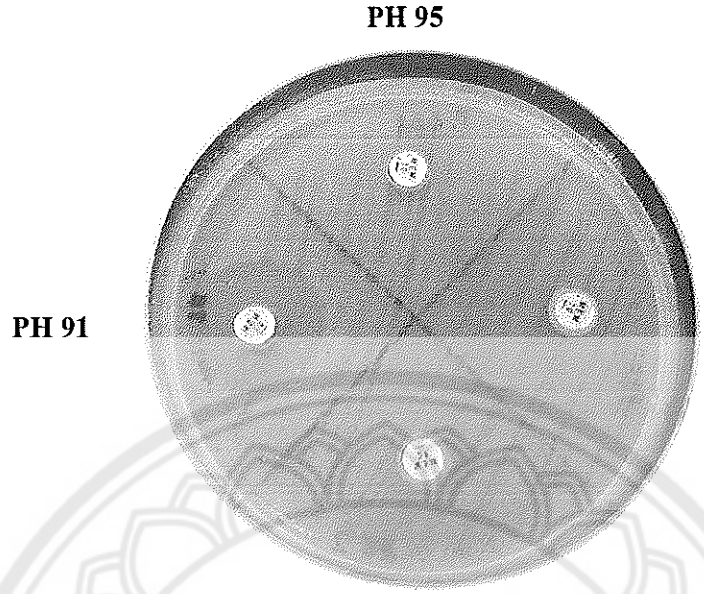


Figure 26 Representative the positive result of mCIM includes, PH95, PH104, SWH36 respectively, and presence of the pinpoint colonies within a 16–18 mm zone in an isolate of PH91

2. Genotypic detection of carbapenemase gene

Genotypic detection of carbapenemase gene was an additionally test to confirm the presence of carbapenems resistance genes. This study, the molecular method was performed to identify the precise carbapenemase gene of carbapenemase-producing, colistin resistant *Enterobacteriaceae* in the final of experimental study using AMR direct flow chip kit (Master Diagnóstica). DNA Flow Technology for The detection of antibiotic-resistant markers by multiplex PCR and reverse hybridization

In this present study, a total of 7 isolates of carbapenemase-producing, colistin resistant *Enterobacteriaceae*, the *NDM* was found in 4 isolates of *E. cloacae* PH95, *E. cloacae* PH104, *K. pneumoniae* UH063 and *K. pneumoniae* SH10. In addition, the extended-spectrum β-lactamase genes, *CTX-M* was found in *E. cloacae* PH91. Otherwise, 2 isolates of *E. cloacae* SWH36, *K. pneumoniae* UH064, the carbapenemase genes were not detected.

Table 11 Detection of carbapenemase production by phenotypic and genotypic methods

Code	Organism	Detection of carbapenemase production			
		Phenotypic detection			Genotypic detection
		MHT	Carba NP	mCIM	AMR direct flow chip kit
PH 91	*E. cloacae*	Positive	Positive	Positive	ESBL (CTX-M)
PH 95	*E. cloacae*	Negative	Positive	Positive	NDM
PH 104	*E. cloacae*	Negative	Positive	Positive	NDM
SWH 36	*E. cloacae*	Positive	Positive	Positive	ND
UH 63	*K. pneumoniae*	Negative	Positive	Positive	NDM
UH 64	*K. pneumoniae*	Negative	Positive	Negative	ND
SH 10	*K. pneumoniae*	Negative	Positive	Positive	NDM

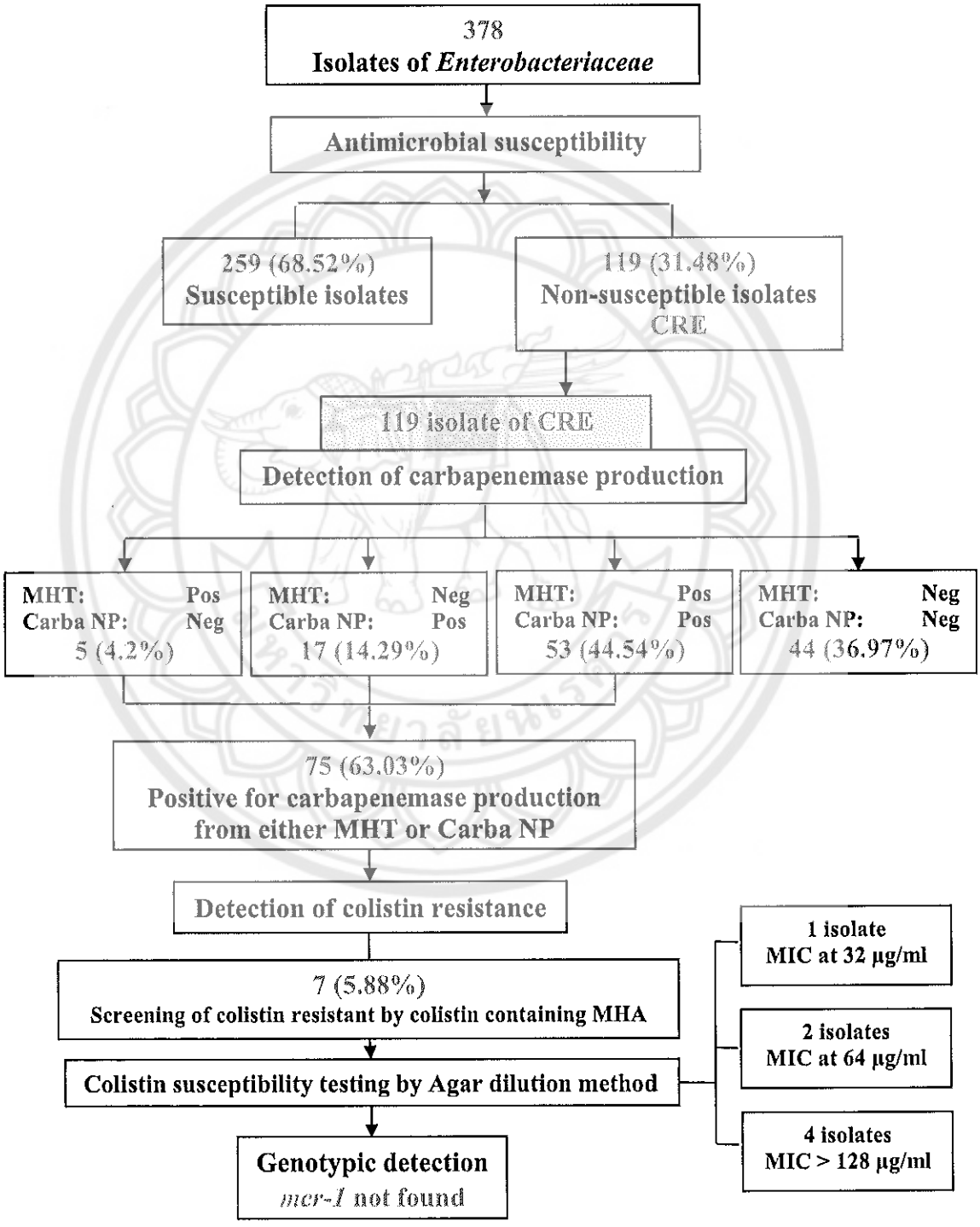
ND: β-lactam resistant and the carbapenemase gene not detected

Table 12 Correlation of carbapenems and colistin resistance in carbapenems resistant *Enterobacteriaceae* from 7 clinical microbiology laboratories located in the lower north region of Thailand.

Code	Organism	Antimicrobial susceptibility testing							Detection of carbapenemase production			Genotypic detection	
		Carbapenems					Colistin						
		Zone diameter (mm)				Interpretation	MIC (µg/ml)	Interpretation	MHT	Carba-NP	mCIM	Carbapenemase gene	*mcr-1* detection
		MEM	ETP	DOR	IMP								
PH 091	*E. cloacae*	6 (R)	6 (R)	6 (R)	6 (R)	Carbapenems resistant	64	Colistin resistant	Positive	Positive	Positive	ESBL (CTX-M)	Negative
PH 095	*E. cloacae*	6 (R)	6 (R)	6 (R)	6 (R)	Carbapenems resistant	>128	Colistin resistant	Negative	Positive	Positive	NDM	Negative
PH 104	*E. cloacae*	6 (R)	6 (R)	6 (R)	6 (R)	Carbapenems resistant	>128	Colistin resistant	Negative	Positive	Positive	NDM	Negative
SWH 036	*E. cloacae*	26 (S)	22 (S)	22 (S)	20 (I)	Imipenem intermediated	>128	Colistin resistant	Positive	Positive	Positive	ND	Negative
UH 063	*K. pneumoniae*	13 (R)	10 (R)	14 (R)	16 (R)	Carbapenems resistant	32	Colistin resistant	Negative	Positive	Positive	NDM	Negative
UH 064	*K. pneumoniae*	12 (R)	10 (R)	23 (R)	15 (R)	Carbapenems resistant	64	Colistin resistant	Negative	Positive	Negative	ND	Negative
SH 010	*K. pneumoniae*	6 (R)	10 (R)	9 (R)	13 (R)	Carbapenems resistant	>128	Colistin resistant	Negative	Positive	Positive	NDM	Negative

SUMMARY REPORTS

Detection of carbapenems and colistin resistance in carbapenems resistant *Enterobacteriaceae* by phenotypic and genotypic methods



CHAPTER V

DISSCUSSION

Multidrug resistance in *Enterobacteriaceae* are now spreading increasingly and becoming a major concern in public health worldwide. The emerging of CRE is one of the most important emerging antibiotic resistance traits correlate with the production of the carbapenem-hydrolysing β-lactamases. The emergence and global spread of carbapenemase producers in *Enterobacteriaceae* has been reported and possibly carried this resistance either on chromosome or acquired via plasmid mediated resistance that spread rapidly among *Enterobacteriaceae* family. The CRE have been emerged to different parts of the world and mostly identified belonging to main molecular classes includes class A, B and D β-lactamases. The situation of CRE in Asia, the over all regions were approximately 37.5% of their contributed epidemiology data, including the continuingly increasing of ESBL-producer and high-level carbapenem resistance among *Enterobacteriaceae* (163). For epidemiology of CRE in Thailand, The surveillance of CRE among clinical isolates of *Enterobacteriaceae* from the hospitals in Thailand during 2012-2013, the CRE prevalence in each regions were 21.9% (52/237) in the north, 27.4% (65/237) in the Northeastern, 24.5% (58/237) in the Central, 9.3 % (22/237) in Bangkok, 6.3 % (15/237) in the East and 10.6% (25/237) in the south of Thailand. Moreover, the previous study revealed the prevalence of CRE among 12,741 clinical isolates of *Enterobacteriaceae* at the largest University hospitals in Thailand was found 1.4% (181 isolates) (84). The overall epidemiology data, given the fact that the prevalence of CRE was increasing during the past decade.

In this present study, we determined the prevalence of CRE from 7 hospitals located in the lower north region of Thailand. A total of 378 clinical isolates of *Enterobacteriaceae*, 119 isolates were non-susceptible to carbapenems, showed as approximately 31.48% of the CRE prevalence among isolate in this study. Overall of CRE isolates were detected for carbapenemase production, the results showed that, just 5 isolates (4.2%) shown positive results for only MHT. Meanwhile, 17 isolates (14.29%) shown positive results for only Carba-NP. The other isolates, 53 isolates

(44.54%) shown positive result for both MHT and Carba-NP as well as 44 isolates (36.97%) shown negative result for both tests. Interestingly, the positive isolates for Carba-NP were mostly *K. pneumoniae.* It is possible that among these isolates maybe positive for class B carbapenemase thus, the yielded low sensitivity and specificity of MHT can cause for a false-negative results. Accordingly, the prevalence of CRE among clinical isolates of *Enterobacteriaceae* from the hospitals in Thailand during 2012-2013, the CRE prevalence in the north was 21.9% (52/237) with mostly NDM-1 has been identified from isolates (6). In addition, a recent study, the prevalence of carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* from Bangkok, Thailand, and their detection by the Carba-NP and mCIM tests revealed a total of 223 isolates (77.70%) carried at least one of carbapenemase genes. Moreover, all most of isolates, 160 isolates (71.75%) were found NDM gene, of which NDM-1 was detected approximately 95.63% (164). Therefore, the MHT result has a limited capability to detect of carbapenemase production and the results should be deliberately interpreted.

This uncorrelated result, referring to the different in terms of sensitivity and specificity of methods. MHT has variable sensitivity and specificity and in addition, they has some limitation, the high levels of expression of AmpC coupled with decreased permeability may be elucidate as carbapenems hydrolyzing enzyme and give rise to false positive interpretation. Also, weakly positive and false negative results are occasionally found in *MBLs*, and especially NDM-1. From previous study, showed that MHT was not well correlated with the genotypic detection of CRE (165). Other carbapenemase detection is Carba-NP test, with the advantages of cost-effective, rapid and reproducible (154). Nevertheless, they may yield invalids results in some OXA-producing strain. With a recent study, OXA-48-like group especially OXA-232 were found 87.5% (91/104) as the unique predominant type of carbapenemase gene in Bangkok, Thailand (164). Therefore, the high prevalence and spreading of OXA-type carbapenemases with a low activity and slow hydrolysis of enzyme may affect to the invalids results in this study. In addition, the efficacy of the lysis buffer in protein extraction may affect the yield of bacterial enzymatic solution. This study used an in-house bacterial protein extraction reagent containing Tris HCl, following the protocol of Carba-NP reagent preparation (154). For this reason, poor quality of in-house lysis buffer can cause a low efficacy of bacteriolyic activity to break cells. Therefore, the

important factors to be consider whether the lysis buffer are the quality of buffer includes a specific pH range, the function of salt to establish an ionic strength and the suitability to target enzyme. Several studies used the commercial extraction buffer (B-PER II), which is used to obtain high yield of enzyme production.

Another issue of concern was the screening criteria of CRE to detect the carbapenemase production as above-mentioned methods. The protocol of previous guidance to performing the screening test for suspected carbapenemase production in CRE before confirmatory test was done to be certain the result. The last protocol of screening test was performed when using interpretive criteria according to CLSI M100-S20 with the zone size of ertapenem and meropenem as 19-21 mm and 16-21 mm, respectively. In this study, the inclusion criterion of CRE to perform the detection of carbapenemase production was different from the screening test protocol. The isolates of *Enterobacteriaceae* were preliminary determined for the susceptibility to all carbapenems that showed non-susceptible to at least a carbapenem agent were selected as sample in the detection of carbapenemase production, when using the zone diameter interpretive criteria listed in CLSI M100-S24. Nevertheless, the screening even the confirmatory tests of carbapenamse production by MHT were behind the times and abrogated due to the low sensitivity and specificity of method. Nevertheless, the selection criterion of CRE to perform the detection of carbapenemase production by accepting to all non-susceptible isolates may affect to increase the number of samples due to the fact that the screening by susceptibility test using disk diffusion method was only qualitative methods. For this reason, some weakly resistant isolate that shown non-susceptible but inhibition zone was larger or interrelated intermedited and susceptible zone even though a single carbapenem was not a turely carbapenamase producer.

All of the carbapenemase detection methods as mentioned-above indicating that some have good specificity and sensitivity but none of them approaches 100%. To resolve a defect of these phenotypic assay, a new specific phenotypic method was recently described, the carbapenem inactivation method (CIM) was usually described in 2015. Then, the CIM was developed to modified carbapenem inactivation method (mCIM) by CLSI working group. This test has high sensitivity and specificity for the detection of a variety of carbapenemase, with equal or better sensitivity for the

detection of OXA-48-type carbapenemase in *Enterobacteriaceae*. Nevertheless, the mCIM be not able to distinguish between serine-based carbapenemases and MBLs, therefore the eCIM was established. The eCIM employs EDTA, in conjunction with the mCIM assay to differentiate serine from MBLs. However, mCIM with or without eCIM testing is not currently recommended for routine use (166). In this presence study, the mCIM was added in final of the experimental study for suspected the carbapenemse genes. Following the previously results, mCIM showed positive results in the clinical isolates which negative for carbapenemase detection by MHT. Futhermore, the genotypic assay was also performed to confirm the presence of carbapenemase gene. A total of tested isolates, *NDM* and the extended-spectrum β-lactamase gene, *CTX-M* were detected. Evidently, the results of phenotypic detection can be preliminary guidelines for carbapenemase classification. However, as in all phenotypic assays, carbapenemase detection still requires genotypic assay for the precise identification of carbapenamase gene. Overall of epidemiology and supporting data, the increasing of CRE and the use of other antibiotic include β-lactam is ineffective.

Colistin was reintroduced into clinical practice for the treatment of multidrug resistant gram negative bacteria. Moreover, colistin has emerged as a last therapeutic option to against the life-threatening infections of carbapenem resistant gram negative bacteria. Nevertheless, the increasing of colistin usage has led to the emergence of colistin resistance in isolates of gram negative bacilli and CRE pathogen (7).

From previous study, colistin resistant in gram negative bacteria has evolved chromosomal encoded mechanism (9). The susceptibility strains may alter to a resistant phenotype through modification of LPS charge (167). The mechanism is associated with two-component systems by the alteration of LPS via lipid A modification. The development of colistin resistance can occur through mutation or adaption mechanism (87). Very recently reported, the emergence of *mcr-1* plasmid mediated colitin resistance via horizontal gene transfer has been readily spread through bacterial population, a world first routine surveillance project on antimicrobial resistance in commensal *E. coli* from food animal in China (9).

This present study was interested in a point of major epidemiological concern and the development of colistin resistance among CRE isolates. Within overall 75

carbapenemase producing strains (63.03%), 7 isolates (5.88%) includes, 4 isolates of *E. cloacae* and 3 isolates of *K. pneumoniae* were resistant to colistin and showed MICs ranging from 32 to ≥ 128 µg/ml. with the higher MIC level might be explained by the accuracy of susceptibility methods. Several data suggest good concordance between agar dilution and microbroth dilution. However, the diffusion of colistin is poorly diffuse through solid agar and the higher MICs have been reported using agar dilution (168). Therefore, it is possible that the susceptibility results may actually 2 to 4 fold higher (8). There remain many factors regarding colistin susceptibility, including the effect of the sulphate and sulphomethyl derivertive, anion content and pH on susceptibility test results. Nevertheless, in a part of genetic detection, a total of 7 colistin resistant isolates were negative to *mcr- 1* detection. From this result indicating that the plasmid-mediated colistin resistance gene, *mcr- 1*, might not correlated with these resistant strains.

In case of non *mcr-1* mediated colistin resistance, the development of colistin resistant mainly through the mutation of regulatory system among the main genetic factor mediating colistin resistant is attributed to a two-component system includes, *PmrA/ PmrB* and *PhoP/PhoQ* (167). Interestingly, previous study point out the evident that, clinical use of colistin may not be the only reason for an emergence of colistin resistant and showed that the resistant isolate can colonize in healthy individuals or no exposed person as well. Therefore, the factors besides colistin treatment use are related to the occurrence of colistin resistance in human (11). The development of colistin resistance exists even though the absence of colistin exposure both in healthy individuals and patient. This kind of phenomenon might explain by the affecting of "cross-resistance" between host cationic antimicrobial peptide (LL-37 and lysozyme) and polymyxin anbibiotic. This tolerance or resistance is occur as a result of exposure to similary substance (170).

Moreover, the horizontal transmission of acquired resistant from livestock to human is another reason that is possibly occurring (171). The genetic movement of insertion sequence within the genome and plasmids of bacteria is a common driver of diversity and genetic evolution in context of antibiotic resistance (11). The inactivation of *mgrB* gene regulator is associated with colistin resistance though the different forms includes, truncation with various ISs, nonsense mutation leading to premature stop

codon or missense mutation (11). From recently report in Italy, the isolates of *Enterobacteriaceae* can become resistant to colistin through interruption of *mgrB* by a plasmid-borne insertion sequence (IS) (172). These insertion includes, IS5-like insertion and the same loci insertion of the isolates of *Enterobacteriaceae* has an IS1 insertion in *mgrB,* as reported in Spain and Thailand (173).

From the study of correlation of β-lactamase production and colistin resistance among *Enterobacteriaceae* from a global surveillance program has been reported, the large collection of *Enterobacteriaceae* isolates, of 306 (1.6%) isolates without intrinsic resistance were resistant to colistin antibiotic. Moreover, with over two-third of *Enterobacter spp.* were more commonly resistant to colistin. The second most common species was *K. pneumoniae* (2.4%) and the lower percentage of colistin resistant were observed among *Citrobacter spp.* and *E. coli* in range of 0.1% to 0.6%, respectively. Compared to the colistin resistance of other genera, isolate of *Enterobacter spp.* were mose commonly resistant to colistin (8). The incidence of colistin resistance among carbapenamase producing *Enterobacteriaceae* has showed the reginal distribution of resistance as similar as in several regions include, 1.8% in Europe, 1.5% in Latin America, 1.4% in Middle East-Africa, 1.3% in North America, and 1.3% in Asia pacific. The interesting data revealed that, 482 carbapenemase producing *Enterobacteriaceae* from overall collection isolates, 58 isoalates were resistant to colistin and the rate of colitin susceptibility was reduced to 88.0% among isolates that did not express a carbapenemase enzyme. (8). In addition, previous study of worldwide emergence of colistin resistance in *K. pneumoniae*, a total of 212 stool samples were collected from healthy human and patient in Thailand. All of isolates, 14 isolates were resistant to colistin giving prevalence of 6.6% in range of MICs 4 to 64 mg/l (11).

A recent study, *mcr-1* gene was found in colistin resistant isolates from humans in Thailand. From all over 317 *Enterobacteriaceae* clinical isolates during 2014-2017, all isolates were screened for the *mcr-1* gene by PCR. The prevalence of *mcr-1* gene in colistin resistant isolates were 29.7% (11/37) in *E. coli* and 1.4 % (4/280) in *K.pneumoniae* with MICs of 4-32 μg/ml and 4-<128 μg/ml, respectively. Another factor of interest was the specific primer sequence for screening mcr-1 gene by PCR using forward5'GTGTGGTACCGACGCTCGG 3' and reverse 5' CAAGCCC

AATCGGCGCATC-3' that designed for specific part of *mcr-1* amplicon 460 bp. The difference of primer design is a major factor in genotypic detection. With the variation of target gene in different host organism and individual condition of PCR technique thus, a specific primer design is very significant for *mcr-1* detection.

In the present study, the results revealed that the isolates of carbapenemase producing colistin-resistant *Enterobacteriaceae* were not related to the plasmid-mediated colistin resistance gene, *mcr- 1*. Although, In case of non *mcr-1* mediated, the hypothesis of the development of colistin resistant in most of *Enterobacteriaceae* isolates are possible associated with the inactivation of regulatory system within chromosomal of bacterial strain has been supported. However, the molecular mechanism mediated colistin resistance among these isolates should be investigated to prove this hypothesis in further study.

Although, the finding of colistin resistance among CRE was low prevalence and infections of humans with *mcr-1* producing *Enterobacteriaceae* were rarely found (13-14). Nevertheless, arising of plasmid-borne antibiotic resistance genes harbor mobile genetic element either ISs or plasmid transfer between strains within the gut is a potential meachanism of indiresct acquisition of colistin and other antibiotic resistance as well (11). Furthermore, high mortality rate have been observed among patients with infection due to colistin resistant CRE (174). With the combination of resistance mechanism and the therapeutic options are currently limited. Therefore, it is necessary to continually monitor the activity of drug treatment to retrain the emergence of carbapenemase producing colistin-resistant *Enterobacteriaceae*.

CHAPTER VI

CONCLUSION

In conclusion, this present study revealed the prevalence of CRE from 7 hospitals clinical microbiology laboratory includes, Naresuan university hospital, Budhachinnaraj hospital, Srisangworn hospital, Phetchabun hospital, Wichian Buri hospital, Uthaithani hospital, Sawanpracharak hospital, entirely located in the lower north of Thailand. The CRE prevalence was approximately 31.48% and all of CRE isolates were detected for carbapenemase production by phenotypic methods. The detection of carbapenemase production revealed that, 5 isolates (4.2%) shown positive results for only MHT. Meanwhile, 17 isolates (14.29%) shown positive results for only Carba NP test. The other isolates, 53 isolates (44.54%) shown positive result for both MHT and Carba NP test as well as 44 isolates (36.97%) shown negative result for both tests. In total of 75 carbabenemase producing strains, of 7 isolates (5.88%) were resistant to colistin with MICs ranging from 32 to $\geq$ 128 µg/ml. In addition, *NDM* and the extended-spectrum β-lactamase gene, *CTX-M* were detected among the carbapenemase-producing, colistin resistant *Enterobacteriaceae* isolate. However, the involvement of Carbapenemase-producing *Enterobacteriaceae* in colistin resistant isolates was not related to the plasmid-mediated colistin resistance gene, *mcr- 1*. Therefore, the molecular mechanism mediated colistin resistance among these isolates should be investigated for the precise identification to prove this hypothesis in further study.

Nevertheless, this found is preliminary prevalence with the increasing of antibiotic combination resistance is becoming a major public health concern. The information documented in this study may offer significant help to be careful and appropriate use.of antibiotic along with empirical antibiotic management. In addition, the restriction of the antibiotic use in veterinary, particularly colistin use should be seriously reappraisal to preserve the last resort antibiotic to against MDR for patient with severe infection. Therefore, it is necessary to continually monitoring the

emergence of carbapenemase-producing colistin resistant *Enterobacteriaceae* to prevent an uncontrollable pandemic.

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APPENDIX

Research Instrument

1. **Culture media**
   - MacConkey agar
   - Mueller-Hinton agar
   - Tryptic soy broth
   - Blood agar
   - Nutrient agar
2. **Biochemical test**
   - Triple sugar iron agar (TSI)
   - Motile Indole Lysine (MIL)
   - Citrate
   - Malonate
   - Urease
3. **Chemicals and reagents**
   - 0.85% Normal saline
   - 70% Alcohol
   - Imipenem, Ertapenem, Meropenem and Doripenem disks
   - colistin powder
   - 10 mM Zinc sulfate heptahydrate solution
   - 0.5% Phenol red solution
   - 0.1N Sodium hydroxide solution
   - 10% HCl
   - Tris HCl buffer
   - Imipenem-cilastatin powder
4. **Instruments**
   - Biological safety cabinet class II
   - Incubator
   - Autoclave
   - Analytical balance
   - Hotplate
   - Vortex

Water bath

Bunsen burner

Cold room

5. Equipments

Erlenmeyer flask 1000 ml

Erlenmeyer flask 500 ml

Erlenmeyer flask 125 ml

Volumetic flask 250 ml

Volumetic flask 50 ml

Cylinder 25 ml

Beaker 100 ml

Beaker 50 ml

Duran bottles 500 ml

Serological pipette 10 ml

Serological pipette 2 ml

Serological pipette 1 ml

Petri dish

Test tube 13×100

Amber glass bottle

96 well microtiter plate

Microcentrifuge tube

Test tube rack

Auto-pipette 20-200 µl

Auto-pipette 100-1000 µl

Forcep

Loop

Needle

Tip

Pasture pipett

Stirring Rod

Swab

Rubber bulb

Bacterial identification

In total of 384 non-duplicated clinical isolates from 7 separate hospitals clinical microbiology laboratories were cultured and identified by biochemical methods. This identification included 378 isolates (98.43%) of *Enterobacteriaceae* and 6 isolates (1.56%) of non-*Enterobacteriaceae*. A Total of identified isolated are separately reported in each hospital and presented in table 6 as follows

Bacterial identification in clinical isolates from Naresuan University hospital by biochemical test (18 isolates)

Bacterial	Results	
	Isolates	percentage
E. coli	3	16.66
K. pneumoniae	12	66.66
C. freundii	2	11.11
Enterobacter spp.	1	5.55
Total of *Enterobacteriaceae*	18	100
Total	**18**	

Bacterial identification in clinical isolates from Budhachinnaraj hospital by biochemical test (34 isolates)

Bacterial	Results	
	Isolates	percentage
E. coli	7	14.70
K. pneumoniae	25	76.47
E. cloacae	2	5.88
Total of *Enterobacteriaceae*	34	100
Total	**34**	

Bacterial identification in clinical isolates from Srisangworn hospital by biochemical test (51 isolates)

Bacterial	Results	
	Isolates	percentage
E. coli	31	60.78
K. pneumoniae	10	19.60
E. cloacae	3	5.88
P. mirabilis	2	3.92
M. morganii	2	3.92
C. diversus	1	1.96
Salmonella spp.	1	1.96
Enterobacter spp	1	1.96
Total of *Enterobacteriaceae*	51	100
Total	**51**	

Bacterial identification in clinical isolates from Phetchabun hospital by biochemical test (145 isolates)

Bacterial	Results	
	Isolates	percentage
E. coli	85	58.62
K. pneumonia	35	24.13
E. cloacae	13	8.97
P. mirabilis	3	2.07
C. freundii	3	2.07
S. marcesens	2	1.38
Salmonella spp.	1	0.69
Total of *Enterobacteriaceae*	142	97.93
A. hydrophila	2	1.38
A. veronii	1	0.69
Total of non-*Enterobacteriaceae*	3	2.07
Total	**145**	

Bacterial identification in clinical isolates from Wichian Buri hospital by biochemical test (52 isolates)

Bacterial	Results	
	Isolates	percentage
E. coli	27	51.92
K. pneumoniae	15	28.85
Enterobacter spp	4	7.69
P. mirabilis	2	3.85
C. diversus	1	1.92
S. marcesens	1	1.92
Total of *Enterobacteriaceae*	50	96.15
A. baumannii	2	3.85
Total of non-*Enterobacteriaceae*	2	3.85
Total	52	

Bacterial identification in clinical isolates from Uthaithani hospital by biochemical test (65 isolates)

Bacterial	Results	
	Isolates	percentage
E. coli	39	60
K. pneumoniae	15	23.08
C. freundii	2	3.08
M. morganii	1	1.54
P. mirabilis	2	3.08
E. cloacae	1	1.54
Enterobacter spp.	1	1.54
Salmonella group B	2	3.08
Salmonella group D	1	1.54
Total of *Enterobacteriaceae*	64	98.46
A. baumannii	1	1.54
Total of non-*Enterobacteriaceae*	1	1.54
Total	65	

Bacterial identification in clinical isolates from Sawanpracharak hospital by biochemical test (19 isolates)

Bacterial	Results	
	Isolates	percentage
E. coli	7	36.84
K. pneumoniae	9	47.37
E. cloacae	3	15.79
Total of *Enterobacteriaceae*	19	100
Total	19	

Zone of carbapenems susceptibility testing of *Enterobacteriaceae* from Naresuan University hospital by disk diffusion method (18 isolates)

Antibiotics	Results		
	Zone diameter (mm)	Isolates	Percentage (%)
Meropenem			
Susceptible (S) (≥23 mm)	26-30	13	72.22
Intermediate (I) (20-22 mm)	-	-	-
Resistance (R) (≤19 mm)	10-14	5	27.78
Ertapenem			
Susceptible (S) (≥22 mm)	22-31	13	72.22
Intermediate (I) (19-21 mm)	-	-	-
Resistance (R) (≤18 mm)	8-10	5	27.78
Doripenem			
Susceptible (S) (≥23 mm)	23-29	13	72.22
Intermediate (I) (20-22 mm)	-	-	-
Resistance (R) (≤19 mm)	11-14	5	27.78
Imipenem			
Susceptible (S) (≥23 mm)	21-28	13	72.22
Intermediate (I) (20-22 mm)	-	-	-
Resistance (R) (≤19 mm)	13-16	5	27.78

Zone diameter of carbapenems susceptibility testing of *Enterobacteriaceae* from Budhachinnaraj hospital by disk diffusion method (34 isolates)

Antibiotics	Results		
	Zone diameter (mm)	Isolates	Percentage (%)
Meropenem			
Susceptible (S) (≥23 mm)	26	1	5.88
Intermediate (I) (20-22 mm)	-	-	-
Resistance (R) (≤19 mm)	6-17	33	94.12
Ertapenem			
Susceptible (S) (≥22 mm)	27	1	2.94
Intermediate (I) (19-21 mm)	-	-	-
Resistance (R) (≤18 mm)	6-14	33	97.06
Doripenem			
Susceptible (S) (≥23 mm)	26	1	2.94
Intermediate (I) (20-22 mm)	-	-	-
Resistance (R) (≤19 mm)	6-18	33	97.06
Imipenem			
Susceptible (S) (≥23 mm)	23-24	2	2.94
Intermediate (I) (20-22 mm)	-	-	-
Resistance (R) (≤19 mm)	6-14	32	97.06

Zone diameter of carbapenems susceptibility testing of *Enterobacteriaceae* from Srisangworn hospital by disk diffusion method (51 isolates)

Antibiotics	Results		
	Zone diameter (mm)	Isolates	Percentage (%)
Meropenem			
Susceptible (S) (≥23 mm)	23-33	44	86.27
Intermediate (I) (20-22 mm)	21-22	5	9.80
Resistance (R) (≤19 mm)	13-18	2	3.92
Ertapenem			
Susceptible (S) (≥22 mm)	22-35	49	96.08
Intermediate (I) (19-21 mm)	-	-	-
Resistance (R) (≤18 mm)	10-14	2	3.92
Doripenem			
Susceptible (S) (≥23 mm)	23-33	38	74.51
Intermediate (I) (20-22 mm)	20-21	11	21.57
Resistance (R) (≤19 mm)	18-19	2	3.92
Imipenem			
Susceptible (S) (≥23 mm)	23-29	34	66.66
Intermediate (I) (20-22 mm)	20-22	14	27.45
Resistance (R) (≤19 mm)	14-18	3	5.88

Zone diameter of carbapenems susceptibility testing of *Enterobacteriaceae* from Phetchabun hospital by disk diffusion method (142 isolates)

Antibiotics	Results		
	Zone diameter (mm)	Isolates	Percentage (%)
Meropenem			
Susceptible (S) (≥23 mm)	23-35	121	85.21
Intermediate (I) (20-22 mm)	21-22	2	1.40
Resistance (R) (≤19 mm)	6-19	19	13.38
Ertapenem			
Susceptible (S) (≥22 mm)	22-33	113	79.58
Intermediate (I) (19-21 mm)	19-21	8	5.63
Resistance (R) (≤18 mm)	6-18	21	14.79
Doripenem			
Susceptible (S) (≥23 mm)	23-35	123	86.62
Intermediate (I) (20-22 mm)	20	1	0.70
Resistance (R) (≤19 mm)	6-18	18	12.68
Imipenem			
Susceptible (S) (≥23 mm)	23-35	123	86.62
Intermediate (I) (20-22 mm)	22	2	1.40
Resistance (R) (≤19 mm)	6-18	17	11.97

Zone diameter of carbapenems susceptibility testing of *Enterobacteriaceae* from Wichian Buri hospital by disk diffusion method (50 isolates)

Antibiotics	Results		
	Zone diameter (mm)	Isolates	Percentage (%)
Meropenem			
Susceptible (S) (≥23 mm)	23-32	47	94.00
Intermediate (I) (20-22 mm)	20-22	3	6.00
Resistance (R) (≤19 mm)	-	-	-
Ertapenem			
Susceptible (S) (≥22 mm)	22-32	40	80.00
Intermediate (I) (19-21 mm)	19-21	2	4.00
Resistance (R) (≤18 mm)	11-18	8	16.00
Doripenem			
Susceptible (S) (≥23 mm)	23-31	49	98.00
Intermediate (I) (20-22 mm)	22	1	2.00
Resistance (R) (≤19 mm)	-	-	-
Imipenem			
Susceptible (S) (≥23 mm)	23-30	47	94.00
Intermediate (I) (20-22 mm)	22	1	2.00
Resistance (R) (≤19 mm)	16-18	2	4.00

Zone diameter of carbapenems susceptibility testing of *Enterobacteriaceae* from Uthaithani hospital by disk diffusion method (64 isolates)

Antibiotics	Results		
	Zone diameter (mm)	Isolates	Percentage (%)
Meropenem			
Susceptible (S) (≥23 mm)	28-37	58	90.62
Intermediate (I) (20-22 mm)	-	-	-
Resistance (R) (≤19 mm)	6-16	6	9.37
Ertapenem			
Susceptible (S) (≥22 mm)	25-38	57	89.06
Intermediate (I) (19-21 mm)	-	-	-
Resistance (R) (≤18 mm)	6-15	7	10.93
Doripenem			
Susceptible (S) (≥23 mm)	26-39	59	90.62
Intermediate (I) (20-22 mm)	-	-	-
Resistance (R) (≤19 mm)	6-18	5	9.37
Imipenem			
Susceptible (S) (≥23 mm)	23-46	58	90.62
Intermediate (I) (20-22 mm)	-	-	-
Resistance (R) (≤19 mm)	6-18	6	9.37

Zone diameter of carbapenems susceptibility testing of *Enterobacteriaceae* from Sawanpracharak hospital by disk diffusion method (19 isolates)

Antibiotics	Results		
	Zone diameter (mm)	Isolates	Percentage (%)
Meropenem			
Susceptible (S) (≥23 mm)	31-41	7	36.84
Intermediate (I) (20-22 mm)	22	1	5.26
Resistance (R) (≤19 mm)	6-19	11	57.89
Ertapenem			
Susceptible (S) (≥22 mm)	25-35	7	36.84
Intermediate (I) (19-21 mm)	-	-	-
Resistance (R) (≤18 mm)	6-16	12	63.15
Doripenem			
Susceptible (S) (≥23 mm)	31-37	7	36.84
Intermediate (I) (20-22 mm)	22	1	5.26
Resistance (R) (≤19 mm)	6-17	11	57.89
Imipenem			
Susceptible (S) (≥23 mm)	27-36	8	42.10
Intermediate (I) (20-22 mm)	20	1	5.26
Resistance (R) (≤19 mm)	13-19	10	52.63

Detection of carbapenemase production

The detection of carbapenemase production by MHT and Carba-NP test

Naresuan University hospital (18 isolates) 5 isolates non-susceptible to carbapenems

Code	Organism	Carbapenemase detection methods	
		MHT	Carba NP
NH 003	*K. pneumoniae*	positive	positive
NH 004	*K. pneumoniae*	Positive	Positive
NH 005	*K. pneumoniae*	Positive	Positive
NH 011	*K. pneumoniae*	positive	positive
NH 014	*K. pneumoniae*	positive	positive

Budhachinnaraj hospital (34 isolates) 33 isolates non-susceptible to carbapenems

Code	Organism	Carbapenemase detection methods	
		MHT	Carba NP
BH 001	*E. coli*	positive	positive
BH 002	*K. pneumoniae*	positive	positive
BH 003	*K. pneumoniae*	positive	positive
BH 004	*K. pneumoniae*	positive	positive
BH 005	*K. pneumoniae*	positive	positive
BH 008	*K. pneumoniae*	positive	positive
BH 009	*K. pneumoniae*	positive	positive
BH 010	*K. pneumoniae*	positive	positive
BH 011	*K. pneumoniae*	positive	positive
BH 012	*K. pneumoniae*	positive	positive
BH 014	*E. coli*	positive	positive
BH 015	*E. coli*	positive	positive
BH 016	*K. pneumoniae*	positive	positive

Code	Organism	Carbapenemase detection methods	
		MHT	Carba NP
BH 017	*K. pneumoniae*	positive	positive
BH 018	*E. cloacae*	positive	positive
BH 020	*E. coli*	positive	Positive
BH 022	*K. pneumoniae*	positive	positive
BH 024	*K. pneumoniae*	positive	positive
BH 025	*K. pneumoniae*	positive	positive
BH 026	*K. pneumoniae*	positive	positive
BH 027	*K. pneumoniae*	positive	positive
BH 028	*K. pneumoniae*	positive	positive
BH 037	*K. pneumoniae*	positive	positive
BH 038	*K. pneumoniae*	positive	positive
BH 040	*E. coli*	positive	positive
BH 041	*E. coli*	negative	negative
BH 042	*K. pneumoniae*	positive	positive
BH 043	*K. pneumoniae*	positive	positive
BH 044	*E. cloacae*	positive	positive
BH 045	*E. coli*	positive	positive
BH 046	*K. pneumoniae*	positive	positive
BH 047	*K. pneumoniae*	positive	positive
BH 048	*K. pneumoniae*	positive	positive

Srisangworn hospital (51 isolates) 22 isolates non-susceptible to carbapenems

Code	Organism	Carbapenemase detection methods	
		MHT	Carba NP
SWH 001	*E. coli*	negative	negative
SWH 003	*M. moganii*	negative	negative
SWH 004	*Enterobacter spp.*	negative	negative
SWH 005	*E. coli*	negative	negative
SWH 007	*E. coli*	negative	negative
SWH 008	*P. mirabilis*	negative	negative
SWH 009	*K. pneumoniae*	negative	negative
SWH 010	*K. pneumoniae*	negative	negative
SWH 014	*E. coli*	negative	negative
SWH 020	*E. coli*	uninterpretable *	positive
SWH 022	*K. pneumoniae*	negative	negative
SWH 025	*E. coli*	negative	negative
SWH 030	*E. coli*	negative	negative
SWH 031	*E. coli*	negative	negative
SWH 032	*E. coli*	negative	negative
SWH 036	*E. cloacae*	positive	positive
SWH 037	*M. moganii*	negative	negative
SWH 038	*K. pneumoniae*	negative	negative
SWH 041	*E. coli*	negative	negative
SWH 046	*E. cloacae*	negative	negative
SWH 054	*E. coli*	negative	negative
SWH 055	*K. pneumoniae*	negative	negative

Phetchabun Hospital (142 isolates) 29 isolates non-susceptible to carbapenems

Code	Organism	Carbapenemase detection methods	
		MHT	Carba NP
PH 002	*E. coli*	positive	positive
PH 004	*E. coli*	negative	negative
PH 031	*E. cloacae*	negative	negative
PH 032	*C. freundii*	positive	positive
PH 034	*C. freundii*	positive	positive
PH 044	*E. coli*	negative	negative
PH 052	*E. cloacae*	negative	negative
PH 053	*E. cloacae*	negative	negative
PH 054	*E. cloacae*	negative	negative
PH 062	*E. coli*	negative	negative
PH 065	*K. pneumoniae*	negative	negative
PH 070	*Salmonella* gr.D	negative	negative
PH 071	*K. pneumoniae*	negative	negative
PH 078	*E. coli*	positive	positive
PH 088	*E. cloacae*	negative	positive
PH 091	*E. cloacae*	positive	positive
PH 095	*E. cloacae*	negative	positive
PH 102	*E. cloacae*	positive	positive
PH 104	*E. cloacae*	negative	positive
PH 106	*K. pneumoniae*	negative	positive
PH 115	*E. coli*	positive	positive
PH 127	*K. pneumoniae*	negative	negative
PH 129	*E. coli*	negative	positive
PH 131	*K. pneumoniae*	positive	negative
PH 133	*E. coli*	negative	positive
PH 135	*E. coli*	negative	positive

Code	Organism	Carbapenemase detection methods	
		MHT	Carba NP
PH 146	*E. coli*	negative	positive
PH 148	*E. coli*	positive	positive
PH 149	*E. coli*	positive	negative

Wichian Buri hospital (50 isolates) 11 isolates non-susceptible to carbapenems

Code	Organism	Carbapenemase detection methods	
		MHT	Carba NP
VH 001	*K. pneumoniae*	negative	negative
VH 012	*E. coli*	negative	negative
VH 014	*E. cloacae*	positive	negative
VH 016	*Enterobacter spp.*	negative	negative
VH 017	*E. cloacae*	negative	negative
VH 018	*E. coli*	negative	negative
VH 019	*E. coli*	negative	negative
VH 032	*E. coli*	negative	negative
VH 041	*K. pneumoniae*	negative	negative
VH 050	*Enterobacter* spp.	negative	negative
VH 051	*P. mirabilis*	negative	negative

Uthaithani hospital (64 isolates) 7 isolates non-susceptible to carbapenems

Code	Organism	Carbapenemase detection methods	
		MHT	Carba NP
UH 060	*K. pneumoniae*	negative	positive
UH 061	*Enterobacter* spp.	negative	negative
UH 062	*K. pneumoniae*	positive	positive
UH 063	*K. pneumoniae*	negative	positive
UH 064	*K. pneumoniae*	negative	positive
UH 085	*E. coli*	negative	positive
UH 086	*K. pneumoniae*	negative	positive

Sawanpracharak hospital (19 isolates) 12 isolates non-susceptible to carbapenems

Code	Organism	Carbapenemase detection methods	
		MHT	Carba NP
SH 001	*K. pneumoniae*	positive	negative
SH 002	*E. coli*	positive	positive
SH 003	*K. pneumoniae*	negative	positive
SH 004	*K. pneumoniae*	positive	negative
SH 005	*E. cloacae*	positive	positive
SH 006	*E. coli*	negative	positive
SH 007	*E. cloacae*	positive	positive
SH 008	*K. pneumoniae*	negative	negative
SH 009	*K. pneumoniae*	positive	positive
SH 010	*K. pneumoniae*	negative	positive
SH 011	*K. pneumoniae*	positive	positive
SH 012	*E. cloacae*	positive	positive

The detection of colistin resistant

Agar dilution method for colistin susceptibility testing

(According to the EUCAST breakpoint for *Enterobacteriaceae*)

Code	Organism	Colistin concentration in agar dilution (µg/ml)											MIC
		0.125	0.25	0.5	1	2	4	8	16	32	64	128	
Phetchabun Hospital (142 isolates)													
PH 91	*E. cloacae*	+	+	+	+	+	+	+	+	+	NG	NG	**64**
PH 95	*E. cloacae*	+	+	+	+	+	+	+	+	+	+	+	**>128**
PH 104	*E. cloacae*	+	+	+	+	+	+	+	+	+	+	+	**>128**
Srisangworn hospital (51 isolates)													
SWH 036	*E. cloacae*	+	+	+	+	+	+	+	+	+	+	+	**>128**
Uthaithani hospital (64 isolates)													
UH 063	*K. pneumoniae*	+	+	+	+	+	+	+	+	NG	NG	NG	**32**
UH 064	*K. pneumoniae*	+	+	+	+	+	+	+	+	+	NG	NG	**64**
Sawanpracharak hospital (19 isolates)													
SH 010	*K. pneumoniae*	+	+	+	+	+	+	+	+	+	+	+	**>128**

Organism	Colistin concentration in agar dilution (µg/ml)											MIC
	0.125	0.25	0.5	1	2	4	8	16	32	64	128	
Negative control *E. coli* ATCC 25922	+	+	+	NG	NG	NG	NG	NG	NG	NG	NG	**1**
Positive control *E. coli* NCTC 13846	+	+	+	+	+	+	NG	NG	NG	NG	NG	**8**

*MIC target value for quality control strains are 0.25-2 µg/ml for *E. coli* ATCC 25922 susceptible strain and 4 µg/ml but should only occasionally be 2 or 8 µg/ml for *E. coli* NCTC 13846 resistant strain.