DETECTION OF CARBAPENEMASE PRODUCTION IN CARBAPENEM-RESISTANT ENTEROBACTERIACEAE BY MODIFIED CARBA NP TEST

BORE KONG

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By BORE KONG

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Medical Technology of Naresuan University

Oral Defense Committee

..... Chair

(Associate Professor Dr. Pitak Santanirand, Ph.D)

Noppadon Jumroon

..... Advisor

(Dr. Noppadon Jumroon. Ph.D)

P. Panty

..... Co – Advisor

(Dr. Panyupa Pankhong, Ph.D)

..... Internal Examiner

(Dr. Jirapas Jongitwimol, Ph.D)

Approved

.....

(Associate Professor Paisarn Muneesawang, Ph.D.)

Dean of the Graduate School

19 JUN

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Author	Bore Kong
Advisor	Noppadon Jumroon, Ph.D.
Co-advisor	Panyupa Pankhong, Ph.D.
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ABSTRACT

The prevalence of carbapenem resistance in *Enterobacteriaceae* (CRE) continues to increase in the world. The production of carbapenemase is the most importance mechanism of this resistance. Most recently, in phenotypic-based techniques for detecting carbapenemase enzymes, there are many methods such as Carba NP test, Modifed Hodge test (MHT) and other combined disk tests. Unfortunately, these methods cannot classify the classes of carbapenemases. So the modified Carba NP test is established to detect the classes of carbapenemase enzymes by adding the inhibitors of carbepenemase enzymes such as clavulanic acid (CVA) to inhibit the activity of class A, dipicolinic acid (DPA) to inhibit the activity of class B and both inhibitors (DPA and CVA) to detect class D of carbapenemase.

The sensitivity and specificity of the modified Carba NP test were 66.66 % and 100 %, respectively. There were 33 CRE isolates among 178 isolates from Wichianburi and Phetchabun hospitals, Phetchabun province. Ten of 33 CRE isolates were positive to MHT and Carba NP tests. All of these isolates were to class B of carbapenemase enzymes by the modified Carba NP test.

The modified Carba NP test is a novel, easy and fast method to classify the carbapenemase enzymes. It is easy to perform by using 96-well plates and adding inhibitors (clavulanic acid and/or dipicolinic acid) in the solution of the Carba NP test to differentiate the class of carbapenemase enzymes. Besides being easy, this method

can read or interpret the result between 15 and 90 minutes while the combined-disc test or disc-synergy test have spent 16 to 18 hours.

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ABBREVIATIONS

APB	=	3-aminophenylboronic acid
CVA	=	Clavulanic acid
CFU	=	Colony Forming Unit
°C	=	Degree Celsius
DPA	=	Dipicolinic acid
DOR	=	Doripenem
ESBL	=	Extend-spectrum β-lactamase
ETP	=	Ertapenem
IMI	=	Imipenem
KPC	=	*Klesiella pneumoniae* Carbapenemase
MER	=	Meropenem
MHT	=	Modified Hodge test
MIC	=	Minimal inhibitory concentration
MHA	=	Mueller Hinton agar
MAC	=	MacConkey agar
NDM	=	New Delhi metallo-β-lactamase
NaOH	=	Sodium Hydro-oxide
ml	=	Milliliter
mg	=	Milligram
IMI	=	Imipenem hydrolyzing enzymes
OXA	=	Oxacillinase
PCR	=	Polymerase chain reaction
µg	=	Microgram
µl	=	Microliter
VIM	=	Verona integrin encoded metallo-β-lactamase

CHAPTER I

INTRODUCTION

Background

Multidrug resistance is rapidly increasing in the world and caused by variety of bacterial species. This problem makes strongly concern about community-acquired and nosocomial infection (1). One of the most multidrug resistances is *Enterobacteriaceae*. *Enterobacteriaceae* is the most common pathogens in community and hospital-acquired infections, such as urinary and gastrointestinal tract infections. The infection increasingly causes the severity and mortalities (2). Carbapenem is considered as the last choice and highly effective drugs to treat of multidrug-resistant bacterial infection especially in *Enterobacteriaceae* (3).

However, carbapenem-resistant *Enterobactericeae* has been reported increasing amount multidrug-resistant bacterial infection. Resistance to carbapenem has been mostly noticed by producing of carbapenemase enzymes comparing to other mechanisms such as efflux pump and outer membrane alterations (4). Carbapenemases are the groups of β-lactamase enzymes which make an resistance to gram-negative bacilli including *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp (4).

Carbapenemase was firstly discovered in 1993 with gene bla_{NmcA} and now there are many genes of carbapenemase to be charesterized (5). They are classified into three classes according to molecular method such as class A, class B and class D.

To detect carbapenemase enzymes by phenotypic-based techniques, there are many methods such as Carba NP test, Modifed Hodge test (MHT) and other combined disk tests (2). All these methods differently have advantages and disadvantages. Carba NP test has many benefits such as cheap, rapid, reproducible and highly sensitive and specific (2). But Carba NP test cannot differentiate the classes of carbapenemase enzyems. While Modified Hodge test is inexpensive, easy, simple test and high sensitive results to class A and class D of carbapenemase enzymes (6). But MHT has a limitation to class B of carbapenemase and it cannot be used to classify the carbapenemase enzymes classification. Therefore, the further studies or research have

to study in detail to find a new method that is easier, simpler, and more reliable to detect carbapenemase enzymes and also can classify the classes of carbapenemase enzymes as well.

In this study, the detection of carbapenemase enzymes was studied by using phenotypic methods and comparing the results of each method for carbapenemase detection. Moreover, the classes of Carbapenemase would be classified by the newly method called Modified Carba NP test.

Objective of Study

The purposes of this study were:

1. To develop a new method to identify the classes of carbapenemase enzymes, called "Modified Carba NP test".

2. To detect carbapenemase production in carbapenem-resistant *Enterobacteriaceae* by phenotypic methods, such as Carba NP test, Modified Hodge test and Modified Carba NP test to find carbapenemase enzymes and classify into the classes of carbapenenmases on collected isolates from targeted hospitals at Phetchabun province, Thailand.

3. To detect the prevalence of carbapenemase enzymes in Phetchabun province, Thailand.

Scope of Study

This study focused on finding carbapenemase enzymes and the classes of enzymes in 128 carbapenem-resistant *Enterobacteriaceae* isolates from Phetchabun hospital and 50 carbapenem-resistant *Enterobacteriaceae* isolates from Wichianburi hospital by Modified Hodge test, Carba NP test and Modified Carba NP test. These two hospitals are located at Phetchabun province, Thailand.

Research Hypothesis

According to Ambler classification, carbapenemase has three classes such as class A, class B and class D. But the activity of each class is not the same. Some classes have high enzyme activity while other classes have low enzyme activity.

Meanwhile, CLSI guideline recommends two methods (Carba NP test and Modified Hodge test) to detect carbapenemase enzymes. But these methods cannot classify or identify the classes of carbapenemase enzymes.

Therefore, Modified Carba NP test should be developed to classify and identify the classes of carbapenemase enzymes.

CHAPTER II

LITERATURE REVIEW

Antimicrobial drugs

Antimicrobial drugs are groups of drugs using to treat the infections of microorganisms. The purpose of antimicrobial therapy is the elimination or the destruction toxicity of microorganisms without harmful to human host cells (7).

Classification of antimicrobial drugs is divided into five groups according to its mechanical actions (Figure 1), such as cell wall synthesis inhibitors (β-lactam), cell membrane function inhibitors (Polymyxins), Protein synthesis inhibitors (Tetracyclines, Aminoglycocsides, Macrolides and others), Nucleic acid synthesis inhibitors (Fluoroquinolones) and Antimetabolites (Sulfonamides) (7).

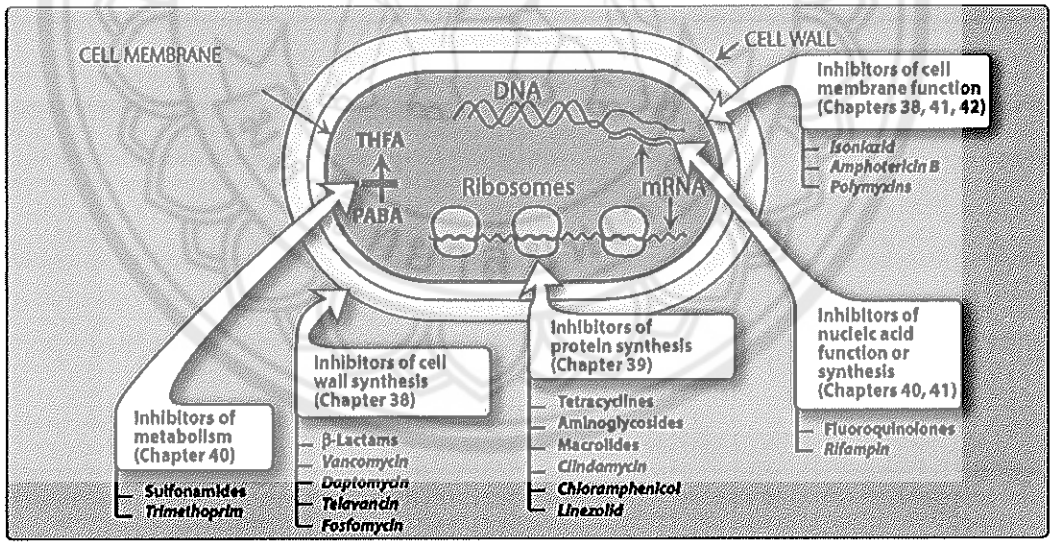


Figure 1 Actions of antimicrobial drugs (7)

β-lactam

β-lactam is a drug, which inhibits cell wall of microorganisms. It is time-dependent killing antimicrobial drug and has been divided into four groups including penicillins, cephalosporins, carbapenems and monobactams (Figure 2) (8).

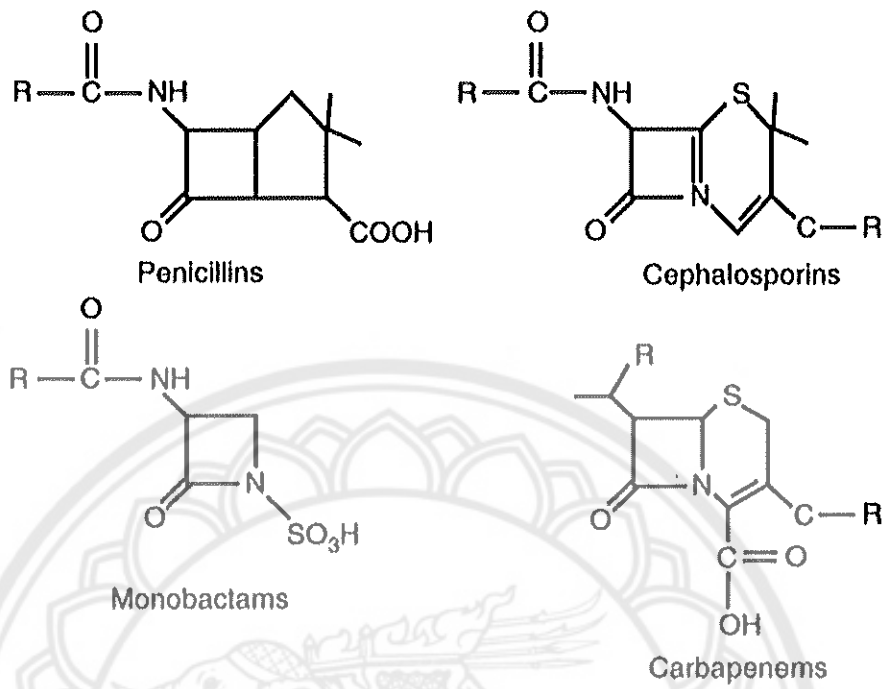


Figure 2 Structure of β-lactam groups (9)

Penicillins

Although penicillin has highly resistance rate comparing to other β-lactam groups, penicillin is regarded as most widely effective and the least toxic drugs (7,10). Basic structure of penicillins has thiazolidine ring attaching to a β-lactam ring and adding secondary amino group (RNH-) (Figure 3).

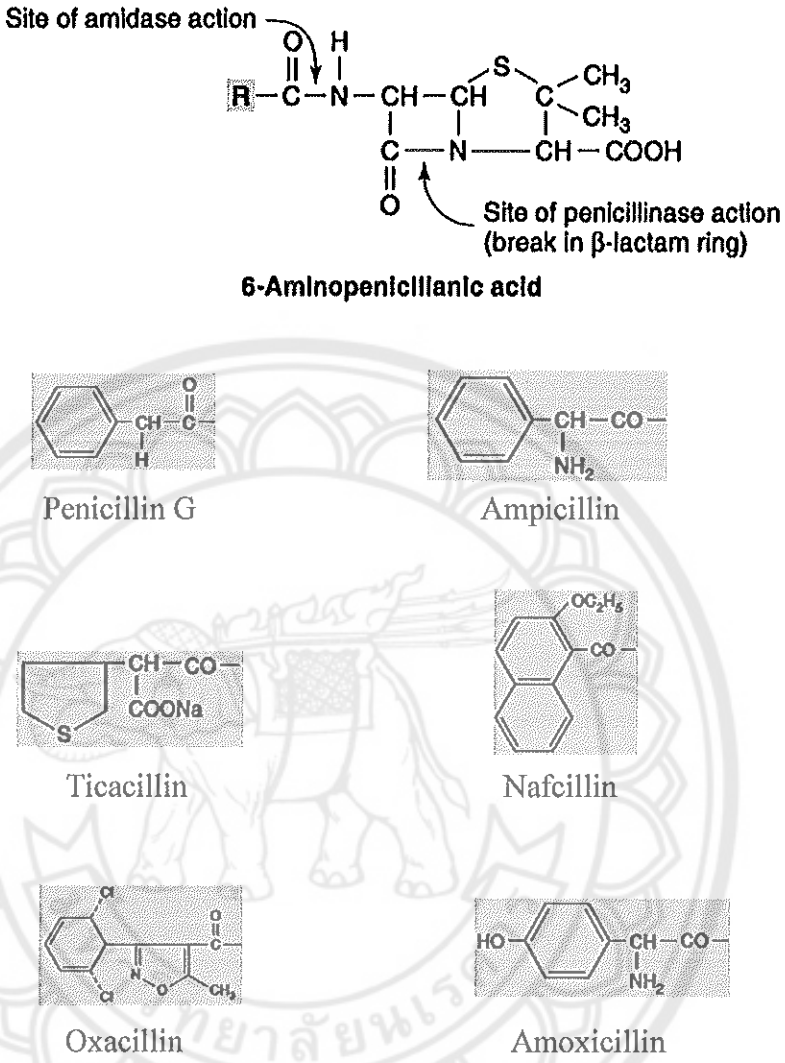


Figure 3 Structure of penicillin (11)

1. Mechanism of Action

Penicillin-binding proteins (PBPs) are the enzymes to inhibit the cell wall synthesis. These enzymes not only prevent cell wall synthesis but also destroy or break bacteria. However, changing of PBPs causes resistance to penicillin. Meanwhile, transpeptidase is inhibited by penicillin and stops cross-linking between peptidoglycan chains. Penicillin also destroys the action of autolysins in remodeling cell wall synthesis (Figure 4) (7).

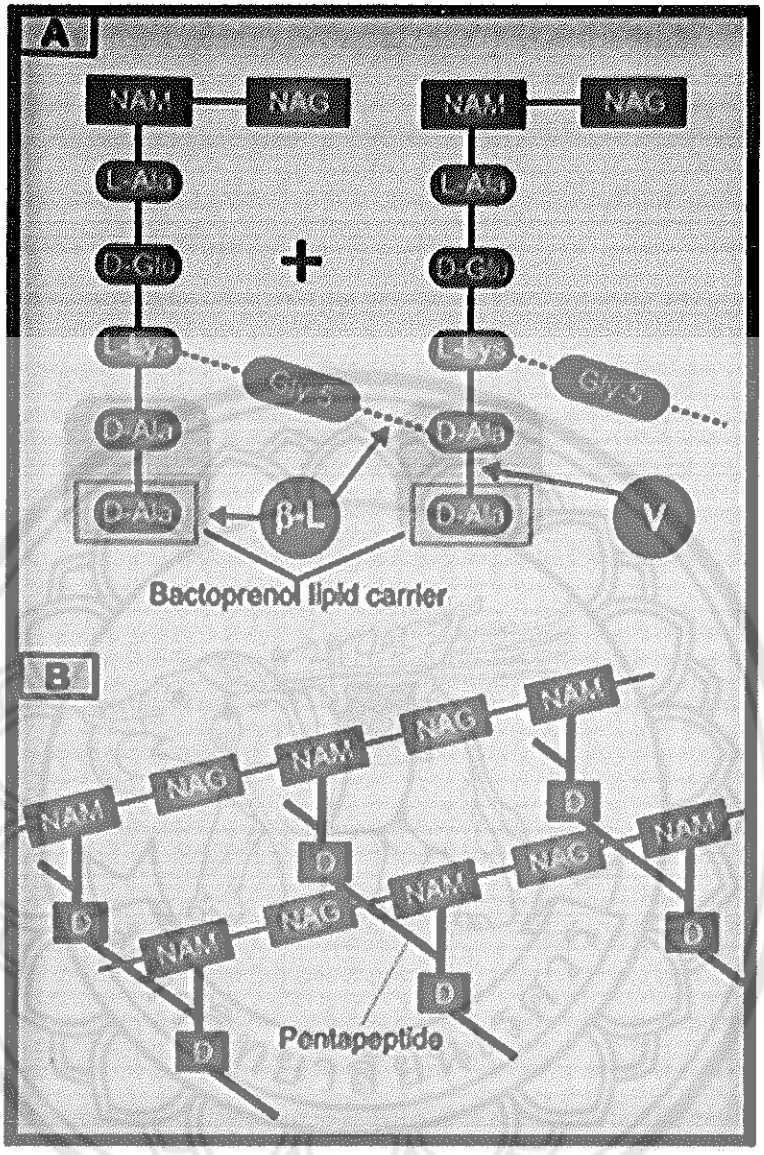


Figure 4 Bacterial cell wall synthesis and structure. A: Cross linking of peptidoglycan. B: highly cross-linked peptidoglycan strands in bacterial cell wall (7)

2. Classification of penicillin

There are three groups of penicillins:

Penicillins G: this group is easily hydrolyzed by β-lactamases and good for anti-gram-positive organisms (12).

Antistaphylococcal penicillins: they have good activity against staphylococci and streptococci but they do not properly use for gram-negative cocci, anaerobic, enterococci and staphylococci β-lactamases (12).

Extended-spentrum penicillins (ampicillin): this group can destroy against gram-negative organisms but they also hydrolysis by β-lactamases as well (12).

3. Penicillin resistance

Resistance to penicillin occurs by activity of β-lactamase, decreased permeability to the drugs and altered PBPs. β-lactamase is the enzyme to hydrolysis the β-lactam ring and inhibits activity of bactericidal of penicillin.

While, decreasing of antibiotic penetration through the outer cell membrane of the bacteria can inhibit or reduce the amount of drug reaching the target PBPs. The last mechanism is altered PBPs. Lower affinity of penicillin can decrease the concentration of penicillin and reduce effect to inhibit bacteria's activity (Figure 5) (7).

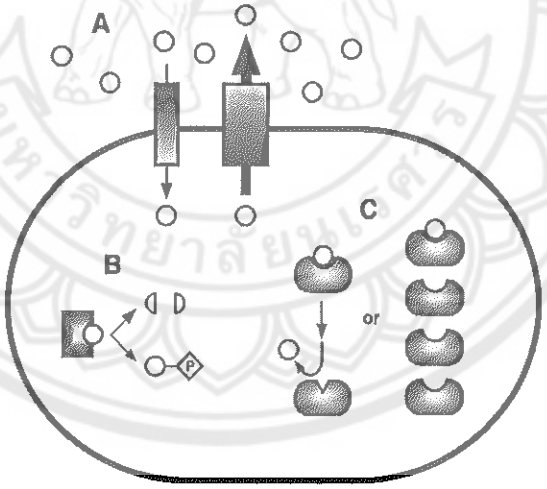


Figure 5 Mechanism of Penicillin resistances. A: decreasing uptakes or increasing efflux. B: Inactivation of antibiotics by hydrolysis or modification. C: Alteration of targets and reduce the affinity for antibiotics or overproduction of target (13)

4. Pharmacokinetic

The absorption of penicillin is not completely absorbed and decreases by food. So penicillin is recommended to take with empty stomach. The excretion is rapidly through the kidney while well distributed throughout the host body (7). The normal half-life of penicillin G is approximately 30 minutes. But in the renal failure, it may prolong as long as 10 hours.

5. Adverse Reaction

Hypersensitivity is approximately 5 percent among patients such as rashes and anaphylaxis. Cross-allergic reactions can happen to all groups of β-lactam (7).

Cephalosporins

The first source of cephalosporin is from cephalosporium fungi. They form by nucleus of 7-aminocephalosporanic acids while penicillins have 6-aminopenicillanic acids (Figure 6) (11).

The mechanism of action of cephalosporins is similar to penicillins, such as binding to specific PBPs, cell wall inhibition and activate autolystic enzyme. However, poor permeation of bacteria by drugs, lack of PBPs and β-lactamase enzyme are factors contributing in resistance to cephalorsporins (11).

Cephalosporins are classified into four generations:

A. First-generation cephalosporins are good against gram-positive cocci but except *Enterococci* and MRSA. This group has cephalexin, cephradine and cefadroxil.

B. Second-generation cephalosporins are better than first-generation activity against gram-positive cocci and gram-negative rods (*Klebsiella* and *Proteus* but not *P. aeruginosa*) (11). There are cefoxitin and cefotetan in second-generation cephalosporins.

C. Third-generation cephalosporins are mostly active against methicillin susceptible staphylococci but they are weakly active against other gram-positive cocci. Third-generation has cefoperazone, cefotaxim, ceftriaxone, ceftrizoxime and ceftazindime.

D. The last generation is four-generation cephalosporins. It is good action against *Enterobacter*, *Citrobacter* species, MRSA and penicillin-resistant *S.*

pneumonia as well. This four-generation have cefepime, ceftaroline and ceftobiprole (11).

Side effects of cephalosporins are fever, skin rashes, nephritis, granulocytopenia, hemolytic anemia and anaphylaxis. There are 5 percent cross-allergy between cephalorsporins and penicillins (11).

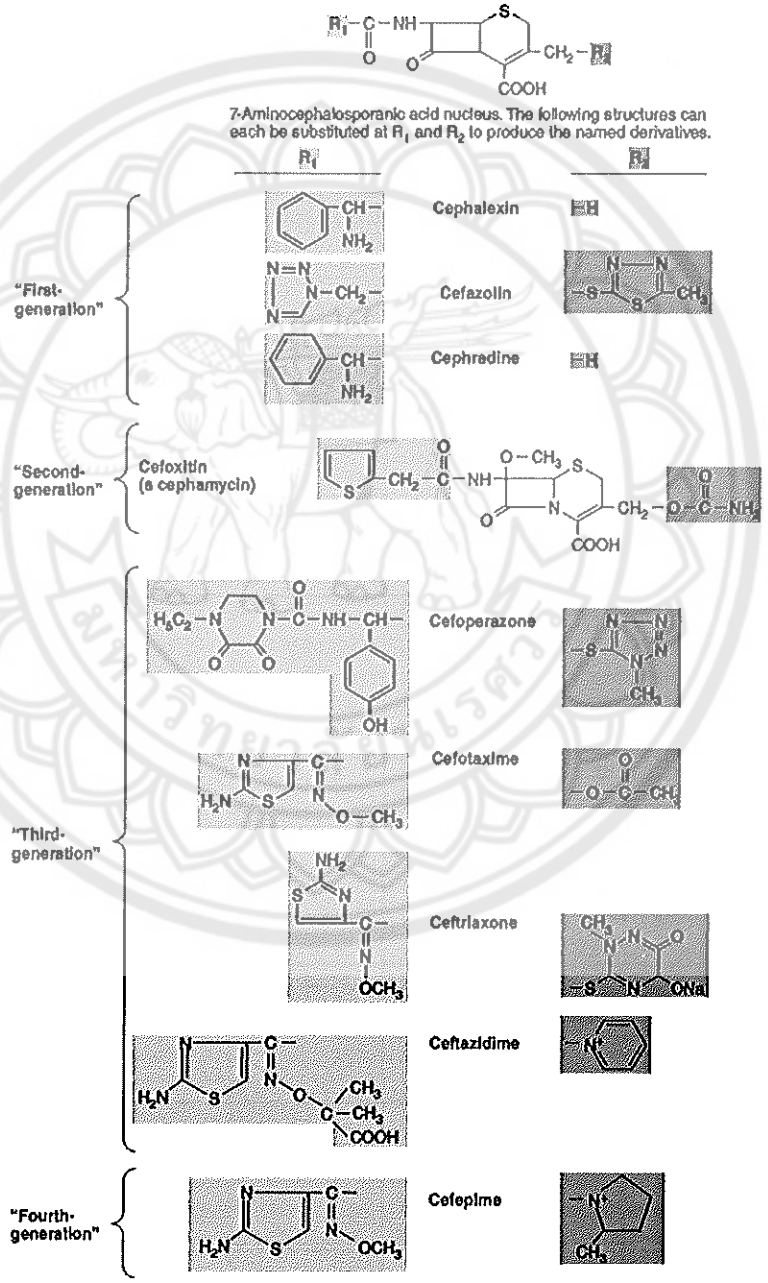


Figure 6 Structure of cephalosporin (11)

Monobactam

There is only Aztreonam in Monobactam group, which is using in clinical use. Monobactams are the monocyclic β-lactams with monocyclic nucleus.

Aztreonam is good action on gram-negative aerobes like *P. aeruginosa.* Bush and Jacoby group 1 and 2 plasmid-encoded and chromosmally-encoded β-lactamase do not hydrolyze it. But ESBLs and some carbapenemase enzymes hydrolyze it.

Like other β-lactams, Aztreonem can cause skin rash, nausea, diarrhea and eosinophilia. But it has minimal cross-reaction with other β-lactams and it is safe for patients who are allergic to penicillins or cephalosporins (14).

Carbapenems

Carbapenems are β-lactam; differ from penicillins at thiazolidine ring instance by sulfur atom (Figure7) (7). Carbapenems have imipenem, meropenem, ertapenem and doripenem. Imipenem is the first drug of carbapenem, is good for many gram-negative rods, gram-positive organisms and anaerobes. Imipenem is resistant to β-lactamase and penetrates well in body tissues and fluids, including CSF. Moreover, carbapenems may be indicated against to multidrug-resistance organisms.

Carbepenems could give adverse effects such as vomiting, diarrhea, skin rashes and reactions at infusion sites. Imipenem has also cross-reaction with penicillins. It means patients who are allergic to penicillin may also be allergic with imipenem as well.

Meropenem is similar to imipenem. Ertapenem is good for complicated infection treatment but it is not good against *Enterococcus* species, *P. aeruginosa* and glucose nonfermenting gram-negative rods. While, doripenem is better than imipenem against *P. aeruginosa* but equal to meropenem (11).

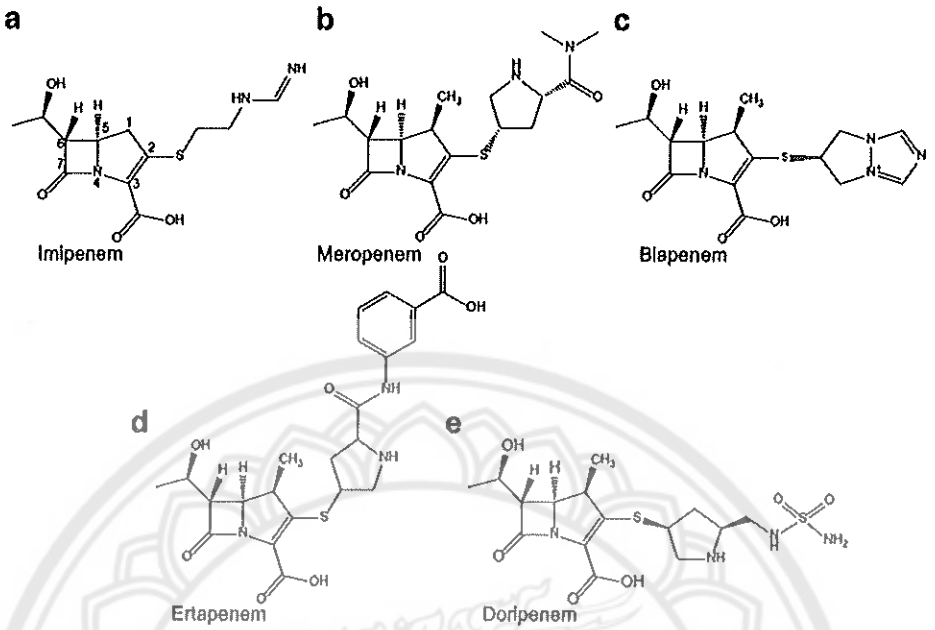


Figure 7 Basic structure of carbapenem (15)

β- lactamase inhibitors

β-lactamase inhibitors are hydrolyzed β-lactam ring by enzyme or acid and destroy the activity of β-lactam activity. There are clavulanic acid, sulbactam and tazobactam (Figure 8) (7).

Clavulanic acid is a nature weak antimicrobial agent and inhibits penicillinases from *staphylococci* and many groups 2 of β-lactamases from gram-negative bacteria. Clavulanic acid is synergistically used with penicillins and cephalosporins against β-lactamase-producing organisms such as *Staphylococci, Klebsiellae, H. influenza, M. catarrhalis, N. gonorrheae, E. coli, Proteus spp.* Moreover; this clavulanic acid inhibits *ESBLs*, plasmid-mediated *TEM, SHV* and *CTX-M* β-lactamase, producing from cephalosporin-resistant *K. pneumoniae* and *E. coli.* But the action of clavulanic acid is still not clear. Clavulanic acid is used as combination with other penicillin such as amoxicillin and ticarcillin. These combination drugs are well absorbed in gastrointestinal tract and give adverse effects, such as diarrhea, abdominal cramps, nausea and vomiting (14).

Sulbactam is a semisythetic 6-desamino-penicillin sulfone and is weak antibacterial agents. Although sulbactam action is not better than clavulanic acid, but sulbactam can inhibit plasmid and chromosomal-mediated β-lactamase, producing from *S. aureus, many Enterobacteriaceae, H. influenzae, M. catarrhalis, Neisseria* spp., *Legionella* spp., members of the *B. fragillis group, Prevotella* spp., *porphyromonas* spp., *and Mycobacterium* spp. Sulbactam is used with penicillins and cephalosporins against β-lactamase-producing organisms. The combination of ampicillin-sulbactam gives the side effects including nausea, diarrhea, skin rash and transient eosinophilia (14).

Tazobactam is a suicide β-lactamase inhibitor and is a penicillanic acid sulfone derivative structurally related to sulbactam. Tazobactam is playing role against *ESBLs*-producing *Enterobactericeae* such as *E. coli, Klebsiella* spp., and *P. mirabilis*. Moreover, it inhibits action of β-lactamase in *Staphylococci, H. influenza, N. gonorrheae, B. fragilis* group, *Prevotella* spp., and *Porphyromonas* spp. Piperacillin-tazobactam combination gives a high concentrations in intestinal mucosa, lung and skin (14).

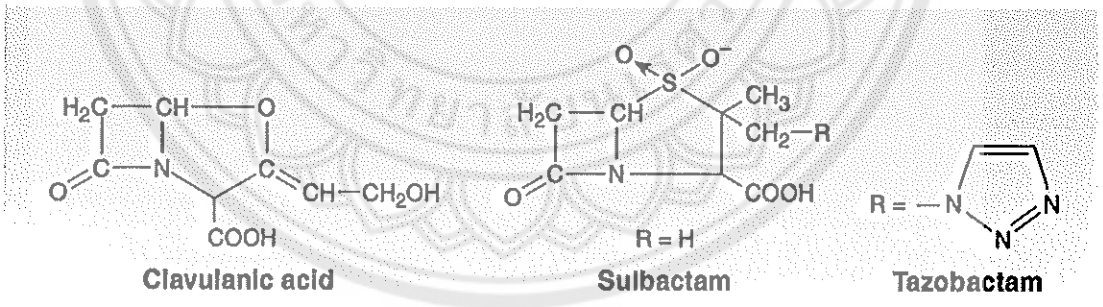
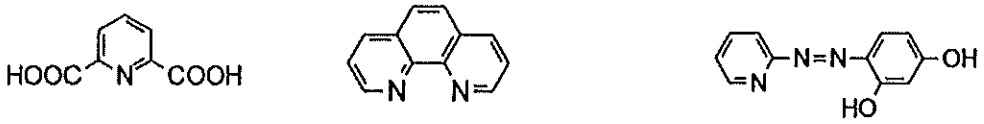


Figure 8 Structure of β-lactamase inhibitor (16)

Dipicolinic acid (DAP) is a zinc chelator using to generate apoprotein from a variety of zinc-dependent enzymes. DPA is a highest metallo-β-lactamase inhibitor comparing to other inhibitors such as EDTA, O-phenanthioline (OP), 4-(2-pyridylazo) resorcinol (PAR) and Zincon sodium salt. Moreover, DPA is also a superior chelator to rapidly remove of metal ions (Figure 9) (17-18).



Dipicolinic acid (DPA) O-Phenanthioline (OP) 4-(2-pyridylazo) resorcinol (PAR)

EDTA Zincon sodium salt

Figure 9 Structure of chelators (17)

Boronic acid compounds (mainly 3-amino-phenylboronic acid) have directly action on active-site of serine-type-β-lactamase. They do not inhibit on β-lactam structure. 3-amino-pheylboronic acid (APB) is used to inhibit class A and class C of β-lactamase enzymes such as KPC (class A) and CTX-M (class C) (Figure 10) (19).

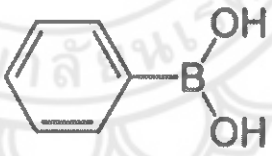


Figure 10 Structure of Boronic acid (19)

Mechanism of β- lactam resistance

There are four mechanisms of β-lactam resistance. First is inactivation of antibiotic by β-lactamase, second is the modification of target penicillin-binding protein (PBPs), third is an impaired penetration of drug to target PBPs and forth is an efflux pump (13,16).

1. Alteration of PBPs

Methicillin-resistant *staphylococci* and penicillin-resistant *pneumococci* and *enterococci* produce low affinity PBPs for binding β-lactam antibiotic. Therefore, β-lactam antibiotics are resistance to these organisms (14,16).

2. Impaired penetration of the drug

This mechanism only occurs in gram-negative organisms. β-lactam antibiotics can pass the outer membrane to enter the bacteria via porins (outer membrane protein channels). When these channels are decreasing or not enough, the poor penetration of β-lactam antibiotics into bacteria cells could be low (16).

3. Efflux pumps

Efflux pumps have cytoplasmic and periplasmic protein components, which produce gram-negative organisms. These efflux pumps can pump out β-lactam antibiotics from the periplasm to outer membrane (Figure 11) (16).

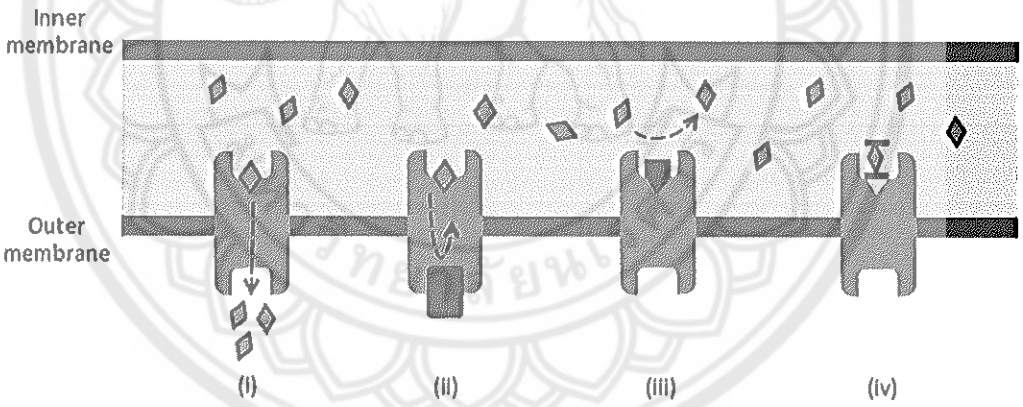


Figure 11 Bacteria efflux pumps (as in number ii) (20)

4. β- lactamase production

β-lactamase enzyme production is the most common mechanism amount other mechanisms. Many organisms, such as *Staphylococcus aureus*, *Haemophilus influenza* and *Escherichia coli* produce narrow specificity β-lactamase, which is resistance to penicillins and cephalosporins. Other β-lactamase such as AmpC, produced by *Pseudomonas aeruginosa* and *Enterobacter* spp., and extended-

spenctrum β-lactamase (ESBLs) can hydrolysis cephalosporins and penicillins. Meanwhile, carbapenemase hydrolysis carbapenem drugs (Figure 12) (16).

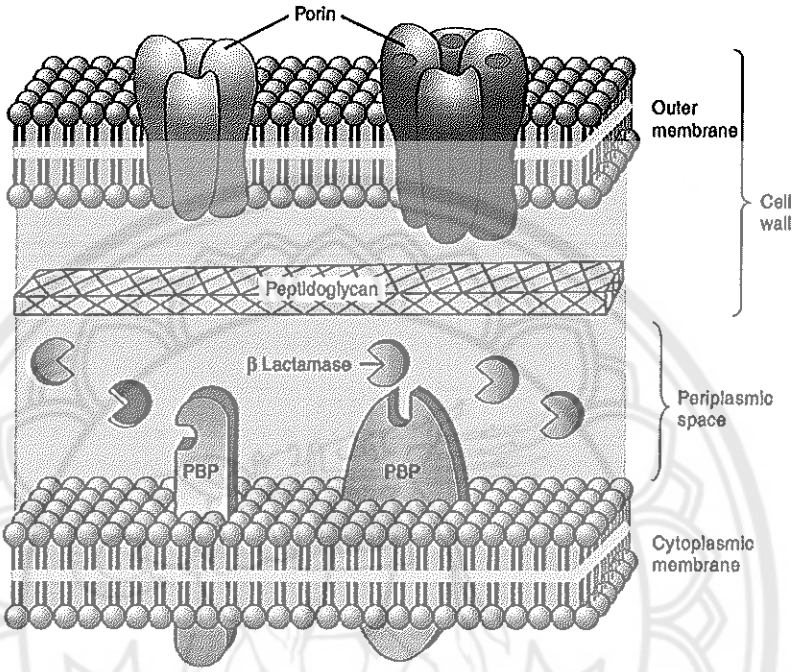


Figure 12 Mechanism of β-lactam resistance (16)

5. β- lactamase enzymes

β-lactamase is the members of a superfamily of active-site serine proteases or D_1D-peptidase. β-lactamase is classified by two systems, Bush-Jacoby classification system and the Ambler classification (AMB) system (Table 1) (14). For Ambler classification class A and class D are amino acid- sequence homology. But Bush-Jacoby classification has 4 groups from group 1 to group 4, which are based on substrate and inhibition profile (Figure 13) (21).

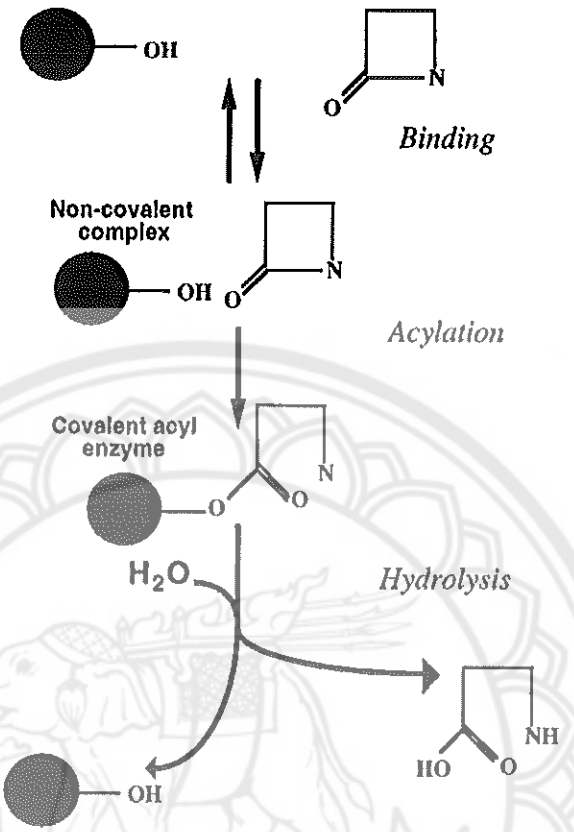


Figure 13 Activity of β-lactamases enzymes (22)

5.1 Ambler or Molecular classification (Figure 15)

5.1.1 Class A serine β-lactamases

The first gene TEM was known in 1963 in *E. coli* isolate. Meanwhile, SHV (sulfhydryl reagent variable) was found firstly in *K. pneumoniae*. These two genes, met on infection of urinary tract, nosocomial respiratory tract and bloodstream, were found on plasmids. Additionally, while newly antibiotic have appeared during the early 1980s, the emergence of extended-spectrum β-lactamases (ESBLs) have also occurred. Penicillin, narrow and extended-spectrum cephalosporins (including the anti-methicillin-resistant *S. aureus* cephalosporin) and monobactam (aztreonam) are hydrolyzed by ESBLs. But unfortunately, ESBLs cannot destroy carbapenem, cephamycin and β-lactamases inhibitor (clavulanic acid and sulbactam) (14,21).

5.1.2 Class B metallo-β-lactamases

These enzymes are Zn2+-dependent β-lactamases and can inhibit penicillins, cephalosporins, carbapenems and β-lactamases inhibitors (21). Class B metallo-β-lactamases divides into three subclasses B1, B2 and B3. Subclass B1 has zine binding at first site of three His residues (His-116, His-118 and His-196) and second site binding to His-263, one Cys-221 and one Asp-120. Subclass B2 is good to hydrolysis only carbapenems and has zine ligands on site 2 where His-116 instead of Asp. The last subclass B3 is broad-spectrum activity to inhibit not only cephalosporins but also carbapenems. Subclass B3 has histidine instead of cysteine ligands in zine binding at site 2 while other two subclasses don't have it (Figure 14) (14,23-24).

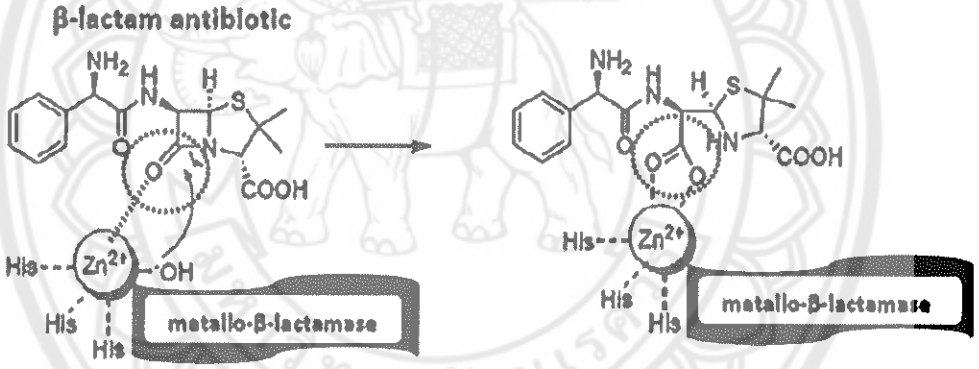


Figure 14 Mechanism of metallo-β-lactamases (24)

5.1.3 Class C β-lactamases

AmpC enzyme is the most genes in class C β-lactamases and can inhibit penicillin, β-lactamases inhibitor, cephalosporins. But it poorly acts against cefepime, cloxacillin, oxacillin an aztreonam. The source of AmpC is from *Enterobactericeae* family such as *Enterobacter* spp., and *Citrobacter* spp. (21).

5.1.4 Class D β-lactamases

This group is recognized as oxacillinase because its action against most oxacillin than other classes. Class D β-lactamase is a good against penicillins, cephalosporins, extended-spectrum cephalosporins (OXA-type ESBLs) and carbapenems (OXA-type carbapenemases) (21). This enzyme has weakly action

against carbapenems. Therefore, OXA β-lactamase needs to combine with other mechanisms like efflux pumps and porin alternation for carbepenem resistance. But OXA β-lactamase is well known as group, which has ability to resist carbapenems (14,25-26).

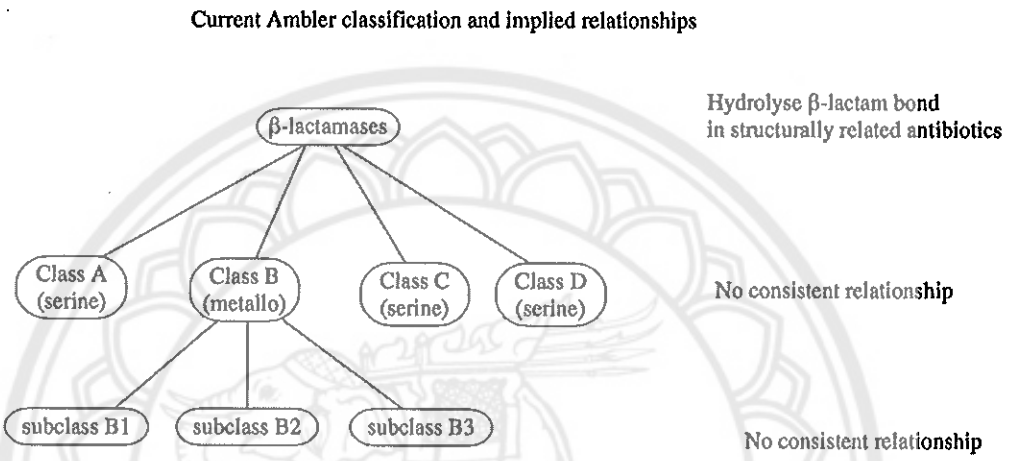


Figure 15 Ambler classification (27)

5.2 Bush-Jacoby functional classification

5.2.1 Group 1 cephalosporinase

Group 1 cephalosporinase is same as molecular class C and is good at against cephalosporins than benzylpencillin. It also inhibits clavulanic acid and cephamycins. Large amount of this Group 1 cephalosporinase enzyme could have activity of resistance to carbapenems (especially ertapenem). In 1989, plasmid-mediated gene AmpC was found in worldwide but it is less than ESBLs. CMY-2 gene is most importance gene in action against β-lactam groups (23,28-29).

5.2.2 Group 2 serine β-lactamase

This group has combination between class A and class D of molecular classification. In this group 2, it is divided into many subgroups such as subgroup 2a, 2b, 2be, 2br, 2ber, 2c, 2ce, 2d, 2de and 2df. Subgroup 2e has ability to inhibit ESBLs, clavulanic acid and tazobactam. Other subgroup 2f is same class A serine carbapenemase from molecular classification (29-30).

5.2.3 Group 3 metallo-β-lactamases

MBLs have ability to hydrolyze carbapenems but poorly hydrolyze monobatams. MBLs are inhibited EDTA and dipicolinic acid. This group divides into three functional subgroups (3a, 3b and 3c) or structure subclasses (B1, B2 and B3). Subgroup 3a has IMP and VIM enzymes, which have been found in Enterobactericeae. Other subgroup 3b is good at hydrolyzing carbapenems (23,29).

5.2.4 Group 4 β-lactamases

In this group, enzymes are not known (31).

Table 1 Classification of β-lactamases (14)

Bush-Jacoby system classification	Major subgroup	Ambler system classification	Main attributes
Group 1 cephalosporin-ases		C (cephalosporin-ases)	Usually chromosomal. resistance to all β-lactamases except carbapenems and not inhibited by clavulanate
Group 2 penicilliases (clavulanic acid susceptible)	2a	A (serine β-lactamases)	Staphylococcal penicillinases
	2b	A	Broad spectrum; TEM-1. TEM-2 and SHV-1
	2be	A	Extended spectrum; TEm and SHV variants predominantly
	2br	A	IRT β-lactamases
	2c	A	Carbenicillin hydrolyzing
	2f	A	Carbapenemmases inhibited by clavulanate
	2d	D (oxacillin hydrolyzing)	Oxacillin-hydrolyzing (OXA)

Table 1 (cont.)

Bush-Jacoby system classification	Major subgroup	Ambler system classification	Main attributes
Group 3 metallo-β-lactamase	3a	B (metaloenzymes)	Zinc-dependent carbapenemases
	3b	B	
	3c	B	
Group 4		Not classified	Miscellaneous enzymes, most not yet sequenced

6. Carbapenemase enzymes

Carbapenemases are enzymes to hydrolyze carbapenems and divide into three classes namely class A, class B and class D.

6.1 Class A carbapenemase

In this class, there are chromosomal-encoded enzymes such as IMI (IMI-1 to IMI-3), Sme (Sme-1 to Sme-3), SFC-1 and NmcA and plasmid-encoded enzymes such as GES (GES-1 to GEM-20) and KPC (KPC-2 to KPC-13). KPC is most common enzymes in this group. Enzymes in this class have action against penicillins, cephalosporins, aztreonam and carbapenems. These enzymes are mostly found in *K. pnuemoniae* and also found in *E.coli*, *Salmonella enterica*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Proteus mirabilis*, *Serratia marcescens*, *Enterobacter cloacae*, *Acinetobacter* spp., *Pseudomonas aeroginosa* and *Pseudomonas putida* (32).

6.2 Class B carbapenemase

This group is metallo-β-lactamases, which two zinc ions binding to active site. It is inhibited by EDTA chelators, Zn^{2+} and other divalent cations. VIM (Verona intergron-encoded metallo-β-lactamases) and IMP are the most enzymes in class B metallo-β-lactamases. NDM (New Delhi metallo-β-lactamases) is an emerged gene and also recently found. VIM has been met in *P. aeroginosa* and *Enterobacteriaceae*. VIM is the most enzymes in this class and spreading over Europe

and Mediterranean such as Greece, Italy and Turkey. Moreover, NDM-1 has been found firstly in India in 2008 but now it is rapidly spreading all around the world. NDM-1 is found in *K. pneumoniae, E.coli, Citrobacter freundii, Enterobacter cloacae, Providencia* spp., and *Morgenella morganii* (32).

6.3 Class D carbapenemase

This class is also named as OXA. Its activity was firstly found in 1993 by Paton et al. OXA is divided into nine subgroups such as OXA-23, OXA-24, OXA-51, OXA-58, OXA-55, OXA-48, OXA-54 and OXA-50. OXA-51 has been found in *A. baumannii*.(30). OXA-48-producing *Enterobactericeae* was firstly found in Turkey in 2001. Then, OXA-48 had spread to Middle East, North Africa and Europe.(32) However, OXA family has weak activity against carbapenems. But this class D can contribute in carbapenems resistance with other mechanism like efflux pumps or overexpression of porin (30).

Enterobacteriaceae

Enterobacteriaceae is gram-negative, non-spore-forming, facultatively anaerobic bacilli. The colony of Enterobacteriaceae can grow on nonselective media such as chocolate agar and blood agar. Colony of this family is large, moist, and gray. Media with sucrose and lactose can allow seeing ability of carbohydrate fermentation. This fermentation can identify by changing of color on medium. For non-fermenting species are differentiated by this color changing. Hektoen (HE) and Xylose-lysine-desoxycholate (XLD) agar are used for identifying hydrogen sulfide producing species because these agars have sodium thiosulfate and ferric ammonium citrate making colonies blacken (33).

There are three antigens on this family such as O antigen (somatic antigen) is on the cell wall; H antigen (flagella antigen) is on surface, structure and responsible for motility; and K antigen (capsular antigen) is only on encapsulated species example *Salmonella enterica* (33).

Most of *Enterobacteriaceae* are commensals in gastrointestinal tract. While some species are also living as free-living organisms in water, soil, sewage and plants (33).

Enterobacteriaceae are divided into two broad categories according to clinical infection: first category is opportunistic pathogens and second is primary pathogens. Opportunistic pathogens are intestinal microbiota of both humans and animals. But it can cause severe infection as extra-intestinal and opportunistic infection when they stay outside their normal body site. For primary pathogens, they occur in immunocompromised hosts or contaminate to soil or water where these organisms are staying. *Shigella* spp., and *Yersinia* spp., are responsible for the primary pathogens (33).

In the family of *Enterobacteriaceae*, there are many bacterial species such as *Escheriachia coli*, *Shigella* spp., *Edwardsiella* spp., *Salmonella* spp., *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Providencia* spp., *Morganella* spp., *Yersinia* spp., *Erwinia* spp., and *Pectobacterium* spp (Figure 16) (33).

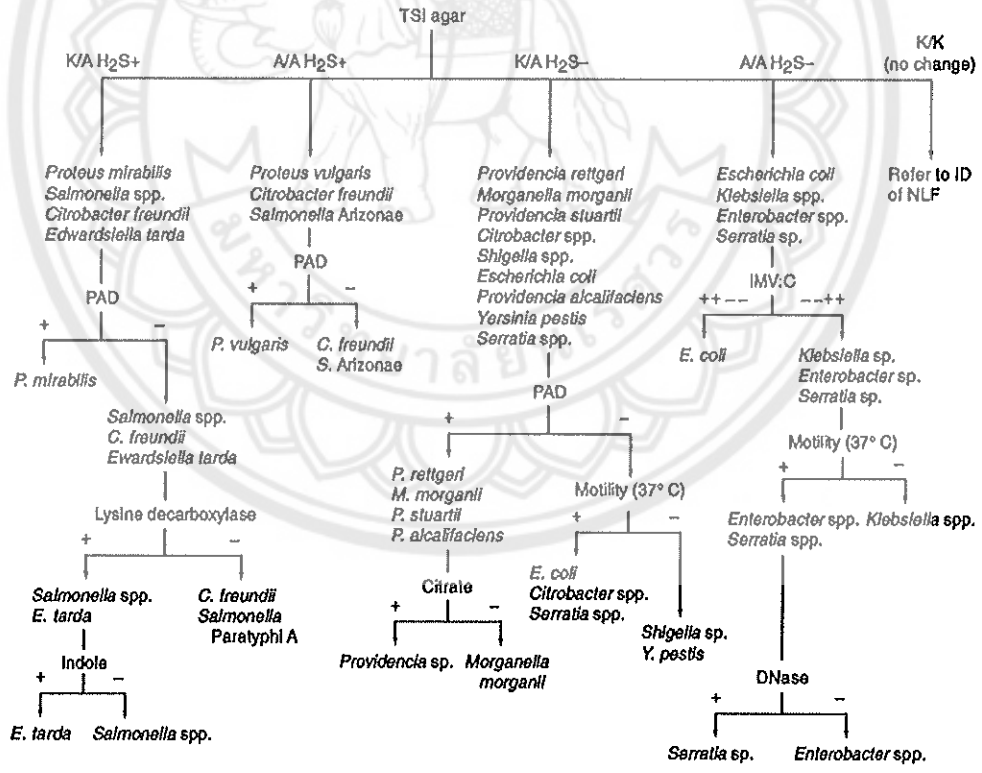


Figure 16 Flow chart for identification of *Enterobacteriaceae* based on Triple sugar iron (TSI) agar, Acid (A), Alkaline (K), Indole methyl red Voge-Proskauer (IMV:C), Nonlactose fermenter (NLF), Phenylalanine deaminase (PAD) (33)

Carbapenem resistance detection

For detection carbapenemase, there are two methods: phenotypic and genotypic methods.

1. Phenotypic methods

CLSI (Clinical and Laboratory Standards Institute) guideline is used as the reference for phenotypic methods to detect carbapenemaes (34).

1.1 Initial screen test

For the first screen, disk diffusion or broth diffusion and E-test are done.

1.1.1 Disk diffusion method

According to CLSI guideline, Mueller Hinton agar is a medium for disk diffusion. 100 mm plate will be used with no more than 6 disks and 150mm plate will be used with 12 disks. Zone diameter should easily measure and visible by the unaided eye. Quality control must be done routinely (35).

1.1.2 Broth dilution methods

There are macrodilution and microdilution methods. For Mueller-Hinton Broth, Mueller-Hinton agar is used as a medium. It uses to test common isolate, rapidly grow aerobic and facultative organisms. For reading MIC (Minimum Inhibition Concentration), QC organism standard will be used as reference (35).

1.1.3 E-test Method

E-test is well known technique for using in antimicrobial susceptibility test (34,36). To interpret for E-test result, CLSI guideline will be used as reference for MICs value (34,37).

1.2 Carbapenemase detection methods

For identifying carbapenemase, the most common methods are Modified-Hodge test (MHT), Carba NP test and inhibitor-based test. Less common methods are immunodiagnostic assays and mass spectrometry. Moreover, genotypic method is gold standard to detect carbapenemase (34,38).

1.2.1 Modified-Hodge test (Figure 17)

This test is a modification test from its original test to detect penicillinase enzyme of *Neisseria gonorrhea* by Hodge et al., in 1978. This clover

leaf-shape test has highly sensitivity for Ambler class A and class D carbapenemase. Moreover, it is easy and cheap methods to detect carbaepenemase enzyme. According to CLSI guideline, MHT uses Mueller-Hinton agar with *E. coli* ATCC 25922 at 0.5 McFarland standards as an indicator. Then, a carbapenem disk is placed on center of the plate and finally streaks the test strains from the disk to periphery. The result will be read after overnight incubation (38-40).

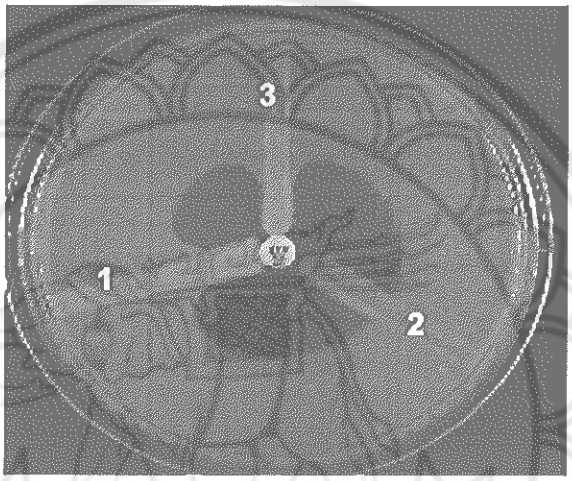


Figure 17 Modified Hodge test (40)

Note: 1: Positive control *K. pneumoniae* ATCC BAA 1705,
2: Negative control *K. pneumoniae* ATCC BAA 1706
3: test isolate is positive result

1.2.2 Carba NP test

It is a method to detect carbapenemase enzyme by using biochemical detection method and tested strain could break down β-lactam ring of imipenem causing color changing of a pH indicator from red to yellow color. The benefit of this test is fast, high sensitivity and specificity. But it cannot differentiate carbapenemaes classes (Figure 18) (38,41-42).

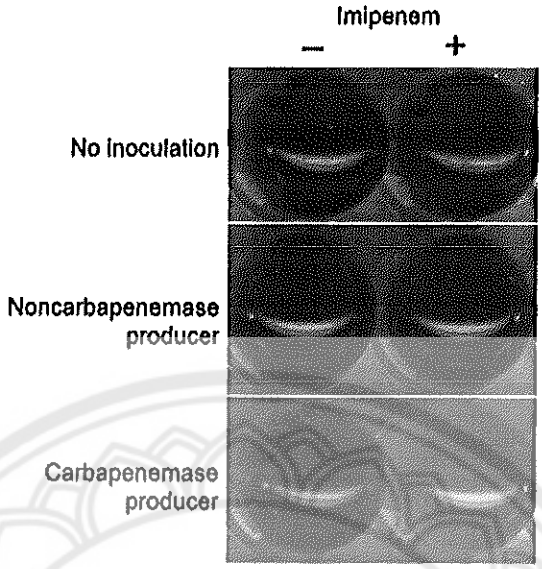


Figure 18 Carba NP test (2)

1.3 Genotypic method

Polymerease chain reaction (PCR) is using as routine in many clinical laboratories mean which phenotypic methods are difficult to detect. There are many type of PCR such as single PCR, multiplex PCR and real-time PCR. The primer sequences for gene encoding carbapenemase are set up. Class A carbapenemase has encoding gene GES and KPC type, OXA-48 is encoding gene for class D and class B has NDM, IMP and VIM (43-45). For multiplex PCR give 100% specificity and 97% sensitivity in detecting KPC, NDM, VIM and OXA-48 according to study of Stuart et al., while real-time PCR has 100% sensitivity and specificity. However, PCR methods are expensive, need high expertise and cannot detect novel unknown genes (38,46).

Newly study

Recently, there are many researchers interesting about studying on detection of carbapenemase in *Enterobacteriaceae*.

In 2011, Birgy et al., studied on phenotypic screening of carbapenemase and associated β-lactamases in carbapenem-resistant *Enterobacteriaceae*. They used two different phenotypic methods for detecting MBL, KPC and other β-lactam resistance mechanisms. Phenotypic method 1 is using carbapenemase inhibitor-impregnated agar. Phenotypic method 2 is using modified version of the CLSI confirmatory test for

ESBL production. The result showed that phenotypic test 1 is good for detection of carbapenemase and associated β-lactam resistance mechanisms (47).

In 2012, Nordmann et al., showed novel Carba NP test on rapid detection of carbapenemase-producing *Enterobactericeae* that the Carba NP test is fast, cheap, reproducible and high sensitivity and specificity (48). The principle of Carba NP test is that hydrolysis of β-lactam ring of imipenem by carbapenemase enzymes and color will change from red to yellow (48).

In 2014, Bartolini et al., showed on his study on Comparison of phenotypic methods for the detection of carbapenem non-susceptible *Enterobacteriaceae* that the combined disc test (Rosco diagnotica KPC and MBL confirm kit) is the most accurately identification method comparing to other phenotypic methods such as MHT and EDTA inhibition test (49).

Van der Zwaluw et al. in 2014 showed on the carbapenem inactivation method (CIM), a simple and low-cost alternative for the Carba NP test to assess phenotypic carbapenemase activity in gram-negative rods that CIM was created to detect carbapenemase enzymes in gram-negative bacilli such as *Enterobacteriaceae* and non-*Enterobacteriaceae*. Conclusion, CIM method is cheap and highly robust phenotypic screening method that can reliably detect carbapenemase activity (50).

In 2015, Hyun Ki et al., studied on further modification of the modified Hodge test for detecting Metallo-β-lactamase (MBL) producing carbapenem-resistant *Enterobactericeae*. They showed that MHT has only 60.6% sensitivity for detecting MBLs, but MHT is better for detecting VIM. Thus, the researchers would like to show about limitation of MHT detecting MBLs (39).

In 2015, Bakour et al., studied on rapid identification of carbapenemase-producing *Enterobacteriaceae, Pseudomonas aeruginosa* and *Acinetobacter baumannii* using a modified Carba NP test (MCNP). MCNP is used to identify three types of bacteria (*Enterobactericeae, Pseudomonas aeruginosa* and *Acinetobacter baumannii*) instead of Carba NP (51).

In 2015, Tsakris et al., studied on evaluation of a new phenotypic OXA-48 disk test for differentiation of OXA-48 carbapenemase-producing *Enterobacteriaceae* clinical isolates. They used imipenem disk and placed two other disks next to imipenem disk. For left side of imipenem disk contains EDTA and right side of

imipenem disk contains EDTA with PBA (Phenyl boronic acid). The result shows that the OXA-48 disk test is an accurate phenotypic test for differentiation OXA-48 isolates from other classes of carbapenemase (52).

CHAPTER III

RESEARCH METHODOLOGY

Population and Sample

Bacterial isolates were divided into control group and clinical (tested) isolates. For control isolates, the positive control group was carbapenemase-producing *Enterobacteriaceae* such as *K. pneumoniae* ATCC BAA 1705 with gene known as bla_{KPC}, *E. coli* ATCC BAA 2452 with gene known as bla_{NDM-1} and *E. coli* ATCC BAA 2523 with gene known as bla_{OXA-48}. Whereas, negative control group was non-carbapenemase-producing *Enterobacteriaceae* isolates such as *E. cloacae* ATCC BAA 1143 with gene known as ESBL (bla_{AmpC}), *K. pneumoniae* ATCC 700603 with gene known as ESBL (bla_{SHV-18}), *E. coli* ATCC 25922 and *K. pneumoniae* ATCC BAA 1706 as in Table 2.

For clinical isolates, there were 128 isolates coming from the Phetchabun hospital and 50 isolates from the Wichianburi hospital, Phetchabun province. All these isolates belong to Dr. Noppadon Jumroom's project P2558C272.

Table 2 Control isolates

No.	Organism	Gene
Carbapenemase-producing *Enterobacteriaceae*		
1	*K. pneumoniae* ATCC BAA 1705	KPC
2	*E. coli* ATCC BAA 2452	NDM-1
3	*E. coli* ATCC BAA 2523	OXA-48
Non Carbapenemase-producing *Enterobactericeae* (ESBL)		
1	*K. pneumoniae* ATCC BAA 1706	No gene
2	*E. cloacae* ATCC BAA 1143	AmpC
3	*K. pneumoniae* ATCC 700603	SHV-18

Research Instruments

1. Medium

1.1 MacConkey agar

1.2 Mueller-Hinton agar

1.3 Trypic soy broth

1.4 Nutrient agar

2. Biochemical test

2.1 Triple sugar iron agar (TSI)

2.2 Motile indole lysine (MIL)

2.3 Citrate

2.4 Malonate

2.5 Urea

3. Reagents and chemical substances

3.1 Normal saline

3.2 Alcohol 70%

3.3 Kovac's indole reagent

3.4 Drug disks (imipenem, ertapenem, meropenem and doripenem)

3.5 E strips (imipenem, ertapenem, meropenem, and doripenem)

3.6 Dipicolinic acid (DPA)

3.7 3-aminophenylboronic acid

3.8 HEPES [4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid]

3.9 DMSO (dimethyl sulfoxide)

4. Equipment and facilities

4.1 Incubator

4.2 Refrigerator

4.3 Biosafety cabinet (Level II)

4.4 Loops

4.5 Needle Loops

4.6 Test tubes (size 13×100mm)

4.7 Plates (size 90×15mm)

4.8 Cryotubes

4.9 Erlenmeyer flask 1000ml and 500ml

4.10 cotton (cover of flask)

4.11 Test tube rasks

4.12 Forceps

4.13 Pipette (serological pipette, pasture pipette and pipette 5ml)

4.14 Swabs

4.15 Foil papers

4.16 96 well plates

4.17 Plastic bags

4.18 Gloves

Methods

This research was performed as in Figure 19.

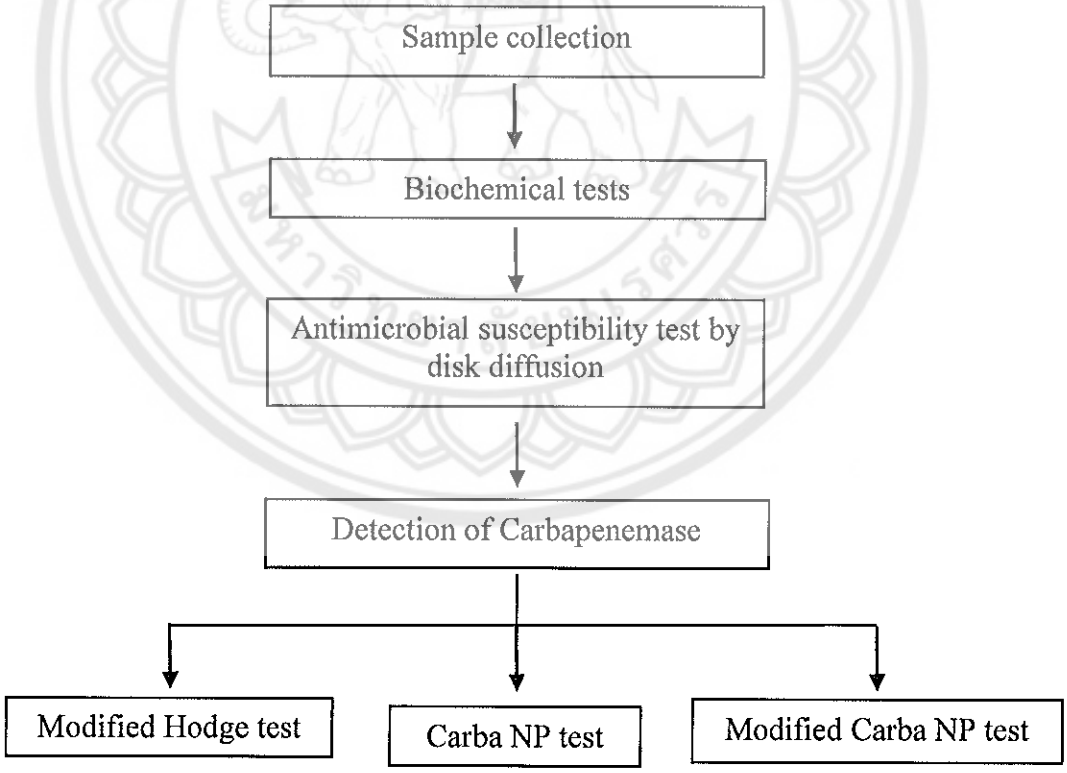


Figure 19 Summary of methods

Research Experiments

1. Clinical bacterial isolates

Clinical bacterial isolates were collected from clinical specimens at the Phetchabun and Wichianburi hospitals and were identified by conventional methods in clinical microbiology laboratory. Only *Enterobacteriaceae* were selected for this study and were sent to the Faculty of Allied Health Sciences, Naresuan University.

Enterobacteriaceae were stabbed in nutrient agar in 1.5 ml cryotube and then packed in zipped lock bags before shipping to Faculty of Allied Health Sciences.

2. Bacterial identification

2.1 Biochemical test

All clinical isolates had been subcultured on MacConkey agar by using loop to pick isolates from nutrient agar in cryotube. Then, these isolates were streaked on MacConkey agar and incubated at 35±2°C for 18-24 hours.

After that, the colonies were identified by conventional biochemical tests such as triple sugar iron (TSI), motility-indole-lysine medium (MIL), citrate, malonate and urease test. Then, the biochemical tests were incubated at 35±2°C for 18-24 hours before interpretation. Biochemical test was performed as in Figure 20.

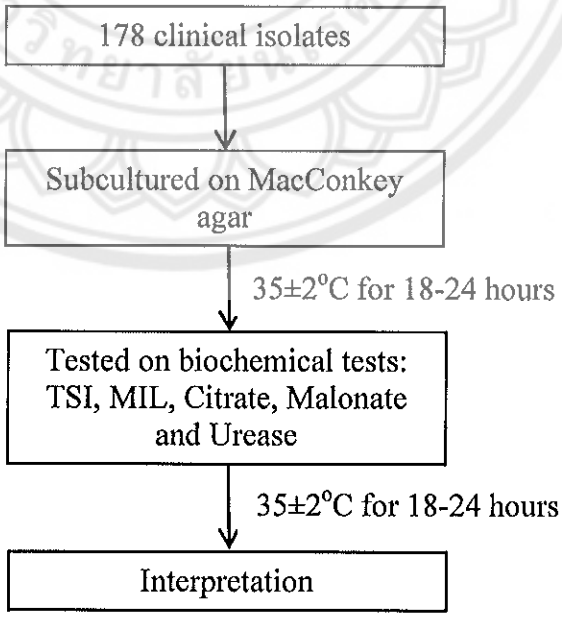


Figure 20 Biochemical test

3. Antimicrobial susceptibility test

Disk diffusion was used for antimicrobial susceptibility test. For disk diffusion, the bacterial suspension of 0.5 McFarland standard was applied on Mueller-Hinton agar and placed imipenem, meropenem, ertapenem and doripenem disks (10 mg) on medium, then incubated at 35±2°C for 16-18 hours. Interpretation was a reading zone diameter according to CLSI guideline M100-S26.

E. coli ATCC 25922 and *P. aeruginosa* ATCC 27853 used as quality control (QC) in disk diffusion. The antimicrobial susceptibility test was performed as in Figure 21.

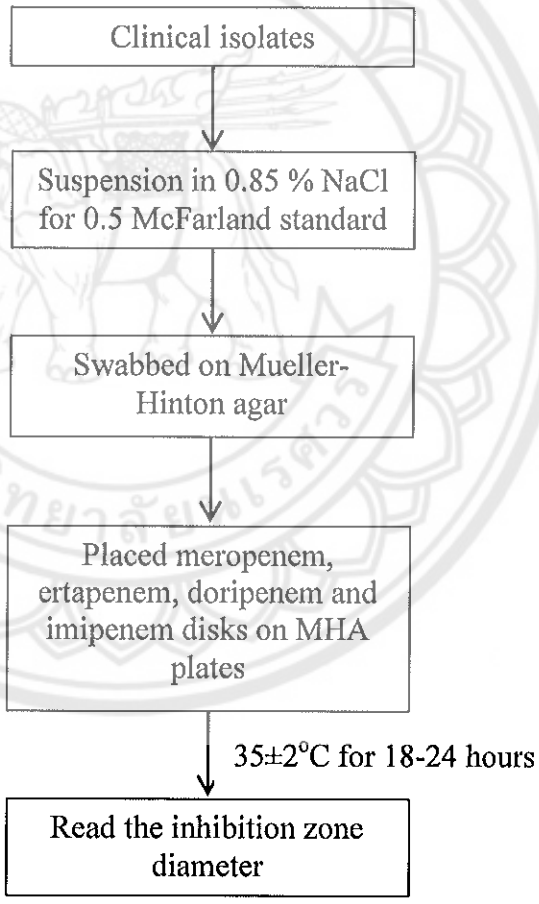


Figure 21 Antimicrobial susceptibility test

4. Carbapenemase detection

4.1 Modified Hodge test

E. coli ATCC 25922 was used as an indicator and the suspension was adjusted to 0.5 McFarland standard in 0.85% NaCl and then diluted to 1:10 for swabbing on MHA according to CLSI M100-S26 guideline. After 3-5 min, 10 mg meropenem was placed on central of plate. Then, tested isolates, positive control and negative control isolates were streaked straightly line from the edge of disk. The plates were incubated at 35±2°C for 16-18 hours. Interpretation was performed by reading the enhancing growth of indicator organism into inhibition zone. If there was an enhancing, it was a positive result. If there was no enhancing growth of indicator organism into inhibition zone, it was a negative result. Modified Hodge test was performed as in Figure 22.

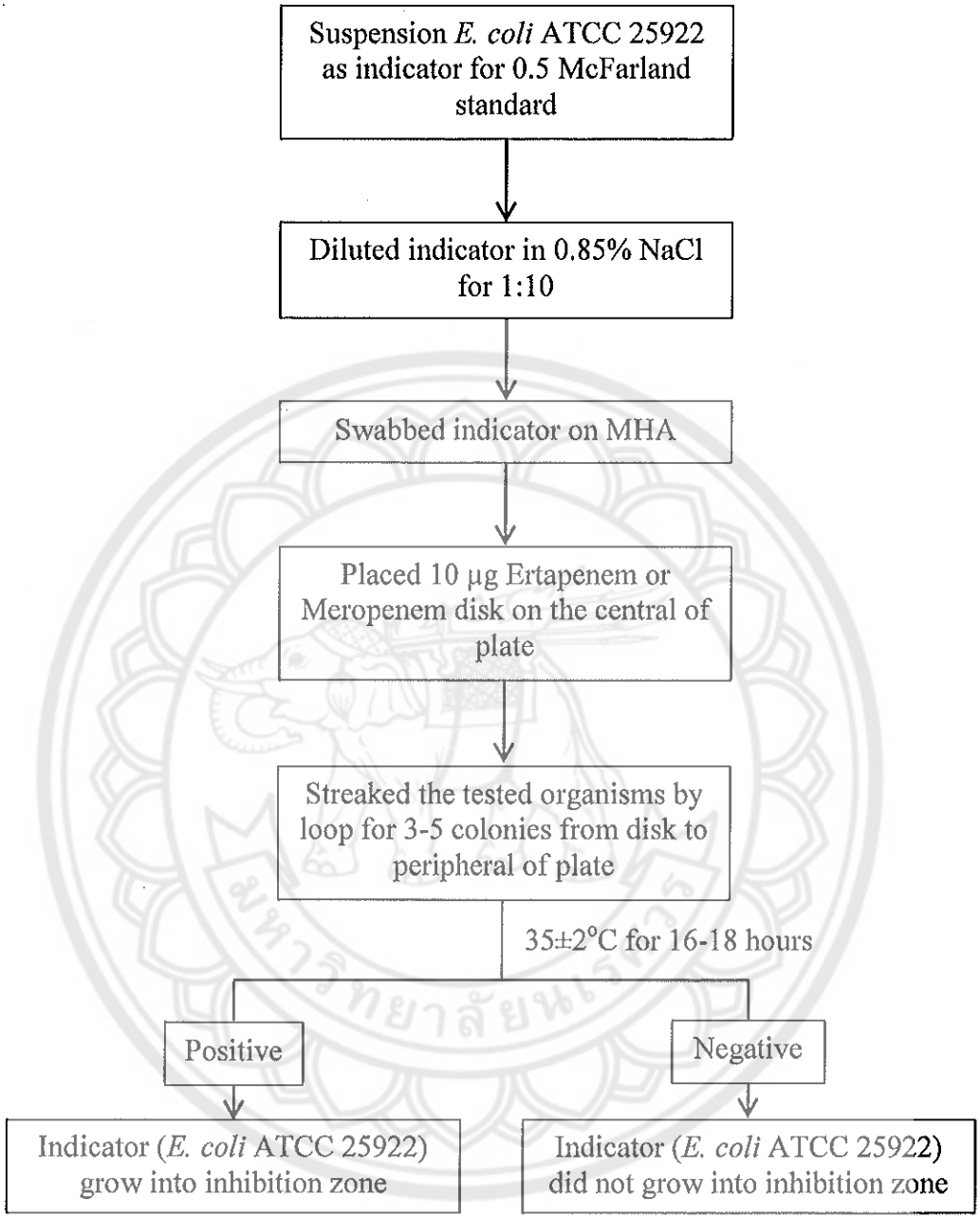


Figure 22 Flow chart of Modified Hodge test

4.2 Carba NP test

According to CLSI M100-S26, two solutions (solution A and B) were prepared with 2 ml of 0.5% Phenol red plus distilled water 16.6 ml then adding 180 µl of 10 mM zinc sulfate and optionally adding 0.1 N NaOH or 10% HCl making pH at 7.8±0.1. Solution B was a solution A with adding 6 mg/ml of imipenem powder. One loopfull of colonies was put in 100 µl micro-tube of lysis buffer. Then, solution A and

solution B were added into two different micro-tubes and incubated at temperature 35±2°C for 2 hours as in Figure 23.

For positive result, the red color of phenol red of solution B was changed into yellow. While the solution B was not change, it showed a negative result.

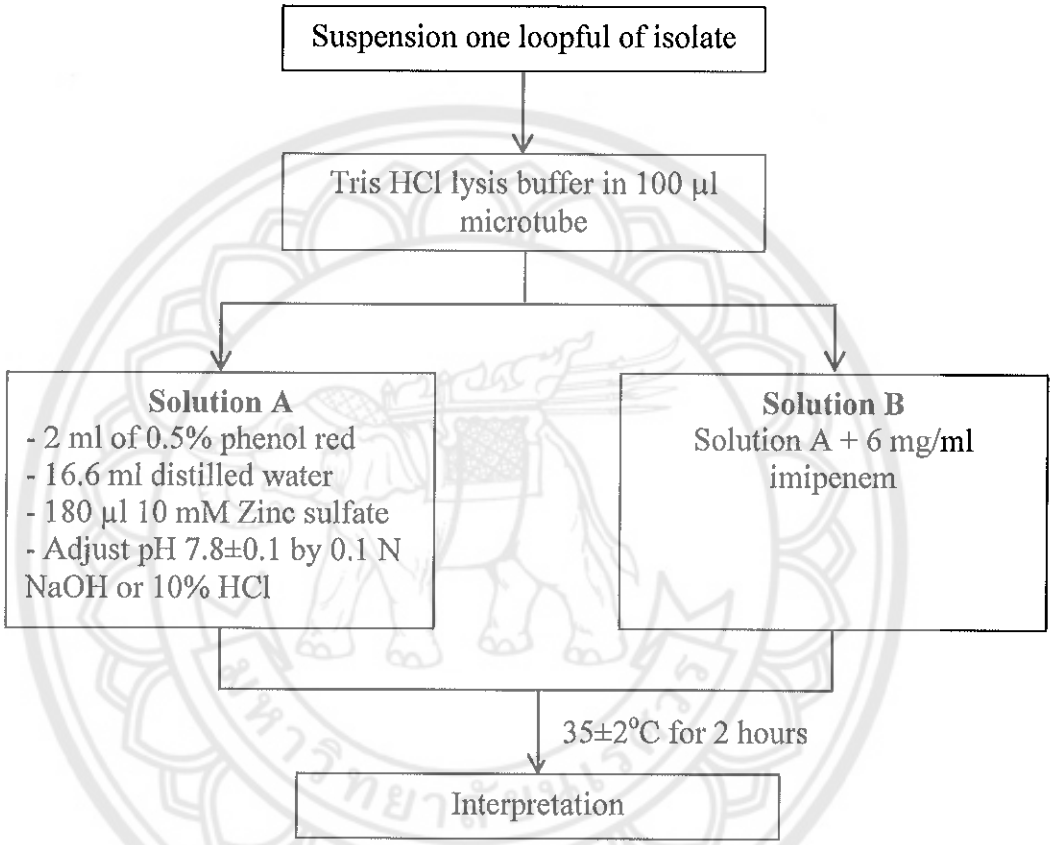


Figure 23 Flow chart of Carba NP test

4.3 Modified Carbapenem Inactivation Method

The modified carbapenem inactivation method (mCIM) has been developed by CLSI guideline M-100 S27 as a phenotypic method for detecting the carbapenemase enzymes. It has a sensitivity and specificity over 99 % to detect the activity of carbapenemase enzymes. If the inhibition zone was between 6-15 mm, it was carbapenemase positive. If the zone was between 16-17 mm, it was intermediate. If the zone was above 19 mm, it was negative result.

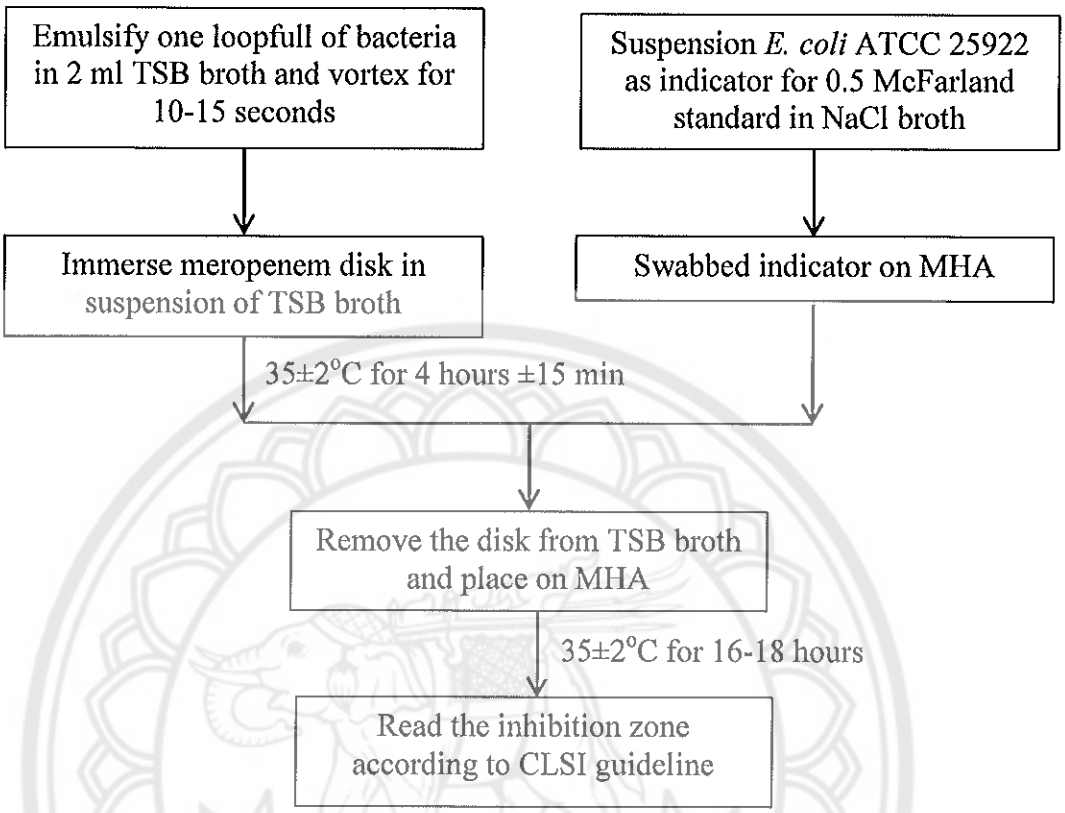


Figure 24 Flow chart of modified Carbapenem Inactivation Method (mCIM)

4.4 Modified Carba NP test

The principle of modified Carba NP test is to add inhibitors such as dipicolinic acid (DPA) and 3-amino-phenylboronic acid (APB) in Carba NP solutions to block the action of carbapenemase enzymes which hydrolyze carbapenem drug (imipenem).

All of these isolate that positive results by MHT and Carba NP test were used in this modified Carba NP test. To prepare the samples, 10 µl of loopfull bacterial colonies from MacConkey agar were added in 100 µl of lysis buffer suspension and centrifuge at 15,500 rpm at room temperature for 10 min. Moreover, the amount of bacteria inoculum was compare to 3 McFarland standard (equal to 9×10^8 CFU/ml) or over 3 McFarland standard. There were five solutions:

1. The solution N (negative control):

1.1 2 ml of 0.5% phenol red solution and added 16.6 ml of clinical laboratory reagent water

1.2 Added 180 µl of 10 mM Zinc sulfate solution

1.3 Adjusted pH to 7.8±0.1 with 0.1 N NaOH

1.4 Stored at 4-8°C in small vial or bottle

2. The solution P (positive control):

2.1 solution N plus 6 mg/ml of imipenem

2.2 stored at 4-8°C for up to 3 days

3. The solution A (Detection of class A carbapenemase):

Solution P added 50 mmol/L dipicolinic acid (DPA)

4. The solution B (Detection of class B carbapenemase):

Solution P added 90 mmol/L 3-aminophenylboronic acid (APB)

5. The solution D (Detection of class D carbapenemase):

Solution P added 50 mmol/L dipicolinic acid (DPA) and 90 mmol/L 3-aminophenylboronic acid (APB)

All reagents and the reaction of Modified Carba NP test were showed as in Table 3.

Five wells of 96-well plate were used and added different reagents. The first well (P well) was positive control. Second well (A well) was detected class A carbapenemase. Third well (B well) was detected class B carbapenemase. Four well (D well) was detected class D carbapenemase. The last well (N well) was a negative control. In each well, there were 10 µl of tested sample and 70 µl of each solution. After that these well were incubated at 35±2°C for 15-90 min as in Figure 25.

Table 3 Reagents and solution of modified Carba NP test

solution	N	P	A	B	D	Per well (70 µl)
Clinical laboratory reagent water	16.6 ml	16.6 ml	16.6 ml	16.6 ml	16.6 ml	58.1 µl
0.5% Phenol red	2 ml	2 ml	2 ml	2 ml	2 ml	7 µl
10mM Zinc sulfate	180 µl	180 µl	180 µl	180 µl	180 µl	0.0063 µl
0.1 N NaOH or 10% HCl	As needed					
Dipicolinic acid (DPA)	No	No	8.356 g/L	No	8.356 g/L	0.584 mg
3-amino-phenylboronic acid (APB)	No	No	No	10.97 g/L	10.97 g/L	7.63 mg
Imipenem	No	6 mg/ml	6 mg/ml	6 mg/ml	6 mg/ml	0.42 mg

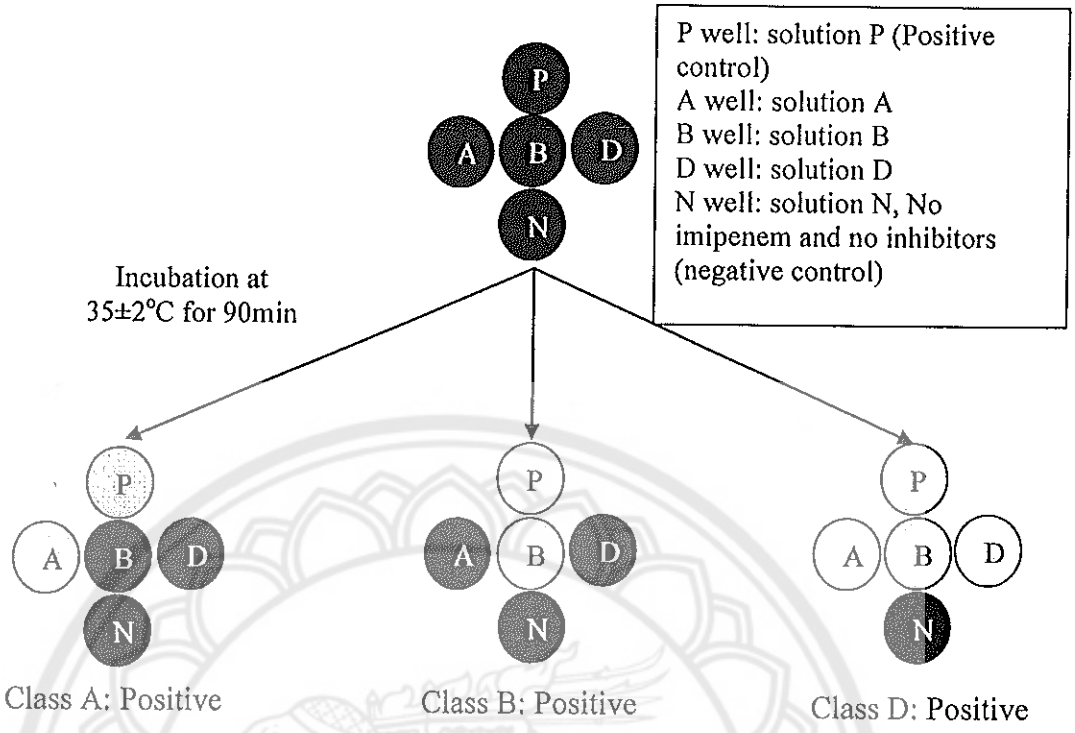


Figure 25 The pattern of 96-well plate for modified Carba NP test

Data Analysis

For statistical analysis, there are two methods.

1. Comparing the modified Carba NP test with control isolates by calculating positive results of this method and calculating the sensitivity and specificity of the modified Carba NP test by comparing to the control groups.

Table 4 Data analysis of the modified Carba NP test

	Enzyme (Carbapenemase)	**No enzyme (No carbapenemase)**
Modified Carba NP test (Positive result)	True Positive (TP)	False Positive (FP)
Modified Carba NP test (Negative result)	False Negative (FN)	True Negative (TN)

Sensitivity = TP/(TP+FN)

Specificity = TN/(TN+FP)

2. Comparing to all of three methods (Modified Hodge test, Carba NP test and Modified Carba NP test) in percentage for clinical isolates.

CHAPTER IV

RESULTS

The prevalence of *Enterobacteriaceae*

In Wichianburi hospital, 50 isolates of *Enterobacteriaceae* were collected from clinical microbiology laboratory including *E. coli* 27 isolates (54%), *K. pneumoniae* 15 isolates (30%), *Enterobacter* spp. 4 isolates (8%), *P. mirabilis* 2 isolates (4%), *C. diversus* 1 isolate (2%) and *S. marcescens* 1 isolate (2%). The results showed in Table 5.

Table 5 The number of *Enterobactericeae* isolated from Wichianburi hospital

Organisms	Number of isolates	Percentage (%)
E. coli	27	54
K. pneumoniae	15	30
Enterobacter spp.	4	8
P. mirabilis	2	4
C. diversus	1	2
S. marcescens	1	2

All these organisms were collected from various clinical samples, such as 18 isolates (36%) from urine, 13 isolates (26%) from hemoculture, 10 isolates (20%) from pus, 8 isolates (16%) from sputum and 1 isolates (2%) from ascetic fluid (Table 6).

Table 6 Type of clinical specimens were collected from Wichianburi hospital

Type of clinical specimen	Number of samples	Percentage (%)
Urine	18	36
Hemoculture	13	26
Pus	10	20
Sputum	8	16
Ascitic fluid	1/50	2

In Phetchabun hospital, there were *E. coli* 73 isolates (57.03%), *K. pneumoniae* 33 isolates (25.78%), *E. cloaceae* 13 isolates (10.15%), *P. mirabilis* 3 isolates (2.34%), *C. freundii* 3 isolates (2.34%), *S. marcescens* 2 isolates (1.56%) and *Salmonella* spp. 1 isolate (0.78%) (Table 7).

Table 7 The number of *Enterobactericeae* from Phetchabun hospital

Organisms	Number of isolates	Percentage (%)
E. coli	73	57.03
K. pneumoniae	33	25.78
E. cloacae	13	10.15
P. mirabilis	3	2.34
C. freundii	3	2.34
S. marcescens	2	1.56
Salmonella spp.	1	0.78

The samples were collected from urine 61 isolates (47.65%), hemoculture 23 isolates (17.96%), sputum 21 isolates (16.40%), swab 16 isolates (12.5%) and body fluid 7 isolates (5.46%) as in table 8.

Table 8 Type of clinical specimens were collected from Phetchabun hospital

Type of clinical specimens	Number of samples	Percentage (%)
Urine	61	47.65
Hemoculture	23	17.96
Sputum	21	16.40
swab	16	12.50
Body fluid	7	5.46

The results of antimicrobial susceptibility test by disk diffusion methods

The antimicrobial susceptibility test of all *Enterobacteriaceae* was examined by disk diffusion which were collected from Phetchabun hospital (128 isolates) and from Wichianburi hospital (50 isolates). The disks of antibiotics were tested in antimicrobial susceptibility test such as imipenem (IMP), meropenem (MEN), ertapenem (ETP) and doripenem (DOR) (Figure 26). The diameter inhibition zone was read and interpreted according to CLSI guideline. The zone diameter of impenem, meropenem and doripenem, ≥ 23 mm is susceptible, 20-22 mm is intermediate and ≤19 mm is resistance. While ertapenem, the zone diameter ≥22 mm is susceptible, the zone 19-21 mm is intermediate and the zone ≤18 mm is resistance.

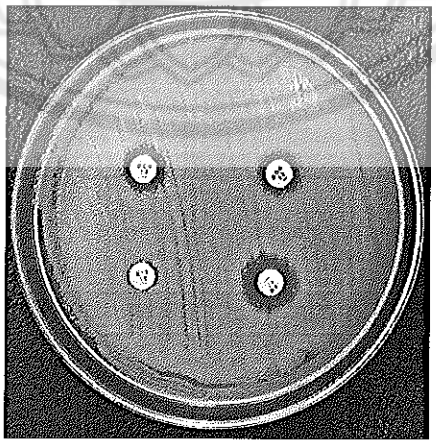


Figure 26 Antimicrobial susceptibility test by disk diffusion: Meropenem (MEM), Ertapenem (ETP), Doripenem (DOR) and Imipenem (IMP)

Fifty isolates of *Enterobacteriaceae* from Wichianburi hospital, 39 isolates (78%) were susceptible to IMP, MEM, ETP and DOR which the zone diameter were between 22-32 mm. Meanwhile, there were 11 isolates (22%) being non-susceptible.

V-001 was resistant to MEM (21 mm), ETP (11 mm) and IMP (18 mm); V-012 was only resistant to ETP (16 mm); V-014 was resistance to ETP (17 mm); V-016 was intermediate to ETP (19 mm) ; V-017 was resistant to ETP (16 mm); V-018 was resistant to ETP (18 mm); V-019 was resistant to MEM (22 mm) and ETP (16 mm); V-032 was resistant to ETP (13 mm); V-041 was resistant to MEM (20 mm), ETP (12 mm) and DOR (22); V-050 was intermediate to ETP (21 mm) and resistant to IMP (16 mm); and V-051 was intermediate to IMP (22 mm) (Table 9). The non-susceptible organisms were *E. coli* 4 isolates (36.36%), *E. cloacea* 2 isolates (18.18%), *Enterobacter* spp. 2 isolates (18.18%), *K. pneumoniae* 2 isolates (18.18%) and *P. mirabillis* 1 isolate (9.09%).

Table 9 The results of 50 clinical isolates from Wichianburi hospital for antibiotic susceptibility test by disk diffusion methods

Interpretation	Number of isolates (%)	Zone diameter (mm)			
		MEM	ETP	DOR	IMP
Susceptible	39 (78%)	23-32	22-32	23-32	23-30
Non-susceptible	11 (22%)	14-22	11-19	22	16-18
Intermediate (IMP)	1 (2%)				22
Intermediate (ETP)	1 (2%)		19		
Resistance (IMP) and Intermediate (ETP)	1 (2%)		21		16
Resistance (MEM, ETP and IMP)	1 (2%)	21	11		18
Resistance (MEN, ETP and DOR)	1 (2%)	20	12	22	
Resistance (MEM and ETP)	1 (2%)	22	16		
Resistance (ETP)	5 (10%)		13-18		

One hundred and twenty eight isolates from Phetchabun hospital were examined by disk diffusion methods for antibiotic susceptible test, there were 106 isolates (82.81%) being susceptible to IMP, MEM, ETP and DOR with zone diameter 23-35 mm. While, 22 isolates (17.18%) were non-susceptible to one or all of IMP, MEM, ETP and DOR. For P-002, P-032, P-034, P-071, P-078, P-091, P-095, P-102, P-104, P-106 and P-115 were resistant to all carbapenems with zone diameter between 6-22 mm. For P-044 and P-062 were resistant to MEM (21-22 mm) and ETP (14-18 mm). For P-065 were resistant to MEM (19 mm), ETR (11 mm) and DOR (20 mm). And the rest of these isolates were intermediate to ETP (19-21 mm) such as P-004 (19 mm), P-031 (20 mm), P-052 (20 mm), P-053 (20 mm), P-054 (20 mm), P-070 (19 mm), P-088 (21 mm) and P-127 (19 mm). These results were showed in Table 10. Among of non-susceptible isolates, there were *E. cloaceae* 9 isolates (40.9%), *E.coli* 6 isolates (27.27%), *K. pneumoniae* 4 isolates (18.18%), *C. freundii* 2 isolates (9.09%) and *Salmonella* spp. 1 isolate (4.54%).

Table 10 The results of 128 clinical isolates from Phetchabun hospital for antibiotic susceptibility test by disk diffusion methods

Interpretation	Number of isolates (%)	Zone diameter (mm)			
		MEM	ETP	DOR	IMP
Susceptible	106 (82.81%)	23-35	22-33	23-36	23-34
Non-susceptible	22 (17.18%)	6-22	6-21	6-20	6-22
Intermediate (ETP)	8 (6.25%)		19-21		
Resistance (MEM, ETP and DOR) and Intermediate (IMP)	1 (0.78%)	16	11	18	22
Resistance (MEN, ETP) and intermediate (DOR)	1 (0.78%)	19	11	20	
Resistance (ETP) and Intermediate (MEM)	2 (1.56%)	19-21	11-18		
Resistance (MEN, ETP, DOR and IMP)	10 (7.81%)	6-19	6-18	6-18	6-14

Totally, there were 11 isolates from Wichiaburi hospital and 22 isolates from Phetchabun hospital being non-susceptible to carbapenems. Therefore, the 33 of non-susceptible isolates were continually detected for carbapenemase enzymes.

The results of carbapenemase detection

In this study, the phenotypic methods of carbapenemase detection were tested by two different standard methods. There were MHT as in Figure 27-28 and Carba NP test as in Figure 29-30.

In the control groups, the results of MHT showed a positive result to *K. pneumoniae* ATCC BAA 1705 with gene of KPC, *E. coli* ATCC BAA 2452 with gene of NDM-1 and *E. coli* ATCC BAA 2523 with gene of OXA-48. While *K. pneumoniae* ATCC BAA 1706 showed a negative control to MHT. *K. pneumoniae* ATCC 700603 with gene of bla_{SHV-18} and *E. cloacae* ATCC BAA 1143 with gene of bla_{AmpC} were an ESBL enzyme producing strains. Their results showed a negative in phenotypic methods of MHT.

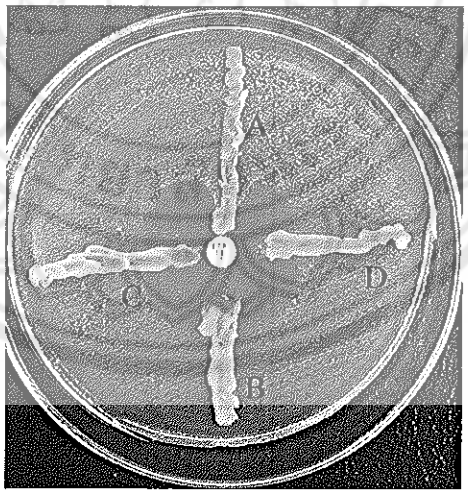


Figure 27 The results of control groups by Modified Hodge test (MHT)

Note: A: Positive control *K. pneumoniae* ATCC BAA 1075
B: Negative control *K. pneumoniae* ATCC BAA 1076
C: *K. pneumoniae* ATCC 700603 (Negative result)
D: *E. cloacae* ATCC BAA 1143 (Negative result)

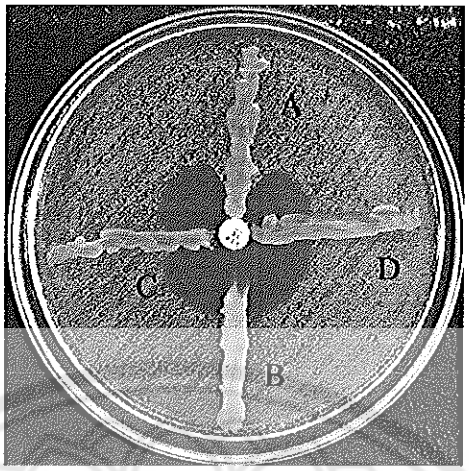


Figure 28 The results of clinical isolates by Modified Hogde test

Note: A: Positive control *K. pneumoniae* ATCC BAA 1075

B: Negative control *K. pneumoniae* ATCC BAA 1076

C: *E. coli* ATCC BAA 2523 (Positive result)

D: *E. coli* ATCC BAA 2452 (Positive result)

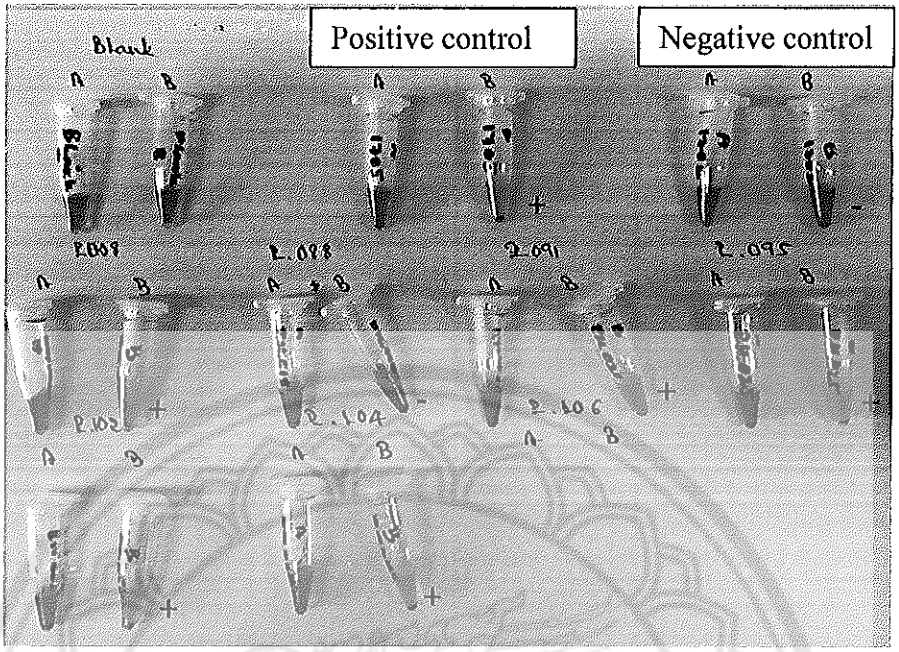


Figure 29 The Carba NP test by micro-tubes

Note: Blank tubes, Positive control (*K. pneumoniae* ATCC BAA 1075),
Negative control (*K. pneumoniae* ATCC BAA 1076)
Clinical isolates (P-008, P-088, P-091, P-095, P-102 and P-104)

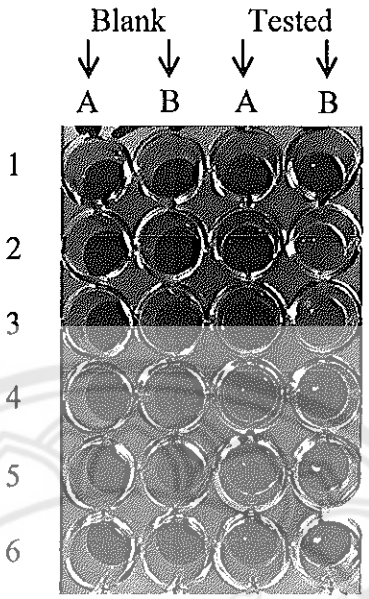


Figure 30 The Carba NP test by using 96-well plate of control groups

Note: 1: Negative control: *K. pneumoniae* ATCC BAA 1076

2: Positive control: *K. pneumoniae* ATCC BAA 1075 (bla_{KPC})

3: Positive: *E. coli* ATCC BAA 2452 (bla_{NDM-1})

4: Positive: *E. coli* ATCC BAA 2523 (bla_{OXA-48})

5: Negative: *K. pneumoniae* ATCC 700603 (bla_{SHV-18})

6: Negative: *E. cloacae* ATCC BAA 1143 (bla_{AmpC})

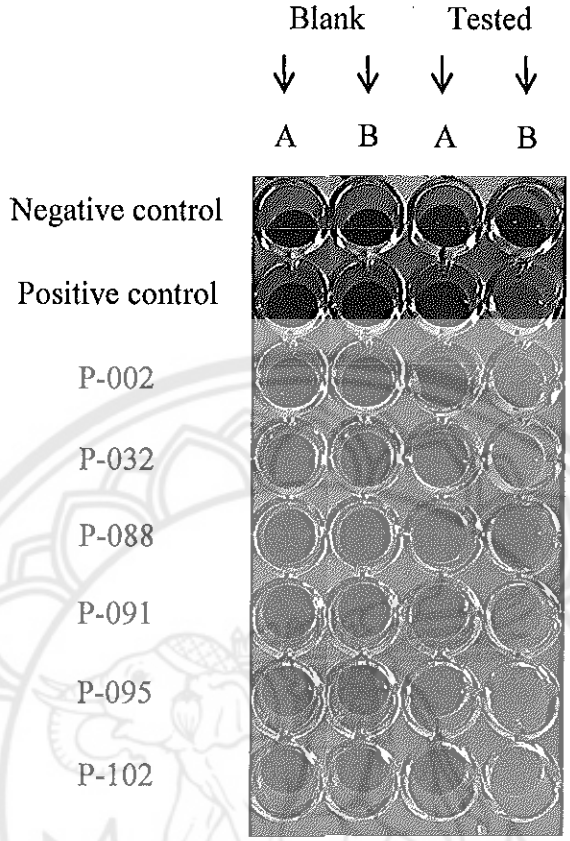


Figure 31 The Carba NP test by using 96-well plate of clinical isolates

Note: The negative control was *K. pneumoniae* ATCC BAA 1076
The positive control was *K. pneumoniae* ATCC BAA 1075
The result were positive for P-002, P-032, P-091, P-095 and P-102
but negative was P-088

For Carba NP test, *K. pneumoniae* ATCC BAA 1705 with gene of bla_{KPC} and *E. coli* ATCC BAA 2552 with gene of bla_{NDM-1} was positive in Carba NP test. While, *E. coli* ATCC BAA 2523 with gene of bla_{OXA-48} , *K. pneumoniae* ATCC BAA 1706, *K. pneumoniae* ATCC BAA 700603 with gene of bla_{SHV-18} and *E. cloacae* ATCC BAA 1143 with gene of bla_{AmpC} were negative result for Carba NP test in Table 11.

In the clinical isolates, the results of MHT was a positive only one isolate (V-014) among of 11 carbapenem-resistant isolates from Wichianburi hospital and that isolate was *E. cloacae* from pus sample. Unfortunately, MHT-positive isolate was detected by Carba NP test. It showed negative result (Table 12).

In Phetchabun hospital, 10 isolates of non-susceptible isolates were tested by MHT. Their results were positive among of 22 carbapenem-resistant isolates, such as P-002 (*E. coli*), P-032 (*C. freundii*), P-034 (*C. freundii*), P-078 (*E. coli*), P-091 (*E. cloacae*), P-095 (*E. cloacae*), P-102 (*E. cloacae*), P-104 (*E. cloacae*), P-106 (*K. pneumoniae*) and P-115 (*E. coli*). At the same time, all of them were positive in Carba NP test (Table 12 and 13).

Moreover, modified carbapenem inactivation method (mCIM) was examined to detect carbapenemase enzymes. All of control group and some of clinical isolates were tested in this method. The results showed as in Table 11 and 12.

Table 11 The result of control groups by MHT, Carba NP test and mCIM

No	Organism	Gene	Carbapenemase detection		
			MHT	Carba NP test	mCIM (Inhibition zone)
Carbapenemase-producing *Enterobacteriaceae*					
1	*K. pneumoniae* ATCC BAA 1705	KPC	+	+	+ (6 mm)
2	*E. coli* ATCC BAA 2452	NDM-1	+	+	+ (13 mm)
3	*E. coli* ATCC BAA 2523	OXA-48	+	-	+ (6 mm)
Non Carbapenemase-producing *Enterobactericeae* (ESBL)					
4	*K. pneumoniae* ATCC BAA 1706		-	-	- (23 mm)
5	*E. cloacae* ATCC BAA 1143	AmpC	-	-	- (22 mm)
6	*K. pneumoniae* ATCC 700603	SHV-18	-	-	- (20 mm)

*Interpretation of mCIM: Positive result: Inhibition Zone 6-15 mm, Intermediate: Inhibition Zone 16-18 mm and Negative result: Inhibition Zone ≥ 19 mm

Table 12 The result of carbapenemase producing in carbapenem-resistant *Enterobac-teriaceae* from Wichianburi and Phetchabun hospital by MHT, Carba NP test and mCIM

Code	Organism	Modified Hodge test (MHT)	Carba NP test	mCIM (Inhibition zone)
V-001	*K. pneumoniae*	Negative (-)	Negative (-)	Not done
V-012	*E. coli*	Negative (-)	Negative (-)	Not done
V-014	*E. cloacae*	Positive (+)	Negative (-)	Not done
V-016	*Enterobacter* spp.	Negative (-)	Negative (-)	Not done
V-017	*E. cloacae*	Negative (-)	Negative (-)	Not done
V-018	*E. coli*	Negative (-)	Negative (-)	Not done
V-019	*E. coli*	Negative (-)	Negative (-)	Not done
V-032	*E. coli*	Negative (-)	Negative (-)	Not done
V-041	*K. pneumoniae*	Negative (-)	Negative (-)	Not done
V-050	*Enterobacter* spp.	Negative (-)	Negative (-)	Not done
V-051	*P. mirabilis*	Negative (-)	Negative (-)	Negative(22 mm)
P-002	*E. coli*	Positive (+)	Positive (+)	Not done
P-004	*E. coli*	Negative (-)	Negative (-)	Not done
P-031	*E. cloacae*	Negative (-)	Negative (-)	Not done
P-032	*C. freundii*	Positive (+)	Positive (+)	Positive (6 mm)
P-034	*C. freundii*	Positive (+)	Positive (+)	Not done
P-044	*E. coli*	Negative (-)	Negative (-)	Not done
P-052	*E. cloacae*	Negative (-)	Negative (-)	Not done
P-053	*E. cloacae*	Negative (-)	Negative (-)	Not done
P-054	*E. cloacae*	Negative (-)	Negative (-)	Not done
P-062	*E. coli*	Negative (-)	Negative (-)	Not done
P-065	*K. pneumoniae*	Negative (-)	Negative (-)	Not done
P-070	*Salmonella* group D	Negative (-)	Negative (-)	Not done
P-071	*K. pneumoniae*		No growth	

Table 12 (cont.)

Code	Organism	Modified Hodge test (MHT)	Carba NP test	mCIM (Inhibition zone)
P-078	*E. coli*	Positive (+)	Positive (+)	Not done
P-088	*E. cloacae*	Negative (-)	Negative (-)	Not done
P-091	*E. cloacae*	Positive (+)	Positive (+)	Not done
P-095	*E. cloacae*	Positive (+)	Positive (+)	Positive (6 mm)
P-102	*E. cloacae*	Positive (+)	Positive (+)	Positive (6 mm)
P-104	*E. cloacae*	Positive (+)	Positive (+)	Positive (6 mm)
P-106	*K. pneumoniae*	Positive (+)	Positive (+)	Not done
P-115	*E. coli*	Positive (+)	Positive (+)	Positive (6 mm)
P-127	*K. pneumoniae*	Negative (-)	Negative (-)	Negative (20 mm)

*Interpretation of mCIM: Positive result: Inhibition Zone 6-15 mm, Intermediate: Inhibition Zone 16-18 mm and Negative result: Inhibition Zone ≥ 19 mm

Table 13 The results of clinical isolates that positive by both of MHT and Carba NP test

Code	Organism	MHT	Carba NP test
P-002	*E. coli*	+	+
P-032	*C. freundii*	+	+
P-034	*C. freundii*	+	+
P-078	*E. coli*	+	+
P-091	*E. cloacae*	+	+
P-095	*E. cloacae*	+	+
P-102	*E. cloacae*	+	+
P-104	*E. cloacae*	+	+
P-106	*K. pneumoniae*	+	+
P-115	*E. coli*	+	+

The result of the modified Carba NP test

The modified Carba NP test with inhibitor (Dipicolinic acid) showed a good expected result, but the modified Carba NP test with inhibitor (3-aminophenylboronic acid) showed an unacceptable result. Therefore, 3-aminophenylboronic acid was replaced by the clavulanic acid 5 mmol/L dissolved in 0.2 mol/l of phosphate buffer at pH 7.4.

The modified Carba NP test showed a good result in positive control group such as *K. pneunomiae* ATCC BAA 1705 (bla_{KPC}) and *E. coli* ATCC BAA 2452 (bla_{NDM-1}). But *E. coli* ATCC BAA 2532 (bla_{OXA-48}) could not change the color in Carba NP test and the modified Carba NP test. So, it could be a false-negative in Carba NP test and the modified Carba NP test. Beside carbapenemase-producing isolates, the modified Carba NP test showed the negative results to all non-carbapenemase-producing isolates, such as *E. cloacae* ATCC BAA 1143 (bla_{AmpC}), *K. pneumoniae* ATCC 700603 (bla_{SHV-18}) and *K. pneumoniae* ATCC BAA 1706 (Figure 23 or Table 11). Therefore, the sensitivity and specificity of the modified Carba NP test were 66.66 % and 100 %, respectively.

Among 33 isolates of carbapenem-resistant *Enterobacteriaceae*, only 10 isolates were positive by both MHT and Carba NP test. These isolates would continuously be tested by the modified Carba NP test. The result showed that all of these 10 isolates were positive to class B of carbapenemase enzymes. These results were showed in Figure 33 and Table 15.

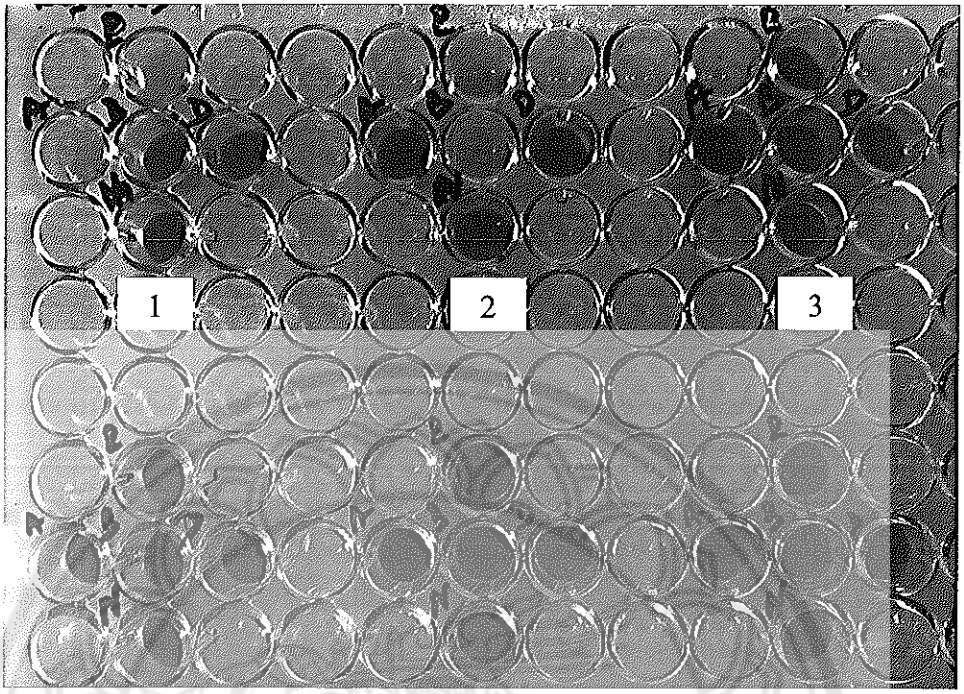


Figure 32 The modified Carba NP test of control groups

Note: 1. *K. pneumoniae* ATCC BAA 1705 (bla_{KPC}): Positive to class A

2. *E. coli* ATCC BAA 2452 (bla_{NDM-1}): Positive to class B

3. *E. coli* ATCC BAA 2523 (bla_{OXA-48}): Negative

4. *K. pneumoniae* ATCC BAA 1076: Negative

5. *E. cloacae* ATCC BAA 1143 (bla_{AmpC}): Negative

6. *K. pneumoniae* ATCC 700603 (bla_{SHV-18}): Negative

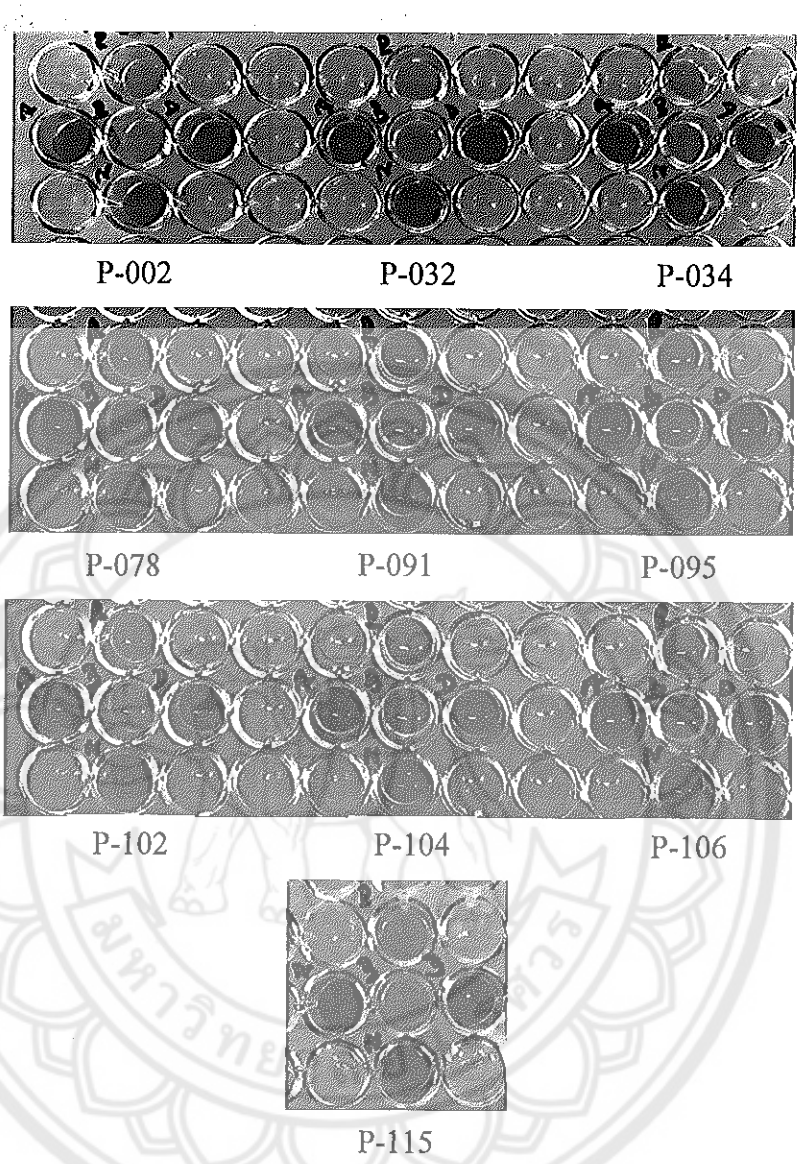


Figure 33 The modified Carba NP test of carbapenemase producing clinical isolates

Note: P well: Positive control, A well: solution A for detection class A, B well: solution B for detection class B, D well: solution D for detection class D and N well: Negative control. P well and B well changed color from red to yellow, they were class B of carbapenemase.

Table 14 Repeatability results of control group by the modified Carba NP test

Day	Time	Modified Carba NP test					
		KPC	NDM-1	OXA-48	*K. pneumoniae* 1706	Amp-C	SHV-18
1	1st	+	+	-	-	-	-
	2nd	+	+	-	-	-	-
	3rd	+	+	-	-	-	-
	4th	+	+	-	-	-	-
	5th	+	+	-	-	-	-
2	1st	+	+	-	-	-	-
	2nd	+	+	-	-	-	-
	3rd	+	+	-	-	-	-
	4th	+	+	-	-	-	-
	5th	+	+	-	-	-	-
3	1st	+	+	-	-	-	-
	2nd	+	+	-	-	-	-
	3rd	+	+	-	-	-	-
	4th	+	+	-	-	-	-
	5th	+	+	-	-	-	-
4	1st	+	+	-	-	-	-
	2nd	+	+	-	-	-	-
	3rd	+	+	-	-	-	-
	4th	+	+	-	-	-	-
	5th	+	+	-	-	-	-
5	1st	+	+	-	-	-	-
	2nd	+	+	-	-	-	-
	3rd	+	+	-	-	-	-
	4th	+	+	-	-	-	-
	5th	+	+	-	-	-	-

Table 15 The results of the modified Carba NP test for carbapenemase producing clinical isolates

Code	Organism	Modified Carba NP test					Reading
		P well	A well	B well	D well	N well	
P-002	*E. coli*	+	-	+	-	-	Class B
P-032	*C. freundii*	+	-	+	-	-	Class B
P-034	*C. freundii*	+	-	+	-	-	Class B
P-078	*E. coli*	+	-	+	-	-	Class B
P-091	*E. cloacae*	+	-	+	-	-	Class B
P-095	*E. cloacae*	+	-	+	-	-	Class B
P-102	*E. cloacae*	+	-	+	-	-	Class B
P-104	*E. cloacae*	+	-	+	-	-	Class B
P-106	*K. pneumoniae*	+	-	+	-	-	Class B
P-115	*E. coli*	+	-	+	-	-	Class B

The result of genotypic method

Some of clinical isolates were sent to the clinical microbiology laboratory of Ramathibodi hospital, Mahidol University for detecting genes by AMR direct flow chip kit (detection of antibiotic-resistant markers by multiplex PCR). There were 6 clinical isolates such as P-032, P-095, P-102, P-105, P-115 and V-051.

The result showed NDM gene were detected in P-032, P-095, P-102, P-105 and P-115, while V-051 was negative by AMR direct flow chip kit (Table 16).

Table 16 The result of genotypic methods by AMR direct flow chip kit for some clinical isolates

Code	Organism	Disk diffusion				Modified Carba NP	Genotypic
		IMI	MER	ETP	DOR		
P-032	*C. freudii*	13 (R)	14 (R)	12 (R)	13 (R)	Class B	NDM
P-095	*E. Cloacae*	6 (R)	6 (R)	6 (R)	6 (R)	Class B	NDM
P-102	*E. Cloacae*	6 (R)	6 (R)	6 (R)	6 (R)	Class B	NDM
P-105	*E. Cloacae*	6 (R)	6 (R)	6 (R)	6 (R)	Class B	NDM
P-115	*E. Cloacae*	10 (R)	6 (R)	14 (R)	13 (R)	Class B	NDM
V-051	*P. mirabili*	22 (I)	29 (S)	29 (S)	27 (S)	Negative	No gene

CHAPTER V

DISCUSSION

The prevalence of carbapenem-resistant *Enterobacteriaceae*

In this study, there were 50 *Enterobacteriaceae* isolates from Wichianburi hospital and 128 *Enterobacteriaceae* isolates from Phetchabun hospital had been collected since August 2015-Deccember 2016. The total of isolates was 178 *Enterobacteriaceae* from both hospitals and there were 79 (44.38%) isolates collected from urine, 36 (20.22%) isolates from hemoculture, 29 (16.29%) isolates from sputum (respiratory system), 26 (14.6%) isolates from wound or pus swabs and 8 (4.5%) isolates from others. These specimens were different from the Taiwanese study of Jann-Tay et al in 2015 showed that there were 39.9%, 35.7%, 13.6%, 7.8% and 4.8% from respiratory, urine, wound swab, hemoculture and others, respectively (53).

These isolates of *Enterobacteriaceae* were tested by many methods such as biochemical test, antimicrobial susceptibility test, carbapenemase detection (Modified Hodge test, Carba NP test and Modified Carba NP test). The results showed that the 11 isolates were non-susceptible to carbapenem in Wichianburi hospital and 22 isolates were non-susceptible to carbapenem in Phetchabun hospital. Therefore, there were 33 (18.54%) *Enterobacteriaceae* isolates which were non-susceptible to carbapenem in the both hospitals (Wichianburi and Phetchabun hospitals). This result showed the prevalence of carbapenem-resistant *Enterobacteriaceae* was higher than other previous study such as Khon Kaen University's study published in 2012 showed the prevalence of carbapenem-resistant *Enterobacteriaceae* was 2.2% among 4818 isolates (54). But according to Yanglin Xu et al showed that there was less than 1% carbapenem-resistant *Enterobacteriaceae* in Thailand (55).

Among of 33 carbapenem-resistant *Enterobacteriaceae*, there were *E. cloacae* 11 isolates (33.33%), *E. coli* 10 isolates (30.30%), *K. pneumoniae* 6 isolates (18.18%), *Enterobacter* spp. 2 isolates (6.06%), *C. freundii* 2 islates (6.06%), *P. mirabillis* 1 isolate (3.03%) and *Salmonella* group D 1 isolate (3.03%). That result was different from Yanglin Xu et al showed that *E. coli* was resistant to imipenem and

meropenem higher than *K. pneumoniae*, *Enterobacter* spp. and *C. freundii*, respectively (55).

The Carbapenemase detection

For carbapenemase detection, the modified Hodge test gave a positive result for 11 isolates among 33 carbapenem-resistant *Enterobacteriaceae* isolates (33.33%). All of 11 MHT-positive isolates also showed a positive result to Carba NP test except one isolate (V-014). The isolate (V-014) could be a false-positive of MHT causing by expectedly *AmpC* producing which more likely resistance to imipenem than other carbapenems (56). Moreover, it could be class D of carbapenemase which was a false-negative of Carba NP test similar to Dortel and Nordmann's study in 2012. Their results showed only 80% specificity to low carbapenemase activity like OXA-48-like (2). Thus, this isolate (V-014) should be done genotypic method for confirming the class of carbapenemase enzymes.

According to CLSI guideline, the duration of incubation is 2 hours (34). For Khon Kaen University's study published in 2012, it showed that the Carba NP test using paper strip test could be positve in 1 minute to 5 minutes (54), but in this study showed that the good incubation period could be between 15 minutes and 90 minutes. For inoculation, this study used the amount of cell less than other study. The amount of cell would compare to equal or over 3 McFarland standard. Meanwhile, CLSI guideline and Fernado Pasteran et al recommended 1 µl loopfull (over 5 McFarland standard) (57).

The modified Carba NP test

The 3-aminophenylboronic acid could not inhibit the activity of carbapenemase enzymes in class A. Previous study, Girgis SM published in 2015 using boronic acid disk for detection of carbapenemase in *K. pneumonia*. In 2014, Hammoudi D reviewed that 3-aminophenylboronic acid could be used to detect class A of carbapenemase enzyme (38), Other studies, 3-aminophenylboronic acid can be detected KPC-producing *K. pneumoniae*, the sensitivity and specificity were 100% and 98%, respectively (52,56,58).

The modified Carba NP test demonstrated the low sensitivity (66.66 %) and high specificity (100 %) to detect the classes of carbapenemase enzymes. Although, it had a low sensitivity but it showed high specificity that were similar to other previous studies (52,56,58). For low-enzyme-activity OXA-48, the modified Carba NP test was not different from the Carba NP test (2). Both of these tests demonstrated a false negative to OXA-48 producing isolates in order that was a reason of the sensitivity of the modified Carba NP test. However, the modified Carba NP test could highly detect class A and class B (100% sensitivity).

Among of 178 isolates collected from Phetchabun province, 10 isolates of class B of carbapenemase producing isolates were detected by the modified Carba NP test and then 5 of these 10 isolates, been confirmed by genotypic method, were NDM gene. Therefore, in this study, the present of class B carbapenemase was not different from Benchamas et al in 2012 showed that an emergence of NDM-1 and IMP-14a-producing *Enterobacteriaceae* in Thailand (54). It was likely that Arpaisri et al in 2016, there were 125 of 137 carbapenemase-producing *Enterobactericeae* and *Pseudomonas* spp. in three different hospitals (Nan hospital, Srinagarind hospital and The Queen Sirikit National Institute of Child Health). NDM and OXA-like genes were detected in these organisms (59).

CHAPTER VI

CONCLUSION

This study showed that 10 among of 128 carbapenem-resistant *Enterobacteriaceae* from Phetchabun province were carbapenemase producing *Enterobacteriaceae* and all of them were class B of carbapenemase enzyme. Therefore, Class B of carbapenemase is the most enzymes to emerge in Thailand comparing to other enzymes.

The modified Carba NP test is a new method and can detect the classes of carbapenemase enzymes. Although, this method have a low sensitivity comparing to combined-disc test and double-disc synergy test but it is easy to perform by using 96-well plate and adding inhibitors (clavulanic acid and/or dipicolinic acid) in the solution of the Carba NP test to differentiate the class of carbapenemase enzymes. Besides being easy, this method can read or interpret the result between 15 to 90 minutes while the combined-disc test or disc-synergy test have spent 16 to 18 hours.

So, the modified Carba NP test is a novel, easy and fast method to classify the carbapenemase enzymes.

Future Perspective and Recommendation

For further confirmation to the modified Carba NP test, the genotypic methods should be done to find or detect the genes of clinical isolates.

The prevalence of carbapenem-resistant *Enterobacteriaceae* and carbapenemase-producing *Enterobacteriaceae* should be studied widely not only in Thailand but also in neighbor of Thailand.

The new drugs should be developed to fight against carbapenemase-producing *Enterobacteriaceae*.

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APPENDIX

Type of specimens from Phetchabun hospital (128 islates)

No	Organism	Specimen
P-001	*E. coli*	Urine
P-002	*E. coli*	Urine
P-003	*E. coli*	Urine
P-004	*E. coli*	Urine
P-005	*E. coli*	Urine
P-006	*E. coli*	Urine
P-007	*E. coli*	Urine
P-008	*E. coli*	Urine
P-009	*E. coli*	Urine
P-010	*E. coli*	Urine
P-011	*E. coli*	Urine
P-012	*E. coli*	Urine
P-013	*E. coli*	Urine
P-014	*E. coli*	Urine
P-016	*E. coli*	Urine
P-017	*E. coli*	Urine
P-018	*E. coli*	Urine
P-019	*E. coli*	Urine
P-020	*E. coli*	Urine
P-021	*E. coli*	Urine
P-022	*E. coli*	Urine
P-023	*E. coli*	Urine
P-024	*E. coli*	Urine
P-025	*E. coli*	Urine
P-026	*K. pneumoniae*	Urine
P-027	*K. pneumoniae*	Urine
P-028	*K. pneumoniae*	Urine

No	Organism	Specimen
P-029	*K. pneumoniae*	Urine
P-030	*E. cloacae*	Urine
P-031	*E. cloacae*	Urine
P-032	*C. freundii*	Urine
P-033	*C. freundii*	Urine
P-034	*C. freundii*	Urine
P-035	*P. mirabilis*	Urine
P-036	*S. marcescens*	Urine
P-037	*E. coli*	Scrotum swab
P-038	*E. coli*	Scrotum swab
P-039	*E. coli*	Peritoneal fluid
P-040	*E. coli*	Bed sore
P-041	*E. coli*	Neck swab
P-042	*E. coli*	Kidney pus
P-043	*E. coli*	Sputum
P-044	*E. coli*	Ascitic fluid
P-045	*E. coli*	Pleural fluid
P-046	*K. pneumoniae*	Finger swab
P-047	*K. pneumoniae*	Hand swab
P-048	*K. pneumoniae*	Sputum
P-049	*K. pneumoniae*	Sputum
P-050	*K. pneumoniae*	Sputum
P-051	*K. pneumoniae*	Sputum
P-052	*E. cloacae*	Foot-heel swab
P-053	*E. cloacae*	Leg swab
P-054	*E. cloacae*	Foot swab
P-055	*P. mirabilis*	Foot swab
P-056	*P. mirabilis*	Coccyx swab
P-057	*S. marcescens*	Peritoneal dialysis
P-058	*E. coli*	Hemoculture

No	Organism	Specimen
P-059	*E. coli*	Hemoculture
P-060	*E. coli*	Hemoculture
P-061	*K. pneumoniae*	Hemoculture
P-062	*E. coli*	Hemoculture
P-063	*K. pneumoniae*	Hemoculture
P-064	*K. pneumoniae*	Hemoculture
P-065	*K. pneumoniae*	Hemoculture
P-066	*K. pneumoniae*	Hemoculture
P-067	*K. pneumoniae*	Hemoculture
P-068	*E. coli*	Hemoculture
P-069	*E. cloacae*	Hemoculture
P-070	*Salmonella* group D	Hemoculture
P-071	*K. pneumoniae*	Sputum
P-072	*K. pneumoniae*	Sputum
P-073	*K. pneumoniae*	Hand swab
P-074	*K. pneumoniae*	Sputum
P-075	*E. coli*	Sputum
P-076	*E. cloacae*	Sputum
P-077	*E. coli*	Hemoculture
P-078	*E. coli*	Urine
P-079	*K. pneumoniae*	Sputum
P-080	*E. coli*	Hemoculture
P-081	*E. coli*	Hemoculture
P-082	*E. coli*	Urine
P-083	*E. coli*	Urine
P-084	*E. coli*	Hemoculture
P-085	*E. coli*	Urine
P-086	*E. coli*	Urine
P-087	*E. coli*	Urine
P-088	*E. cloacae*	Urine

No	Organism	Specimen
P-089	*E. coli*	Urine
P-090	*E. coli*	Urine
P-091	*E. cloacae*	Urine
P-092	*E. coli*	Peritoneal drainage
P-093	*E. coli*	Drainage
P-094	*E. coli*	Urine
P-095	*E. cloacae*	Urine
P-096	*E. coli*	Abdonimal swab
P-097	*E. coli*	Foot swab
P-098	*K. pneumoniae*	Sputum
P-099	*K. pneumoniae*	Sputum
P-100	*K. pneumoniae*	Sputum
P-101	*E. coli*	Sputum
P-102	*E. cloacae*	Back wound swab
P-103	*E. cloacae*	Urine
P-104	*E. cloacae*	Urine
P-105	*E. coli*	Urine
P-106	*K. pneumoniae*	Urine
P-107	*E. coli*	Hemoculture
P-108	*K. pneumoniae*	Sputum
P-109	*E. coli*	Hemoculture
P-110	*E. coli*	Hemoculture
P-111	*E. coli*	Pus
P-112	*K. pneumoniae*	Urine
P-113	*E. coli*	Urine
P-114	*E. coli*	Urine
P-115	*E. coli*	Urine
P-116	*K. pneumoniae*	Sputum
P-117	*K. pneumoniae*	Ascitic fluid
P-118	*E. coli*	Hemoculture

No	Organism	Specimen
P-119	*E. coli*	Hemoculture
P-120	*E. coli*	Urine
P-121	*K. pneumoniae*	Urine
P-122	*E. coli*	Urine
P-123	*K. pneumoniae*	Sputum
P-124	*E. coli*	Urine
P-125	*E. coli*	Hemoculture
P-126	*K. pneumoniae*	Sputum
P-127	*K. pneumoniae*	Urine
P-128	*E. coli*	Sputum

Type of specimens from Wichianburi hospital (50 islates)

No	Organism	Specimen
V-001	*K. pneumoniae*	Urine
V-002	*K. pneumoniae*	Sputum
V-003	*E. coli*	Hemoculture
V-004	*E. coli*	Hemoculture
V-005	*E. coli*	Hemoculture
V-006	*E. coli*	Hemoculture
V-007	*E. coli*	Hemoculture
V-008	*E. coli*	Hemoculture
V-009	*E. coli*	Urine
V-010	*E. coli*	Urine
V-011	*E. coli*	Urine
V-012	*E. coli*	Urine
V-013	*E. coli*	Pus
V-014	*E. cloacae*	Pus
V-015	*P. mirabilis*	Hemoculture
V-016	*Enterobacter* spp.	Pus
V-017	*E. cloacae*	Pus
V-018	*E. coli*	Pus
V-019	*E. coli*	Urine
V-020	*E. coli*	Hemoculture
V-021	*E. coli*	Hemoculture
V-022	*K. pneumoniae*	Sputum
V-023	*K. pneumoniae*	Sputum
V-024	*E. coli*	Urine
V-025	*E. coli*	Urine
V-026	*E. coli*	Urine
V-027	*E. coli*	Urine

No	Organism	Specimen
V-028	*S. marscens*	Urine
V-029	*E. coli*	Urine
V-030	*E. coli*	Urine
V-031	*E. coli*	Urine
V-032	*E. coli*	Urine
V-033	*E. coli*	Urine
V-034	*E. coli*	Urine
V-035	*E. coli*	Pus
V-036	*E. coli*	Pus
V-037	*K. pneumoniae*	Hemoculture
V-038	*K. pneumoniae*	Hemoculture
V-039	*K. pneumoniae*	Hemoculture
V-040	*K. pneumoniae*	Hemoculture
V-041	*K. pneumoniae*	Sputum
V-042	*K. pneumoniae*	Sputum
V-043	*K. pneumoniae*	Sputum
V-044	*K. pneumoniae*	Pus
V-045	*K. pneumoniae*	Pus
V-046	*K. pneumoniae*	Pus
V-047	*K. pneumoniae*	Ascitic fluid
V-048	*Enterobacter* spp.	Sputum
V-049	*P. mirabilis*	Urine
V-050	*C. diversus*	Sputum

Disk diffusion (Zone Diameter)

Phetchabun Hospital (128 isolates)

No.	Organism	Drug susceptibility test			
		Zone diameter (mm)			
		IMP	MEM	ETP	DOR
P-001	*E. coli*	27 (S)	29 (S)	30 (S)	27 (S)
P-002	*E. coli*	14 (R)	14 (R)	10 (R)	15 (R)
P-003	*E. coli*	26 (S)	29 (S)	30 (S)	29 (S)
P-004	*E. coli*	28 (S)	23 (S)	19 (I)	25 (S)
P-005	*E. coli*	24 (S)	28 (S)	23 (S)	26 (S)
P-006	*E. coli*	26 (S)	27 (S)	24 (S)	26 (S)
P-007	*E. coli*	27 (S)	27 (S)	24 (S)	26 (S)
P-008	*E. coli*	25 (S)	29 (S)	29 (S)	28 (S)
P-009	*E. coli*	27 (S)	30 (S)	31 (S)	28 (S)
P-010	*E. coli*	27 (S)	28 (S)	30 (S)	27 (S)
P-011	*E. coli*	31 (S)	32 (S)	33 (S)	30 (S)
P-012	*E. coli*	27 (S)	31 (S)	30 (S)	29 (S)
P-013	*E. coli*	28 (S)	32 (S)	33 (S)	31 (S)
P-014	*E. coli*	29 (S)	31 (S)	33 (S)	30 (S)
P-015	*E. coli*	27 (S)	30 (S)	28 (S)	28 (S)
P-016	*E. coli*	29 (S)	30 (S)	31 (S)	29 (S)
P-017	*E. coli*	26 (S)	30 (S)	29 (S)	28 (S)
P-018	*E. coli*	27 (S)	30 (S)	31 (S)	29 (S)
P-019	*E. coli*	29 (S)	31 (S)	32 (S)	29 (S)
P-020	*E. coli*	27 (S)	30 (S)	31 (S)	28 (S)
P-021	*E. coli*	29 (S)	31 (S)	29 (S)	30 (S)
P-022	*E. coli*	29 (S)	30 (S)	29 (S)	30 (S)
P-023	*E. coli*	27 (S)	29 (S)	30 (S)	30 (S)

No.	Organism	Drug susceptibility test			
		Zone diameter (mm)			
		IMP	MEM	ETP	DOR
P-024	*E. coli*	27 (S)	30 (S)	31 (S)	28 (S)
P-025	*E. coli*	28 (S)	30 (S)	32 (S)	29 (S)
P-026	*K. pneumoniae*	26 (S)	27 (S)	29 (S)	27 (S)
P-027	*K. pneumoniae*	25 (S)	27 (S)	27 (S)	24 (S)
P-028	*K. pneumoniae*	26 (S)	27 (S)	24 (S)	27 (S)
P-029	*K. pneumoniae*	27 (S)	29 (S)	29 (S)	27 (S)
P-030	*E. cloacae*	23 (S)	28 (S)	28 (S)	28 (S)
P-031	*E. cloacae*	23 (S)	26 (S)	20 (I)	27 (S)
P-032	*C. freundii*	13 (R)	14 (R)	12 (R)	13 (R)
P-033	*C. freundii*	28 (S)	32 (S)	33 (S)	30 (S)
P-034	*C. freundii*	22 (I)	16 (R)	11 (R)	18 (R)
P-035	*P. mirabilis*	25 (S)	31 (S)	32 (S)	29 (S)
P-036	*S. marcescens*	23 (S)	25 (S)	27 (S)	25 (S)
P-037	*E. coli*	27 (S)	29 (S)	29 (S)	29 (S)
P-038	*E. coli*	28 (S)	29 (S)	27 (S)	28 (S)
P-039	*E. coli*	28 (S)	30 (S)	31 (S)	29 (S)
P-040	*E. coli*	26 (S)	27 (S)	31 (S)	27 (S)
P-041	*E. coli*	30 (S)	31 (S)	30 (S)	31 (S)
P-042	*E. coli*	31 (S)	34 (S)	33 (S)	32 (S)
P-043	*E. coli*	29 (S)	32 (S)	30 (S)	32 (S)
P-044	*E. coli*	27 (S)	21 (I)	18 (R)	23 (S)
P-045	*E. coli*	27 (S)	27 (S)	29 (S)	26 (S)
P-046	*K. pneumoniae*	26 (S)	29 (S)	30 (S)	27 (S)
P-047	*K. pneumoniae*	27 (S)	27 (S)	28 (S)	27 (S)
P-048	*K. pneumoniae*	24 (S)	29 (S)	23 (S)	28 (S)
P-049	*K. pneumoniae*	27 (S)	30 (S)	32 (S)	28 (S)
P-050	*K. pneumoniae*	27 (S)	30 (S)	29 (S)	28 (S)

No.	Organism	Drug susceptibility test			
		Zone diameter (mm)			
		IMP	MEM	ETP	DOR
P-051	*K. pneumoniae*	29 (S)	30 (S)	31 (S)	28 (S)
P-052	*E. cloacae*	24 (S)	26 (S)	20 (I)	25 (S)
P-053	*E. cloacae*	23 (S)	26 (S)	20 (I)	25 (S)
P-054	*E. cloacae*	22 (I)	25 (S)	20 (I)	25 (S)
P-055	*P. mirabilis*	25 (S)	30 (S)	31 (S)	28 (S)
P-056	*P. mirabilis*	26 (S)	31 (S)	31 (S)	28 (S)
P-057	*S. marcescens*	26 (S)	30 (S)	32 (S)	29 (S)
P-058	*E. coli*	26 (S)	27 (S)	22 (S)	25 (S)
P-059	*E. coli*	27 (S)	30 (S)	29 (S)	28 (S)
P-060	*E. coli*	27 (S)	30 (S)	32 (S)	29 (S)
P-061	*K. pneumoniae*	27 (S)	28 (S)	28 (S)	26 (S)
P-062	*E. coli*	26 (S)	22 (I)	14 (R)	23 (S)
P-063	*K. pneumoniae*	27 (S)	28 (S)	32 (S)	27 (S)
P-064	*K. pneumoniae*	27 (S)	31 (S)	33 (S)	31 (S)
P-065	*K. pneumoniae*	26 (S)	19 (R)	11 (R)	20 (I)
P-066	*K. pneumoniae*	26 (S)	29 (S)	30 (S)	28 (S)
P-067	*K. pneumoniae*	27 (S)	28 (S)	25 (S)	28 (S)
P-068	*E. coli*	28 (S)	32 (S)	33 (S)	30 (S)
P-069	*E. cloacae*	27 (S)	29 (S)	29 (S)	31 (S)
P-070	*Salmonella* group D	27 (S)	26 (S)	19 (I)	27 (S)
P-071	*K. pneumoniae*	6 (R)	6 (R)	6 (R)	6 (R)
P-072	*K. pneumoniae*	28 (S)	25 (S)	27 (S)	27 (S)
P-073	*K. pneumoniae*	30 (S)	30 (S)	29 (S)	26 (S)
P-074	*K. pneumoniae*	31 (S)	31 (S)	28 (S)	28 (S)
P-075	*E. coli*	33 (S)	33 (S)	32 (S)	32 (S)
P-076	*E. cloacae*	27 (S)	22 (S)	28 (S)	25 (S)
P-077	*E. coli*	34 (S)	33 (S)	34 (S)	32 (S)

No.	Organism	Drug susceptibility test			
		Zone diameter (mm)			
		IMP	MEM	ETP	DOR
P-078	*E. coli*	12 (R)	6 (R)	14 (R)	14 (R)
P-079	*K. pneumoniae*	30 (S)	27 (S)	30 (S)	27 (S)
P-080	*E. coli*	32 (S)	27 (S)	31 (S)	29 (S)
P-081	*E. coli*	35 (S)	34 (S)	34 (S)	35 (S)
P-082	*E. coli*	31 (S)	26 (S)	31 (S)	30 (S)
P-083	*E. coli*	32 (S)	30 (S)	32 (S)	30 (S)
P-084	*E. coli*	35 (S)	32 (S)	33 (S)	33 (S)
P-085	*E. coli*	33 (S)	32 (S)	31 (S)	28 (S)
P-086	*E. coli*	35 (S)	32 (S)	31 (S)	32 (S)
P-087	*E. coli*	35 (S)	28 (S)	34 (S)	32 (S)
P-088	*E. cloacae*	31 (S)	21 (I)	31 (S)	28 (S)
P-089	*E. coli*	33 (S)	32 (S)	34 (S)	31 (S)
P-090	*E. coli*	34 (S)	32 (S)	33 (S)	34 (S)
P-091	*E. cloacae*	6 (R)	6 (R)	6 (R)	6 (R)
P-092	*E. coli*	30 (S)	29 (S)	30 (S)	29 (S)
P-093	*E. coli*	31 (S)	29 (S)	30 (S)	30 (S)
P-094	*E. coli*	30 (S)	30 (S)	29 (S)	28 (S)
P-095	*E. cloacae*	6 (R)	6 (R)	6 (R)	6 (R)
P-096	*E. coli*	30 (S)	29 (S)	30 (S)	28 (S)
P-097	*E. coli*	32 (S)	30 (S)	32 (S)	30 (S)
P-098	*K. pneumoniae*	30 (S)	26 (S)	27 (S)	29 (S)
P-099	*K. pneumoniae*	30 (S)	28 (S)	29 (S)	29 (S)
P-100	*K. pneumoniae*	30 (S)	27 (S)	30 (S)	29 (S)
P-101	*E. coli*	32 (S)	30 (S)	32 (S)	31 (S)
P-102	*E. cloacae*	6 (R)	6 (R)	6 (R)	6 (R)
P-103	*E. cloacae*	35 (S)	32 (S)	35 (S)	33 (S)
P-104	*E. cloacae*	6 (R)	6 (R)	6 (R)	6 (R)

No.	Organism	Drug susceptibility test			
		Zone diameter (mm)			
		IMP	MEM	ETP	DOR
P-105	*E. coli*	31 (S)	32 (S)	31 (S)	31 (S)
P-106	*K. pneumoniae*	11 (R)	6 (R)	10 (R)	11 (R)
P-107	*E. coli*	32 (S)	30 (S)	32 (S)	33 (S)
P-108	*K. pneumoniae*	29 (S)	25 (S)	28 (S)	26 (S)
P-109	*E. coli*	32 (S)	27 (S)	33 (S)	30 (S)
P-110	*E. coli*	30 (S)	29 (S)	30 (S)	30 (S)
P-111	*E. coli*	33 (S)	32 (S)	33 (S)	30 (S)
P-112	*K. pneumoniae*	30 (S)	31 (S)	29 (S)	26 (S)
P-113	*E. coli*	31 (S)	27 (S)	31 (S)	30 (S)
P-114	*E. coli*	32 (S)	32 (S)	31 (S)	29 (S)
P-115	*E. coli*	10 (R)	6 (R)	14 (R)	13 (R)
P-116	*K. pneumoniae*	30 (S)	25 (S)	30 (S)	29 (S)
P-117	*K. pneumoniae*	30 (S)	22 (S)	28 (S)	26 (S)
P-118	*E. coli*	30 (S)	30 (S)	30 (S)	28 (S)
P-119	*E. coli*	30 (S)	29 (S)	29 (S)	26 (S)
P-120	*E. coli*	34 (S)	31 (S)	32 (S)	30 (S)
P-121	*K. pneumoniae*	27 (S)	27 (S)	29 (S)	28 (S)
P-122	*E. coli*	30 (S)	30 (S)	30 (S)	27 (S)
P-123	*K. pneumoniae*	34 (S)	30 (S)	36 (S)	29 (S)
P-124	*E. coli*	34 (S)	31 (S)	35 (S)	27 (S)
P-125	*E. coli*	Not grow	Not grow	Not grow	Not grow
P-126	*K. pneumoniae*	31 (S)	26 (S)	29 (S)	27 (S)
P-127	*K. pneumoniae*	24 (S)	19 (I)	25 (S)	26 (S)
P-128	*E. coli*	32 (S)	30 (S)	30 (S)	27 (S)

Disk diffusion (Zone Diameter)

Wichianburi Hospital (50 isolates)

No.	Organism	Drug susceptibility test			
		Zone diameter (mm)			
		IMP	MEM	ETP	DOR
V-001	*K. pneumoniae*	18 (R)	21 (I)	11 (R)	23 (S)
V-002	*K. pneumoniae*	24 (S)	27 (S)	26 (S)	26 (S)
V-003	*E. coli*	27 (S)	29 (S)	27 (S)	28 (S)
V-004	*E. coli*	27 (S)	29 (S)	28 (S)	28 (S)
V-005	*E. coli*	25 (S)	28 (S)	30 (S)	27 (S)
V-006	*E. coli*	27 (S)	30 (S)	31 (S)	28 (S)
V-007	*E. coli*	26 (S)	29 (S)	28 (S)	26 (S)
V-008	*E. coli*	25 (S)	27 (S)	28 (S)	25 (S)
V-009	*E. coli*	27 (S)	30 (S)	32 (S)	29 (S)
V-010	*E. coli*	27 (S)	28 (S)	25 (S)	28 (S)
V-011	*E. coli*	25 (S)	28 (S)	27 (S)	29 (S)
V-012	*E. coli*	23 (S)	24 (S)	16 (R)	26 (S)
V-013	*E. coli*	28 (S)	27 (S)	27 (S)	29 (S)
V-014	*E. cloacae*	24 (S)	26 (S)	17 (R)	25 (S)
V-015	*P. mirabilis*	24 (S)	29 (S)	30 (S)	27 (S)
V-016	*Enterobacter* spp.	25 (S)	26 (S)	19 (I)	26 (S)
V-017	*E. cloacae*	23 (S)	25 (S)	16 (R)	24 (S)
V-018	*E. coli*	25 (S)	24 (S)	18 (R)	25 (S)
V-019	*E. coli*	23 (S)	22 (I)	16 (R)	23 (S)
V-020	*E. coli*	27 (S)	31 (S)	31 (S)	31 (S)
V-021	*E. coli*	27 (S)	31 (S)	32 (S)	29 (S)
V-022	*K. pneumoniae*	25 (S)	28 (S)	26 (S)	26 (S)
V-023	*K. pneumoniae*	27 (S)	27 (S)	26 (S)	30 (S)

No.	Organism	Drug susceptibility test			
		Zone diameter (mm)			
		IMP	MEM	ETP	DOR
V-024	*E. coli*	28 (S)	31 (S)	31 (S)	30 (S)
V-025	*E. coli*	29 (S)	30 (S)	28 (S)	29 (S)
V-026	*E. coli*	29 (S)	31 (S)	31 (S)	29 (S)
V-027	*E. coli*	30 (S)	29 (S)	27 (S)	29 (S)
V-028	*S. marscens*	24 (S)	27 (S)	22 (S)	26 (S)
V-029	*E. coli*	24 (S)	27 (S)	29 (S)	25 (S)
V-030	*E. coli*	26 (S)	29 (S)	32 (S)	27 (S)
V-031	*E. coli*	27 (S)	27 (S)	29 (S)	26 (S)
V-032	*E. coli*	25 (S)	23 (S)	13 (R)	24 (S)
V-033	*E. coli*	24 (S)	26 (S)	31 (S)	25 (S)
V-034	*E. coli*	28 (S)	32 (S)	32 (S)	31 (S)
V-035	*E. coli*	27 (S)	32 (S)	32 (S)	31 (S)
V-036	*E. coli*	25 (S)	29 (S)	24 (S)	29 (S)
V-037	*K. pneumoniae*	27 (S)	32 (S)	32 (S)	31 (S)
V-038	*K. pneumoniae*	27 (S)	28 (S)	30 (S)	29 (S)
V-039	*K. pneumoniae*	27 (S)	30 (S)	32 (S)	28 (S)
V-040	*K. pneumoniae*	27 (S)	29 (S)	30 (S)	27 (S)
V-041	*K. pneumoniae*	27 (S)	20 (I)	12 (R)	22 (I)
V-042	*K. pneumoniae*	29 (S)	30 (S)	31 (S)	29 (S)
V-043	*K. pneumoniae*	26 (S)	29 (S)	30 (S)	27 (S)
V-044	*K. pneumoniae*	27 (S)	29 (S)	30 (S)	28 (S)
V-045	*K. pneumoniae*	25 (S)	27 (S)	25 (S)	25 (S)
V-046	*K. pneumoniae*	25 (S)	27 (S)	27 (S)	26 (S)
V-047	*K. pneumoniae*	27 (S)	28 (S)	29 (S)	27 (S)
V-048	*Enterobacter* spp.	16 (R)	25 (S)	21 (I)	25 (S)
V-049	*P. mirabilis*	22 (I)	29 (S)	29 (S)	27 (S)
V-050	*C. diversus*	27 (S)	29 (S)	32 (S)	27 (S)

How to prepare inhibitors' reagents of the modified Carba NP test

1. Dipicolinic acid solution preparation:

Dipicolinic acid reagent dissolved in 0.1 mol/l of HEPES and adjusted pH 7.8 by 0.1 M NaOH

2. Phenylboronic acid solution preparation:

120 mg of 3-aminophenylboronic acid dissolved in 3 ml of dimethyl sulfoxide (DMSO) and 3 ml of distilled water.

3. Clavulanic acid solution preparation:

5 mmol/l of clavulanic acid dissolved in 0.2 mol/l of phosphate buffer and adjusted pH 7.4

Data Analysis

	Enzyme (Carbapenemase)	**No enzyme (No carbapenemase)**
Modified Carba NP test (Positive result)	2	0
Modified Carba NP test (Negative result)	1	3

Sensitivity = TP/(TP+FN) = 2/(2+1) = 0.6666×100= 66.66%

Specificity = TN/(TN+FP) = 3/(3+0) = 1×100 = 100%