DEVELOPMENT OF DNA LATERAL FLOW DIPSTICK TESTS FOR DETECTION CARBAPENEM RESISTANCE BACTERIA BASED ON NEW DELHI METALLO-BETA-LACTAMASE-1 GENE

SUPPARIT PAKDEETHAI

Graduate School Srinakharinwirot University

2021

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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(Molecular Biology)

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THE DISSERTATION TITLED

DEVELOPMENT OF DNA
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BY

SUPPARIT PAKDEETHAI

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(Assoc. Prof. Dr. Chatchai Ekpanyaskul, MD.)
Dean of Graduate School

ORAL DEFENSE COMMITTEE

..... Major-advisor
(Assoc. Prof. Dr.Somchai Santiwatanakul)

..... Co-advisor
(Prof. Dr.Kosum Chansiri)

..... Chair
(Asst. Prof. Dr.Chamras Promptmas)

..... Committee
(Assoc. Prof.Woraphot Tantisiriwat, MD.)

..... Committee
(Assoc. Prof. Dr.Teeraporn Bureerug)

Title	DEVELOPMENT OF DNA LATERAL FLOW DIPSTICK TESTS FOR DETECTION CARBAPENEM RESISTANCE BACTERIA BASED ON NEW DELHI METALLO-BETA-LACTAMASE-1 GENE
Author	SUPPARIT PAKDEETHAI
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Co Advisor	Professor Dr. Kosum Chansiri

Carbapenem-resistant *Enterobacteriaceae* (CRE), a resistant group of gram-negative bacteria, can produce carbapenemase, destroying carbapenem molecules and causing drug resistances. Among drug-resistant genes, the incidence of New Delhi metallo-beta-lactamases (bla_{NDM-1}), are increasing and causing worldwide drug resistant outbreaks. However, the rapid method for screening and detection of this evidence is necessary to detect the early stages and to help with treatment. For these reasons, this study developed a rapid method of screening detection for carbapenem-resistant bacteria based on bla_{NDM-1}. All specimens were initially isolated for CRE by using a carbapenem disk inhibition test. The methods included the Polymerase Chain Reaction (PCR), Loop Mediated Isothermal Amplification (LAMP), and Recombinant Polymerase Amplification (RPA) assays were conducted for the identification of drug resistance bla_{NDM-1} gene with previous CRE isolates. Then, the amplicon was analytically verified for sensitivity and specificity on a lateral flow dipstick (LFD). The limit of detection (LOD) of PCR-LFD, LAMP-LFD and RPA-LFD assays showed 0.74 ng/ µl, 7.4 pg/ µl and 0.74 ng/ µl, respectively. According to the sensitivity results demonstrated that LAMP-LFD was 100 times more sensitive than PCR-LFD and RPA-LFD. Moreover, the specificity of the tests showed no cross-hybridization with host DNA and other microorganisms. In conclusion, the LAMP-LFD has been developed for the detection of bla_{NDM-1} with efficient, rapid and easy to implement routines. The high sensitivity and early screening detection can help physicians to choose the proper antibiotics to treat CRE bacterial infection and can reduce morbidity and mortality rates.

Keyword : Carbapenem-Resistant Enterobacteriaceae (CRE), New Delhi metallo-beta-lactamase (blaNDM-1)

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CHAPTER I
INTRODUCTION

Antimicrobial resistance (AMR) is one of the major public health problems. Presently, the infection is associated with high mortality, primarily due to delay in effective administration of treatment and the limited optional available treatment [1, 2], causing by a group of drug resistant bacteria. The multidrug-resistant bacteria (MDR) is increasingly found in hospitalized patients with long term stays. Among the resistant drug bacteria, the beta-lactam and carbapenem resistant bacteria groups broadly cause resistance by producing enzyme called Metallo-β-lactamase (for example, New delhi metallo β-lactamase-1 (*NDM-1*)) and carbapenemase (for example, *K. pneumoniae carbapenemase* (*KPC*), and *Oxacillinase* (*OXA*)). In 2009, the Centers for Disease Control and Prevention United States of America or the CDC (Center for Disease Control and Prevention) reported the name of these bacteria as Carbapenem Resistant *Enterobacteriaceae* (CRE). [3, 4]

Surprising, *Enterobacteriaceae* has three major resistant mechanisms to carbapenem such as enzyme production, efflux pumps and porin mutations. However, the enzyme production is the main resistance mechanism that split the β-lactam ring, an important ingredient of carbapenam antibiotic. The β-lactam ring is known to penicillin binding proteins (PBPs), by inhibiting formation of peptidoglycan of *Enterobacteriaceae*. Generally, the CRE bacteria is detected from sputum sample or the blood of a patient but the results have been reported too late. Other antibiotics can be used as drug of choice but it is expensive and causes many side effects. CRE bacteria are commonly colonized at intestinal of patient and can spread to blood stream as well as excrete into feces. Therefore, the CRE early detection can choose the effective treatment to inhibit the infection and spreading of CRE bacteria from feces.

This study is to develop the rapid method for early detection of carbapenem resistant bacteria. This can help the physician to choose the suitable antibiotic to medicate the infection by CRE bacteria and can decrease morbidity and mortality rates.

CHAPTER II
LITERATURE REVIEW

Enterobacteriaceae

Enterobacteriaceae are a family of facultative anaerobic gram-negative, rod-shaped bacteria. They are usually found in human intestinal flora and also cause infection both community-acquired and hospital-acquired.[5] This family is comprised of various genus and species such as *Klebsiella* spp. , *E. coli*, *Citrobacter* spp. , *Enterobacter* spp. , *Salmonella* spp. , *Proteus* spp. , *Morganella* spp. , *Serratia* spp. , *Shigella* spp., *Yersinia* spp., and *Providencia* spp.

The *Enterobacteriaceae* are the most important bacteria causing bloodstream infections, urinary tract infections (UTIs) and healthcare-associated pneumonias.[6] They can normally spread to humans via water and food. However, plasmids and transposons of them are the main transferred way of multiple genetic resistant elements.[7]

Carbapenem

Carbapenem is a group of antimicrobial drugs of which chemical structure called β-lactam ring (Beta-lactam) is against many types of bacteria. [8] Generally, carbapenem is a natural derivative development derived from the *Streptomyces cattleya*. In clinical, this drug is used for treat after other ineffective antibacterial drugs. The effective carbapenem group is proven to treat in clinical, for example Gram positive (+), Gram negative (-), and anaerobic bacteria. Many of these drugs have been clinically approved and are clinically proven to be effective such as:

Imipenem: it must be taken with cilastatin and destroyed by an enzyme in the human kidney called dehydropeptidase.

Ertapenem: the trade name is invanz, has a short-term effect, and are administered within 6 hours prior to hemodialysis.

Doripenem: the trade names are finibax and doribax.

Meropenem: the trade name is merrem.

Panipenem: it must be used with betamipron to stop the absorption of Panipenem into the ureter, and preventing kidney failure.

Biapenem: it is often used to treat anaerobic bacteria infected patients.

There are also other drug groups under research and development such as:

Razupenem: it is a developed drug to treat carbapenem-resistant bacteria.

Tebipenem: it is a developed drug to be oral taken.

Lenapenem and Tomopenem: they are developed to be better efficacy.

Currently, there are a number of outbreaks from the United States and England reporting a group of bacteria resistant carbapenem (carbapenem-resistant bacteria) such as carbapenem-resistant *Enterobacteriaceae* (CRE) in *Klebsiella pneumonia* as same as in Pakistan, Bangladesh, and India [9]. They also have been reported the number of infected patients called as New delhi metallo-β-lactamase (*NDM-1*). [10] which can damage the structure of the β-lactam ring.

The structure of Carbapenem

The structure of Carbapenem is the same as the structure of the Penicillins (penams). The first sulfur atom of the structure is replaced with carbon atom after that the double bonds are formed and called as "carbapenems" [11] (Figure 1).

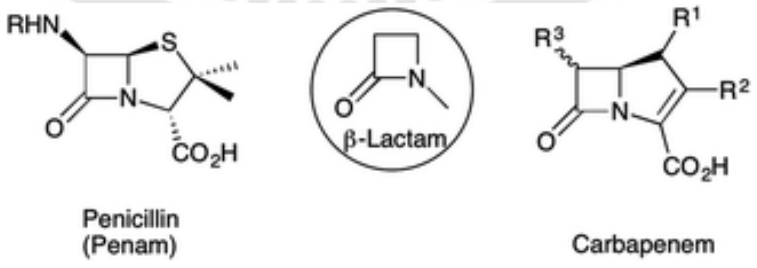


Figure 1 Core structure of carbapenem molecules [10]

Biosynthesis

The Carbapenems is originated from two compounds, malonyl-CoA and glutamate-5-semialdehyde. They are formed as a pentagonal ring structure (five-membered ring) The β-lactam synthetase uses ATP to create the β-lactam to be saturated carbapenem core and then is oxidized to form the carbapenem structure (Figure 2).

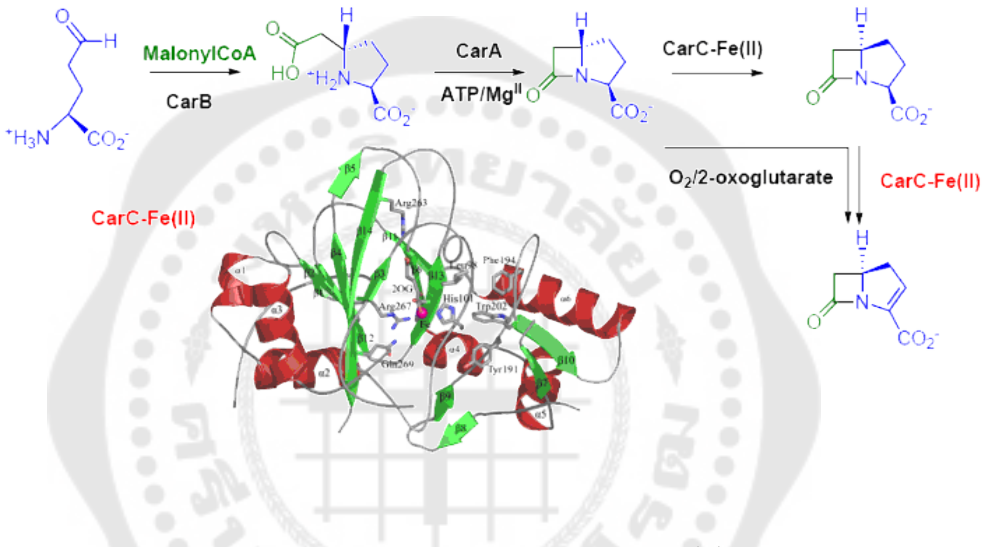


Figure 2 Carbapenem biosynthesis [11]

Gram-negative (-) pathogens

The group of Carbapenems (such as imipenem, doripenem, and meropenem), can kill Gram negative (-) pathogens in the *Enterobacteriaceae* species for example *K. pneumonia, E. coli*, *C. freundii*, *E. cloacae*, *P. mirabilis*, and *S. marcescens*. Moreover *E. coli* and *K. pneumoniae* also can resist to cephalosporins. Not only, *Pseudomonas aeruginosa* and *Acinetobacter* species also have been reported that imipenem, doripenem, and meropenem can inhibit the growth of both but also revealed that they can resist to other antibiotics. [12]

Gram-positive (+) pathogens

Carbapenem can inhibit the growth of Gram positive (+) bacteria in widely range especially *Staphylococcus* species. *Streptococcus* species have been reported

that they are resistant to methicillin and penicillin. However, carbapenem cannot inhibit the growth of Methicillin-resistant *Staphyloccus aureus* or *Enterococcus* infection due to carbapenem cannot bind to penicillin-binding protein (PBP) of this species. [12]

Mechanisms of drug resistance

- Intrinsic resistance mechanism of some antibiotics cannot be used for natural antibiotic groups such as vancomycin in gram negative bacilli and aminoglycoside in anaerobic bacteria.

- Acquired resistance mechanism is a mechanism showing the adaptation of bacteria to eliminate or reduce the effectiveness of antibiotics. [13]

Carbapenem-resistant *Enterobacteriaceae* (CRE)

Carbapenem-resistant *Enterobacteriaceae* (CRE) is gram-negative bacteria and is a resistant group. Because of, they can produce carbapenemase destroying carbapenem molecules and causing to a few moderate drug resistances. *Enterobacteriaceae* drug-resistant causes the morality of many patients, especially infected patients in the bloodstream.

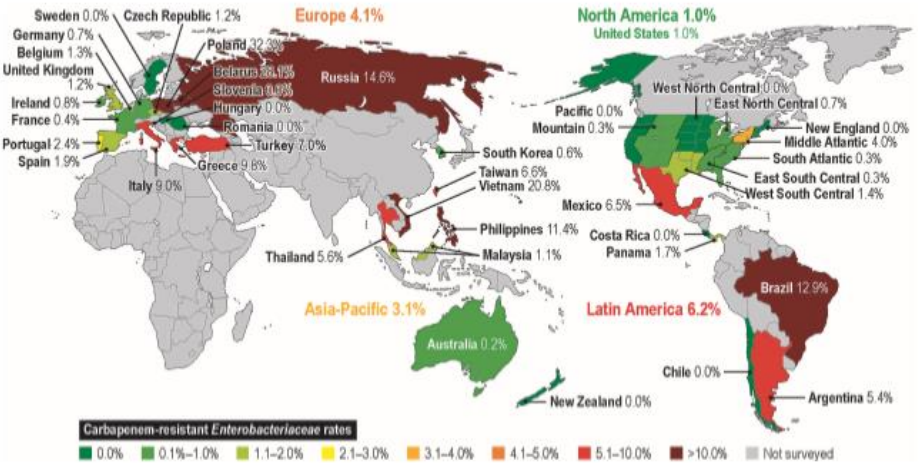


Figure 3 Carbapenem-resistance *Enterobacteriaceae* rates among nations surveyed by the SENTRY Program in 2017 [14]

Mechanism of carbapenem resistance *Enterobacteriaceae*

Generally, carbapenem is classified as β-lactam antibiotic. Carbapenem can inhibit enzyme activity of Transpeptidases (penicillin-binding proteins) preventing the development of peptidoglycan in bacteria. When the PBP is inhibited by Carbapenem. it causes the cells break until die. Therefore, the mechanism is possible to be a resistance mechanisms in Gram-negative bacteria [15]. For example

1. Some resistant bacteria found an active transport process to remove carbapenem from the cells, known as "augmented drug efflux" [16]

2. Resistance of the gene regulation of outer membrane proteins production called as "porins". preventing the drug entry of the bacteria cell [17-20]. The mutation of sequences on the regulated "porin" gene causes to frameshift of gene and changes the amino acid sequence, and then makes structure and function of "porin" differentiation. However, differentiation of porin protein lead to entry of carbapenem and other antibiotics at periplasm of bacterial cells and creates an enzyme that resist to carbapenem and other antibiotics called as plasmid-borne extended-spectrum β-lactamases (ESBL). In case of drug resistant *K. Pneumonia* is inserted or duplicated the sequences into the “porin” gene such as OmpK35 and OmpK36. The deletion of some sequences of OmpK36 gene causes premature end of the translation process causing in the loss of function and structure of the delivery of carbapenem into the cells. The mutation of OmpK35 or OmpK36 on the outer membrane of *K. pneumoniae* causes to carbapenem resistance.

3. Carbapenem-resistant *Enterobacteriaceae* has the ability of enzyme production called as carbapenemases (β-lactamase) [21]. This enzyme can digest the structure of the β-lactam ring, which is an important element of β-lactam antibiotics. The target of this enzyme is Penicillin-binding proteins (PBPs), so that it makes bacteria resist to carbapenem.

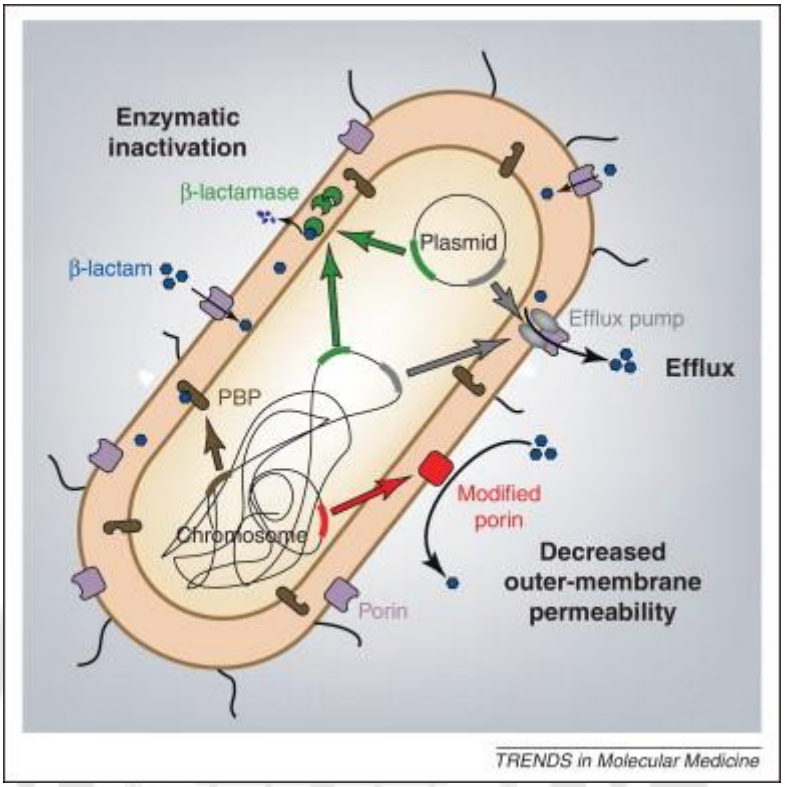


Figure 4 Mechanisms of carbapenem-resistance *Enterobacteriaceae* (CRE) [13]

Table 1 Resistance mechanisms in clinical gram-negative bacteria [22]

Antibacterial drugs	Important resistance mechanisms
Cephalosporin Powerful type	Enzyme production extended-spectrum β-lactamase (ESBL). AmpC β-lactamase production. Excretion of drugs outside the cell.
Beta-lactam and enzyme inhibitors β-lactamase	Enzyme production high β-lactamase (hyperproduction) such as AmpC β-lactamase. Mutations or reduced porin protein intake. Excretion of drugs outside the cell.
Carbapenem	Enzyme production carbapenemase. Mutations or reduced porin protein intake. Excretion of drugs outside the cell.
Quinolone	Mutation of drug action targets. Excretion of drugs outside the cell.

Carbapenemases are divided into 2 groups based on the structure and digested mechanism of β-Lactam ring [23] as follows:

A. Serine-carbapenemases are divided into three classes,

a. Class A carbapenemases[24, 25] : serine at position 70 is an active site requiring to digestion. The structure of β-lactams can be synthesized from chromosome or plasmid of bacteria. There are 4 subgroups.

- *Serratia marcescens* enzymes (*SME*) (3 species found in *S. marcescens*)

- *Imipenemase (IMP)* (Found in *Enterobacter cloacae*)

- *Guiana Extend-Spectrum (GES)* (16 variants are most common in *P. aeruginosa* and some in *E. coli* and *K. pneumoniae*)

- 10 types of *KPC*

b. Class C Cephalosporinases. This group is specific to destroy cephalosporin called cephalosporinase or AmpC β- lactamase[26] . This enzyme is usually found in the plasmid that is moved from chromosome to plasmid. The speedy generation AmpC beta-lactamase involves to the spread of *Enterobacteriaceae*.

c. Class D carbapenemases[27] or *Oxacillinase* (*OXA*) or *OXA* β- lactamases (bla_{OXA}) classified as serine β-Lactamases synthesized from bacterial plasmid. This enzyme has the degradation ability of oxacillin, originated from bla_{OXA} gene. *OXA* β-lactamases is met in both plasmids and chromosomes. Mechanism of drug resistance Class D carbapenemases degrades the structure of β-lactam ring by acyl intermediate progression process.

B. Metallo-carbapenemases[28] or Class B carbapenemases or metallo-β-lactamases (MBL). There are 3 subclasses such as B1, B2, and B3. The sub-structure of β-Lactams depends on zinc at active site[29, 30]. Moreover, the incidence of New Delhi metallo-β-lactamase-1 (*NDM-1*) [31] shows increasing worldwide.

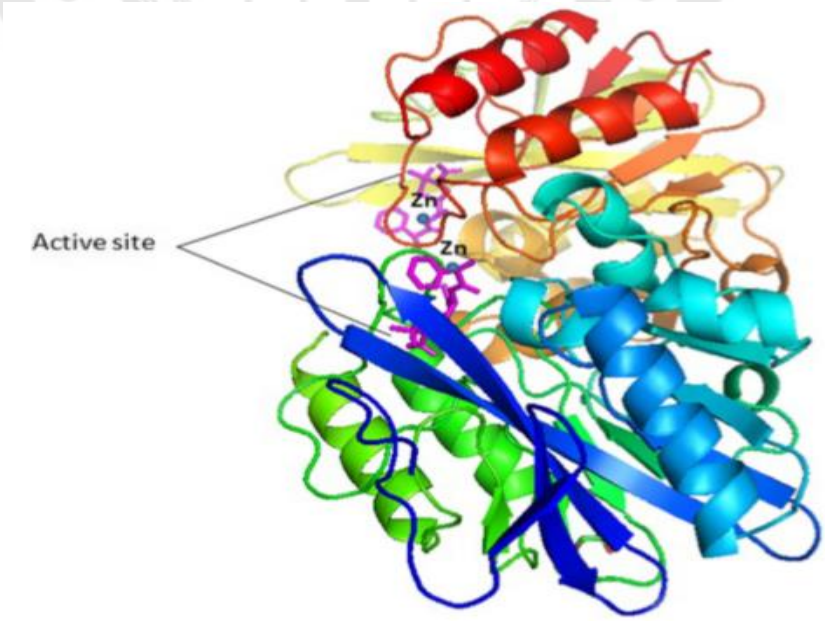


Figure 5 3D structure of NDM-1 protein backbone shown with helices and strands, the two zinc ions at the active sites are shown as blue spheres [32]

In 2008, *Klebsiella pneumoniae* and *Escherichia coli* were found that they produce the New Delhi metallo-β-lactamase 1 (*NDM-1*) isolated from the transferred Swedish patient[10]. The *NDM-1* enzyme is an NDM-1 gene that can destroy beta-lactam antibiotics. Surprising, all lactates, except azetreonam, can also destroy carbapenems.

Currently, *Enterobacteriaceae* carried the bla_{NDM-1} gene is widely epidemic because bla_{NDM-1} gene and can be found in many kinds of bacteria including *Klebsiella oxytoca*, *Citrobacter freundii*, *Enterobacter cloacae*, *Morganella morganii*, *Proteus* spp., and *Providencia* spp.[33].

A schematic representation of Tn125 transposon carried with bla_{NDM-1} gene shows the mutations at various nucleotide locations leading to the occurrence of *NDM* variants.(Figure 6).

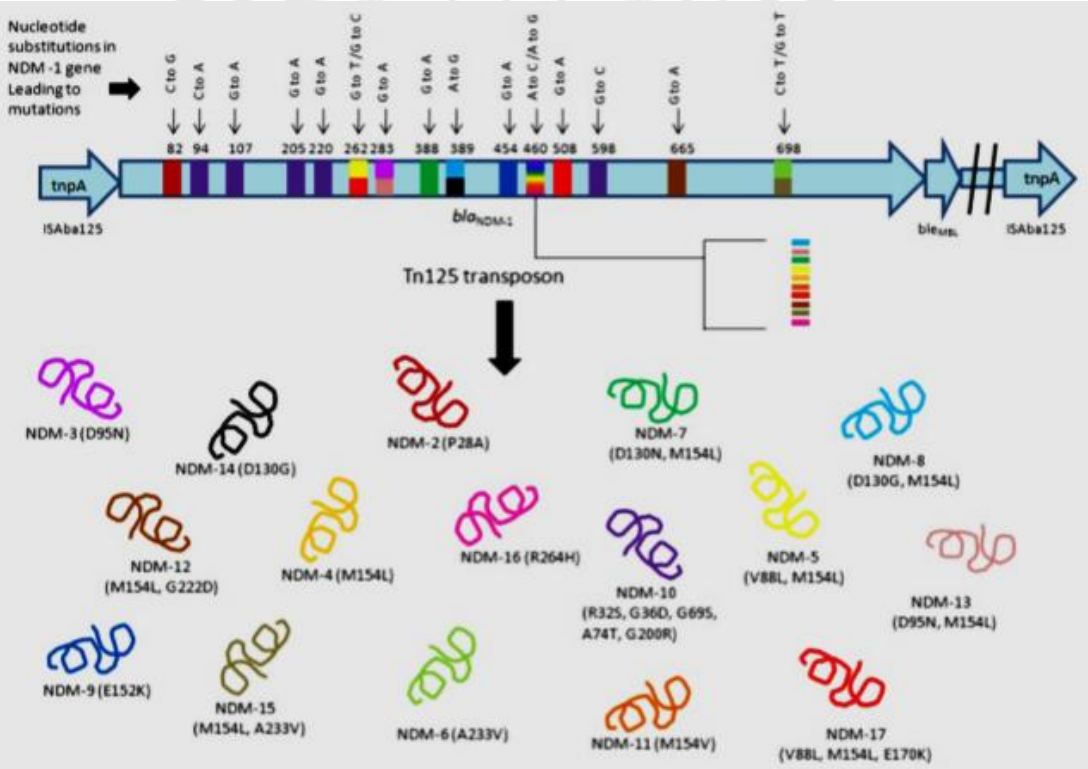


Figure 6 A schematic representation of bla_{NDM-1} gene carrying Tn125 transposon. [32]

Table 2 Genetic variations among the *NDM-1* and its variants and its first source of spread [32]

NDM-1 variants	Amino acid (s) substitution	Source organism (s)
NDM-2	Proline 28 to Alanine	*Acinetobacter baumannii*
NDM-3	Aspartate 95 to Asparagine	*Escherichia coli*
NDM-4	Methionine 154 to Leucine	*Escherichia coli*
NDM-5	Valine 88 to Leucine Methionine 154 to Leucine	*Escherichia coli*
NDM-6	Alanine 233 to Valine	*Escherichia coli*
NDM-7	Aspartate 130 to Asparagine Methionine 154 to Leucine	*Escherichia coli*
NDM-8	Aspartate 130 to Glycine Methionine 154 to Leucine	*Escherichia coli*
NDM-9	Glutamic Acid 152 to Lysine	*Klebsiella pneumoniae*
NDM-10	Arginine 32 to Serine, Glycine 36 to Aspartic acid, Glycine 69 to Serine, Alanine 74 to Threonine, Glycine 200 to Arginine	*Klebsiella pneumoniae*
NDM-11	Methionine 154 to Valine	*Escherichia coli*
NDM-12	Glycine 222 to Aspartic acid and Methionine 154 to Leucine	*Escherichia coli*
NDM-13	Aspartic acid 95 to Asparagine and Methionine 154 to Leucine	*Escherichia coli*

Table 2 (continue)

NDM-1 variants	Amino acid (s) substitution	Source organism (s)
NDM-14	Aspartic acid 95 to Asparagine and	
NDM-15	Methionine 154 to Leucine	*Acinetobacter lwoffii*
	Aspartic acid 130 to Glycine	*Escherichia coli*
NDM-16	Alanine 233 to Valine Methionine	
NDM-17	154 to Leucine	*Klebsiella pneumoniae*
	Arginine 264 to Histidine	*Escherichia coli*

The transfer between bacteria

The gram-negative bacteria can develop, progress and transfer many β-lactam resistance genes via various pathways and mechanisms for example, carbapenem resistance gene. Moreover, Gram-negative bacteria can produce a new type of ESBL from the remaining plasmid-mediated β-lactamases using amino acid replacement. However, bacteria can naturally transfer and develop new genes from other bacteria. For this reason, the production of β-lactamase is increased by modification of *bla* genes at the sequence of regulatory genes and promoter region on the chromosome. Although, bacteria can move *bla* genes via integrons or horizontal transferring between Gram-negative species and other strains but also can be distributed plasmid-mediated carbapenemases to the bacteria and inhibit the production of porin.

The regulated gene of the production of beta-lactamase in gram-negative bacteria can be found on many genetic structures. [34-37] For example: Chromosome: Bacteria have a circular chromosome containing a gene that is necessary for living and involving to resistant genes. This gene located on the chromosome are transferred to the offspring through the vertical gene transfer mechanism.

The plasmid also has a loop genome. The size of plasmid is smaller than a chromosome. There are many rings in each cell and different species in each species. Some of them also have resistance genes located on plasmid. The plasmid can be transmitted during bacterial contact, called conjugative plasmid, allowing horizontal gene transfer mechanism.

The mobile genetic element is a mobile genetic mechanism moving genetic element within the same cell or between cells. Therefore, moving resistant genetic element shows spreading to other cells, such as conjugative plasmid as discussed above, bacteriophage bring bacterial genes to other bacteria cells through the transposon (Tn) processing.

Integron (In) is a genetic region found on the chromosome plasmid or inserted in Tn. However, it is consisting of the genes expressed to integrase enzyme and other genes located in specific position (site-specific recombination). In addition, they are divided into three genetic groups including-class 1, 2, and 3 inserted box for the resistant. Genes For class 1 structure called as class 1 integron, there are not only inserted to 5 genes at In region but also most of them cut off and passed on to other places without In moving (unlike Tn). Therefore, In is a structure involved in the propagation of the enzyme beta-lactamase.

The main genetic structure consists of the creation of enzymes β-lactamase ( ) such as chromosome, plasmid, transposon (Tn), and integrin (In). Tn region is a mobile genetic region between chromosome and plasmid or between bacterial cells. At the end and the middle of Tn regions have insertion of sequence (IS) structures and the resistance gene respectively. Furthermore, Tn moving carries the resistance gene, In region can be found on the chromosome, plasmid, and Tn region determining the integrase enzyme production. [37] (Figure 7).

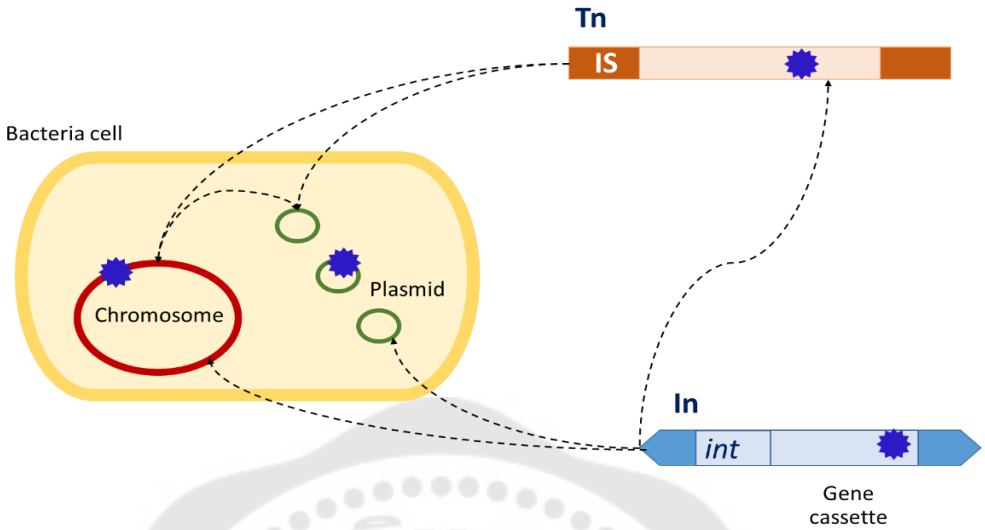


Figure 7 The gene position that determines the creation of enzymes β-lactamase found on the genetic structure of bacteria. [34-35]

CRE Laboratory diagnostic

Phenotype tests for carbapenemase detection in clinical isolates.

1. Agar plate method

Many studies have used the innocuous *E. coli* (Gram-negative) as a test microorganism which can be cultured and handled in a standard laboratory with minimal health risk. The whole plate is then incubated under conditions ideal for microbial growth; that is, 18–24 h at 37°C (for bacteria).

Normally, Agar plate method is used for general cultured bacteria. The most culture media is adding with imipenem (1-2 mg/ L) of because the drug resistant microbes can generate *OXA-48* or *OXA-181* in poor levels. [38]

2. Disc diffusion method

Method disc diffusion is generally used in laboratories of hospital to detect CRE. The specimens can be tested with many discs placed antibiotic on Mueller Hinton agar plates before incubated at 37°C for 12-16 hours. The results are measured from the inhibition zones around antibiotic discs comparing with the Clinical and Laboratory

Standard Institute 2018 (CLSI 2018) guidelines. In Thailand, carbapenem resistance strain must show 1:3 ration of carbapenem antibiotics used in testing. [39]

3. MALDI-TOF MS

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) is a method to determine the protein pattern of drug resistant microbes cultured on a blood agar for 12-14 hours. Additionally, it is used to examine the change in proportion of carbapenem-resistant bacteria producing β-lactamases can break down the construction of β-lactam antibiotics, resulting in mass of antibiotic changed. Therefore, the MALDI-TOF MS-resistant microbial examination takes about 4 minutes-5 hours. However, MALDI-TOF cannot detect the drug-resistant microorganisms destroying the structure of β- lactam antibiotic. Furthermore, the effectiveness of MALDI-TOF are negative, and requires more other tests to confirm the results. [40]

Table 3 Select phenotype tests for carbapenemase detection in clinical isolates.

Test parameter	Agar plate [41]	Disc diffusion [42]	MALDI-TOF MS [43]
Carbapenem-resistant organisms	Generally *Enterobacteriaceae*	*Enterobateriaceae, P.aeruginosa*	*Enterobateriaceae, P.aeruginosa, A.baumannii*
Accuracy	Accuracy varies by test, e.g. *OXA-48* sensitivity 96% and specificity 98%	*Enterobateriaceae* sensitivity 97% and specificity 99%	*Enterobateriaceae* sensitivity 77-100% and specificity 94-100%
Turnaround time	18-24 h	18-24 h	Same-day

4. PCR method

In 1983 polymerase chain reaction or PCR is built and developed by Kary Mullis. The principles of PCR; it replicated and amplified target DNA, master mix of this

reaction which contains: DNA polymerase, nucleotides (A, T, C, G), set of primers and DNA template.The step of PCR is repeated cycles (3 steps/ cycle) – 20 to 30 cycles

Denaturation; heating master mix to 95 °C, and separation of two complementary strands of dsDNA.

Annealing; cool down to lower temperature e.g., 55 °C, primers bind to the complementary DNA, and begin to attach additional complementary nucleotides.

Extension; increase temperature to 72 °C (optimum for *Taq* polymerase), addition of further nucleotides to the extending DNA strand, and breaking of bonds that are not fully complementary.

PCR-based screening methods are a developed method to be rapid detection of the infection. However, it is expensive. In 2007, the University of Virginia Medical Center developed the Nested arbitrary PCR (ARB-PCR) to detect bla_{KPC} plasmid genes for CRE outbreaks. [44]

5. DNA amplification using Loop-mediated isothermal amplification (LAMP)

"LAMP" stands for Loop-mediated Isothermal Amplification. This technology was developed by Notomi et al. It is a very sensitive, easy and time efficient method.

The primers are designed; the 4 sets of primers created from the 6 separated regions of target gene: regions at the 3' side; F3c, F2c and F1c and regions at the 5' side; B1, B2 and B3 (figure 8)

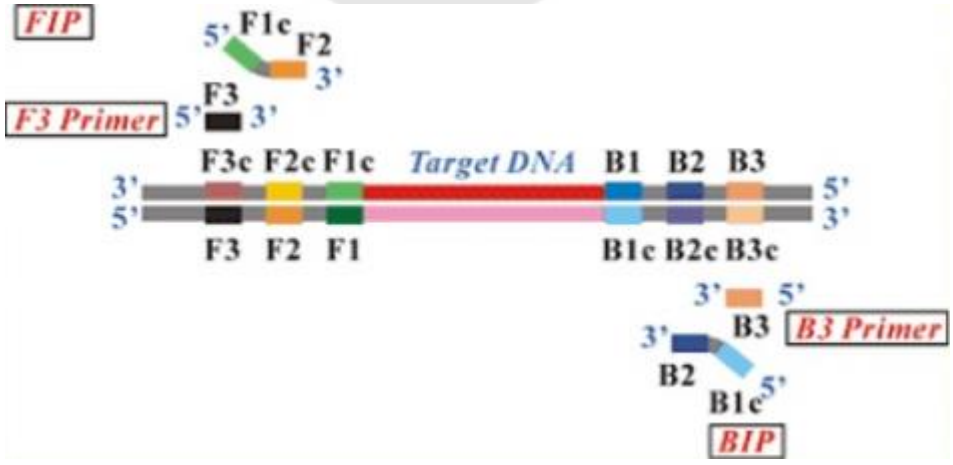


Figure 8 primer of Loop-mediated isothermal amplification (LAMP).[45]

Table 4 Four types of primers based on the 6 distinct regions of target gene

FIP	Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end
F3 Primer	Forward Outer Primer consists of the F3 region that is complementary to the F3c region
BIP	Backward Inner Primer (BIP) consists of the B2 region(at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end
B3 Primer	Backward Outer Primer consists of the B3 region that is complementary to the B3c region

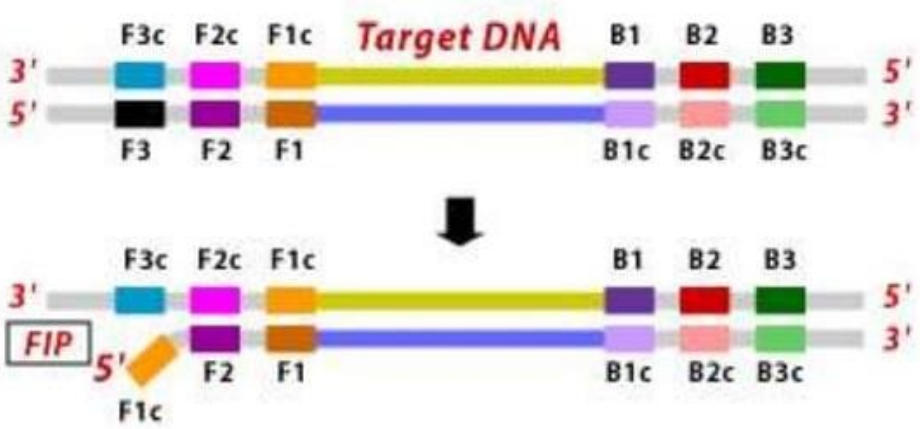
Principle;

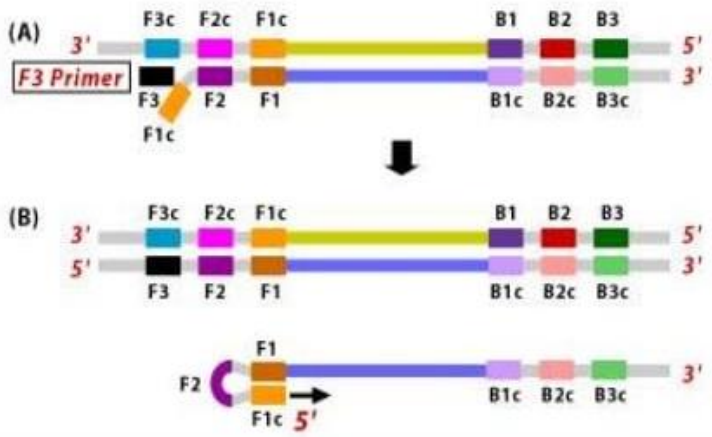
The target gene and the reagents are incubated at a stable temperature at 60-65 ^{o}C, the following reaction steps. (figure 9)

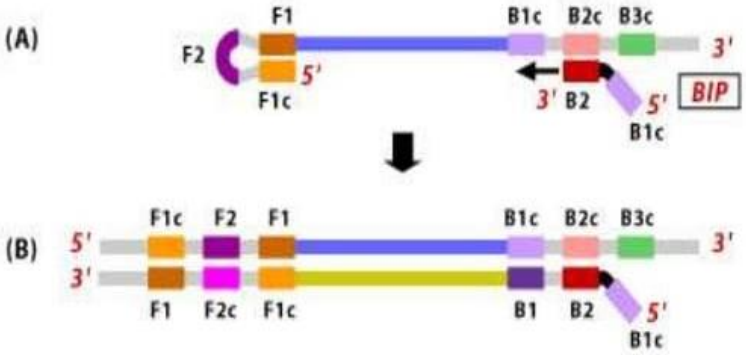
The double stranded target DNA are complemented with one of the LAMP primers, then first DNA synthesis using the *Bst* DNA polymerase with activity such as strand displacement, displacing and releasing. The LAMP method are unlike with PCR method because it has not denaturation step. The proceeding amplification describes from when single strand template DNA are released from the FIP anneals. The DNA polymerase activity is strand displacement. The F2 region of FIP primer synthesized DNA strand complementary from 3' end template DNA. After that the F3c region of the

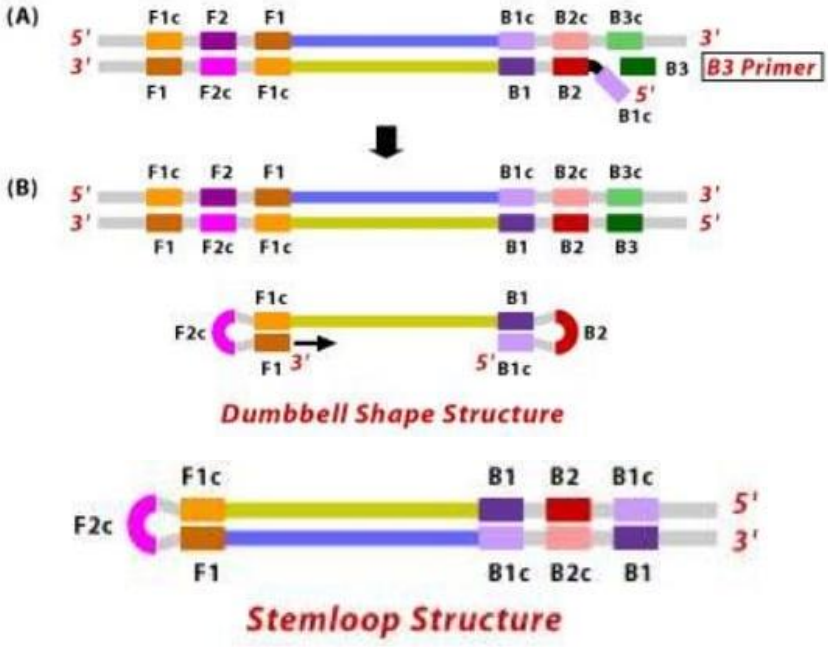
target DNA are hybridized with the outer primer F3 and extends, displacing the FIP connected complementary strand. This displaced strand forms a loop at the 5' end. The BIP primers are complemented with this single stranded DNA with a loop at the 5' end as a template. B2c region of the template DNA are hybridized with B2. DNA synthesis is now started leading to the construction of a complementary strand and opening of the 5' end loop. The B3c region of the target DNA are hybridized with outer primer B3 to and extended, displacing the BIP connected complementary strand. This products from this step is a dumbbell shaped DNA. When extends and opens up the loop at the 5' end, the DNA polymerase add the nucleotides to the 3' end with F1. This formation serves as an originator of LAMP cycling, which is the next stage of LAMP reaction. Thus, a target DNA sequence is amplified 13 fold every half cycle. The final products found are a combination of stem loop DNA with several stem length and several cauliflower like structures with multiple loops.

In the LAMP technique, primers are specific designed to carbapenem resistant genes into the test tube including chemicals and related substrates: Deoxynucleotides (dNTPs), *Bst* DNA polymerase, Primer, PCR buffer, and Magnesium (Mg^{2+}) before incubated at 60-65°C for 30-60 minutes and transferred on Membrane-Based Lateral Flow Biosensor. [45]




(A)
F3c F2c F1c
B1 B2 B3
3'
5'
F3 Primer
F3 F2 F1
B1c B2c B3c
F1c
(B)
F3c F2c F1c
B1 B2 B3
3'
5'
F3 F2 F1
B1c B2c B3c
F1
F2
F1c 5'
B1c B2c B3c
3'


(A)
F1
F2
5'
F1c
B1c B2c B3c
3'
3' B2
5'
BIP
B1c
(B)
F1c F2 F1
B1c B2c B3c
5'
3'
F1 F2c F1c
B1 B2
5'
B1c
3'


(A)
F1c F2 F1
B1c B2c B3c
5'
3'
F1 F2c F1c
B1 B2
B3
B3 Primer
5'
B1c
3'
(B)
F1c F2 F1
B1c B2c B3c
5'
3'
F1 F2c F1c
B1 B2 B3
3'
5'
F1c
B1
F2c
B2
F1 3'
5' B1c
Dumbbell Shape Structure
F1c
B1 B2 B1c
5'
3'
F2c
F1
B1c B2c B1
Stemloop Structure

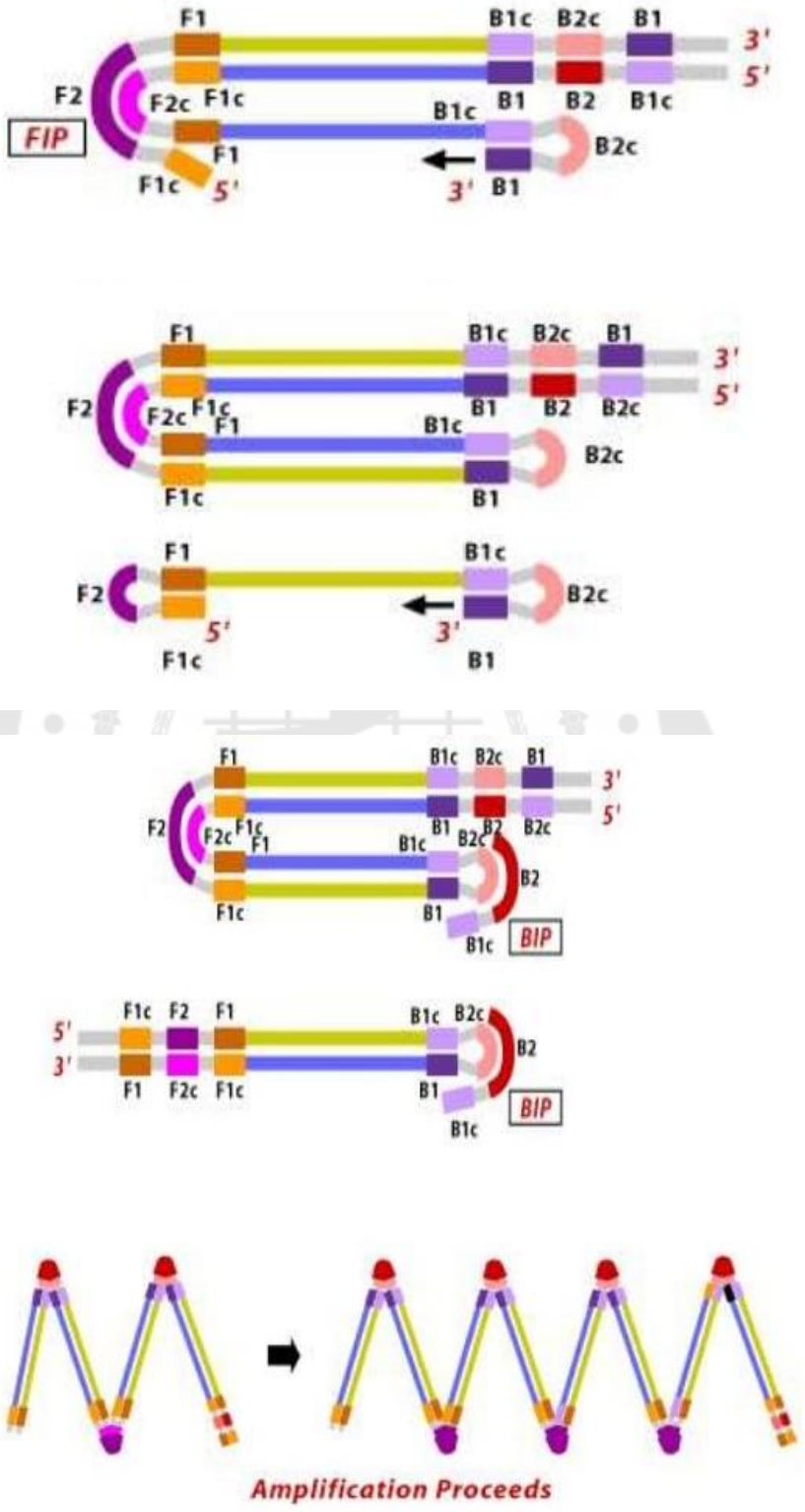


Figure 9 Principle of Loop-mediated isothermal amplification (LAMP) method.[45]

6. DNA amplification using Recombinase Polymerase Amplification (RPA)

RPA is becoming a molecular tool of choice for the rapid, specific, and cost-effective identification of pathogens.

The recombinase polymerase amplification process. A layout of the steps involved in RPA amplification is provided. First, recombinases bind primers pair to complimentary sequence on template DNA. With the aid of single-strand binding protein to bind exposed DNA, the primer is paired and a D-loop is formed. A polymerase extends the primer, eventually displacing the two original strands of template DNA. This process then repeats for amplification. [46] (Figure 10)

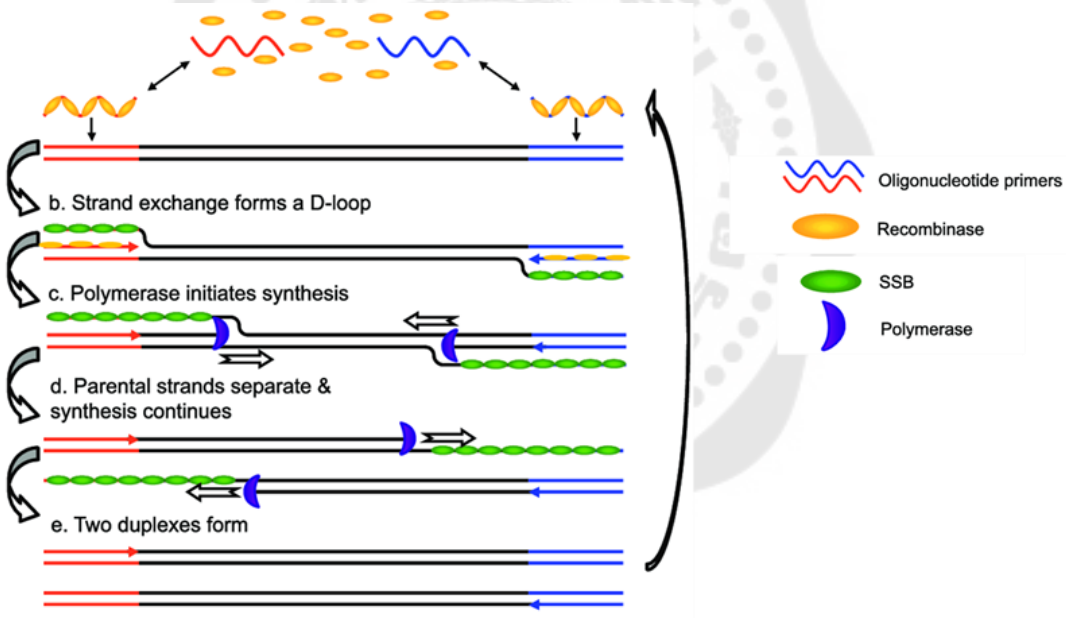


Figure 10 schematic of the recombinase polymerase amplification process.[47]

RPA is a new method using single temperatures to amplify DNA. Primers are specific used to each type of bacterial infection and Incubated at 37°C for 30 minutes before transferred on Membrane-Based Lateral Flow Biosensor. [48]

Table 5 Comparative of molecular methods

Methods	Advantages	Disadvantages
PCR	- High sensitivity and specificity	- Require Thermocycle
LAMP	- High sensitivity and specificity - Rapid - Not require thermal cycle	- Use primer more than usual
RPA	- High sensitivity and specificity - Convenient and rapid - Not require thermal cycle	- Expensive

7. Nucleic acid lateral flow

Nucleic acid lateral flow is a method measured by binding to the nucleic acid based on DNA-DNA hybridization[49, 50], Many research studies have been publicated because it is potential pathogen detection for commercial development and used to be a diagnostic kit for various forensic diseases, agriculture and environment[51-54] based on Nucleic acid lateral flow. The general membrane usually made from the many kinds of materials such as nitrocellulose, polycarbonate, and special biomimetic properties.

Generally, immunoassay is combined with chromatography method called as Nucleic acid lateral flow. Composition of Nucleic acid lateral flow consists of 3 important parts, Pad region, Test line, and Control line (Figure 11):

Part 1. The Pad region is the bottom. It consists of gold particles (Au) that are connected to anti-FITC conjugated with secondary antibody.

Part 2. Test line is attached with biotin ligand or streptavidin (SA).

Part 3. Control line contains anti-antibody (primary antibody)

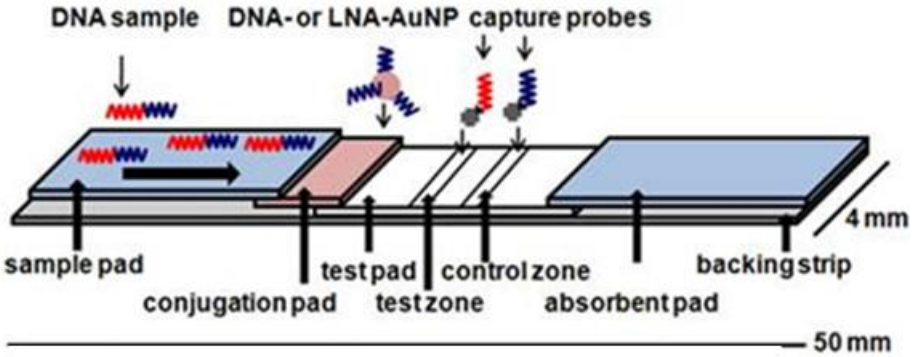


Figure 11 Various components and the function of the DNA sensor in a Nucleic acid lateral flow [55]

Nucleic acid lateral flow method is an easy, convenient, fast, high-specific, and inexpensive method. Moreover, this method can be measured with the naked eye by the color bands, reducing the steps of washing and incubating various substances compared to other methods and does not require the expertise of the experimenters.

CHAPTER III
MATERIALS AND METHODS

This research was approved by the ethics committee of Srinakharinwirot University (SWUEC/E-344/2560).

1. Specimen collection and species identification

Bacteria samples were derived from hospital (HRH Maha Chakri Sirindhorn Medical Center, Srinakharinwirot University, Nakhon Nayok). 110 species of Carbapenem Resistant *Enterobacteriaceae* (CRE) were enrolled in this study, consisting of 97 *Klebsiella pneumoniae*, 10 *Escherichia coli*, 2 *Enterobacter cloacae*, and 1 *Citrobacter freundii*. All 110 species were stored in skim milk and freeze at -50°C. When used for testing, the skim milk was thawed, subcultured and then incubated at 37 °C for 18-24 hours.

2. Antimicrobial susceptibility test

The disk diffusion method were used [57]. Three type of carbapenem sensitivity disc consist of meropenem, imipenem, and ertapenem, the bacterial colony was suspended in 0.9% normal saline and adjusted to 0.5 McFarland. It was spreaded on the Mueller Hinton agar plate and the selected sensitivity disc was put on the plate. The plate was incubated at 37 °C for 18-24 hours. The inhibition zone was measured by comparing to CLSI 2018 (Clinical and Laboratory Standard Institute). The carbapenem resistance strain must show resistant at least one-third of the selected disc according to CLSI 28th ed.

3. Detection of resistance genes by polymerase chain reaction (PCR) and confirmed by sequencing

3.1 Plasmid extraction for the detection of resistance genes with the PrestoTM Mini Plasmid Kit (Geneaid, Taiwan).

3.2 The primer set for PCR were designed by using the data from the Genbank accession numbers are KY074638, MF197908, JX477134, MH673497, MF379694, MF379693, NG_049326, MG773530, NG_049341 and KC539429, for the NDM-1 gene

that controls the production of enzymes β-lactamase, which provides a PCR product approximately as 998 base pairs.

3.3 PCR: Plasmid were extracted to be final volume of 12.5 µl for checking the primer. The suitable conditions were designed and tested. The PCR mixture, including 1.25 µl of 10X PCR buffer, 0.5 µl of dNTP mixture, 0.25 µl of $MgCl_2$, 0.5 µl forward and reverse primer,0.25 µl of *Taq* polymerase (Vivantis, Malaysia) and 0.5 µl of DNA template. The final volume was adjusted to 12.5 µl with deionized water. The PCR amplification were pre-denaturation at 94°C for 2 min, then next step 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 30 sec. The post-extension were incubated at 72°C for 5 min. The amplified products were analyzed by using 0.8% agarose gel electrophoresis.

All sample the results showed that all drugs resistance genes were reproduced by PCR. by using Taq *Pfu* polymerase (Promega, USA) in 50 µl in conditions. The PCR mixture, including 5 µl of 10X PCR buffer Pfu , 1 µl of dNTP mixture, 1 µl forward and reverse primer, 0.5 µl of Taq *Pfu* polymerase (Promega, USA) and 2 µl of DNA template. The final volume were adjusted to 39.5 µl with deionized water. The PCR amplification program pre-denaturation at 94°C for 2 min, then next step 30 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 1 min and extension at 72°C for 1 min. The post-extension were incubated at 72°C for 5 min. The amplified products were analyzed by using 1.8% agarose gel electrophoresis.

The recombinant genomic DNA was performed by to inserting the PCR product into the pGEM T vector based on the ligase method via the ligase enzyme. After that, the recombinant DNA was transformed by using electroporation then was cultured in X-Gal and IPTG plate and incubated at 37 °C for 14 hours. The selected colonies were confirmed by using DNA sequencing.

3.4 DNA sequencing: the PCR product was purified by using the GenepHlowTM Gel / PCR Kit (Geneaid, Taiwan) and measured the concentration by using nanodrop to be final concentration as 50 ng/µl or more before sequencing operated by (Macrogen, Korea).

4. Resistance gene based on PCR, LAMP, and RPA amplification methods

Study of drug resistance genes that are interested, the primers were designed for testing in all 3 methods with the following conditions:

4.1 PCR

PCR amplification was achieved by using primers designed based on bla_{NDM-1} gene (Gen-bank accession on. Genbank; KY074638). The forward and reverse primers were analyzed by using software Primer explorer V5 programed (http://primerexplorer.jp/lampv5e/index.html). The condition of PCR amplification for bla_{NDM-1} gene was prepare in 25 µl reaction containing 4 ng of genomic DNA, 10X PCR buffer, 50 mM $MgCl_2$, 10 mM dNTPs, *Taq* polymerase (Invitrogen, USA) and 10 µM of each primer. The PCR reaction was performed by using C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Ltd.; California, USA). The steps of PCR amplification contained pre-denaturation at 94 °C for five minutes followed by 30 cycles of denaturation at 94 °C for one minute, annealing at 58 °C for one minute and extension at 72 °C for one minute, and finally post-extension at 72 °C for another five minutes. The PCR products were analyzed by using 2% AGE in 0.5X Tris/Borate/EDTA (TBE) buffer at 100 volts. The DNA pattern was observed under UV light by using gel-doc (UVITEC Cambridge). The PCR product is 204 base pair.

4.2 LAMP

LAMP amplification was accomplished by using primers designed based on bla_{NDM-1} gene (Gen-bank accession on. Genbank; KY074638). The forward and reverse primers as well as forward inner and reverse inner primers were analyzed by using software Primer explorer V5 programed (http://primerexplorer.jp/lampv5e/index.html) (Submission patent NO. 2103000755). The condition of LAMP amplification for bla_{NDM-1} gene was prepared in 25 µl reaction containing 4 ng of genomic DNA, 10X thermal, 5M Betaine, 25 mM dNTP, 100 µM $MgSO_4$, *Bst* DNA polymerase (New England Biolabs, Ipswich, MA, USA). 10 µM of outer primer (NDM-1-F3 and NDM-1-B3) and 100 µM of inner primer (NDM-1-FIP and NDM-1-BIP). The reaction mixture was incubated at 63 °C

for 60 minutes by using C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Ltd.; California, USA). It was analyzed by 2% AGE.

4.3 RPA

RPA was achieved by using primers designed based on *bla*$_{NDM-1}$ gene (Genbank accession on. Genbank; KY074638). The forward and reverse primers were analyzed by using software Primer explorer V5 programed (http://primerexplorer.jp/lampv5e/index.html). The condition of RPA for *bla*$_{NDM-1}$ gene was prepared in 50 µl reaction containing 4 ng of genomic DNA, RPA kit (TwistAmp® Basic kit Quick Guide). The reaction mixture was incubated at 33 °C for 20 minutes. The RPA product was isolated by using GenepHIow™ Gel/PCR kit (Geneaid) and analyzed by using 2% AGE. The RPA product is 204 base pair.

5. Lateral Flow Dipstick (LFD) biosensor detection

5.1 FITC-labeling-probe for LFD

Labeled DNA probe was designed to specific with the target DNA about 20-30 bases by software Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0). The FITC-labeling probe was synthesized from Pacific Science. (Submission patent NO. 2103000755)

5.2 DNA probe hybridization

PCR-LFD; After PCR amplification, 9 µl of PCR product was transferred to a microcentrifuge tube and 1 µl of FITC-DNA probe was added. Then, the reaction was incubated at 58 °C for 10 minutes before the addition of 100 µl of running buffer. Finally, the LFD strips (Milenia HybriDtect, Germany) was placed into the hybridization mixture and leave 10 minutes prior to dipping into the water for another 10 minutes to stop reaction.

LAMP-LFD; After LAMP amplification, 9 µl of LAMP product was transferred to a microcentrifuge tube and 1 µl of FITC-DNA probe was added. Then, the reaction was incubated at 63 °C for 10 minutes before the addition of 100 µl of running buffer. Finally, the LFD strips (Milenia HybriDtect, Germany) was placed into the hybridization mixture and leave 10 minutes prior to dipping into the water for another 10 minutes to stop reaction.

RPA-LFD; After RPA amplification, 9 µl of RPA product was transferred to a microcentrifuge tube and 1 µl of FITC-DNA probe was added. Then, the reaction was incubated at 33 °C for 10 minutes before the addition of 100 µl of running buffer. Finally, the LFD strips (Milenia HybriDtect, Germany) was placed into the hybridization mixture and leave 10 minutes prior to dipping into the water for another 10 minutes to stop reaction.

6. Sensitivity test

The analytical sensitivity test was performed by 1:10 serial dilution of the standard DNA prior to testing with PCR, LAMP, and RPA assays. Standard DNA measured the concentration start from 0.74 ng/µl by using nanodrop and performed by 1:10 by Sterile deionized water into 10 times. Every dilution was used as a template for evaluating of the sensitivity of PCR, LAMP, and RPA methods.

7. Specificity test

The analytical specificity test was accomplished by testing the primers against 16 strains separated into two groups including ten strains of non-Enterobacteriaceae *(E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *S. agalactiae* ATCC 12386, *S. pneumonia* ATCC 49619, *E. casseliflavus* ATCC 700327, *A. baumannii* ATCC 196606, *S. aureus* ATCC 29213, *S. pyogenes* ATCC 19615, and *S. maltophilia* ATCC 17666) and six strains of Enterobacteriaceae (positive bla_{NDM-1} gene, *K. pneumonia* ATCC 700603, *S. sonnei* ATCC 25931, *E. coli* ATCC 35218*, P. mirabilis* ATCC 43071, and *E. hormaechei* ATCC 700323), which were used as templates for evaluation of the specificities of PCR, LAMP, and RPA methods.

8. Clinical validation

Sixty samples CRE extracted DNA Bacteria samples were derived from the hospital. All samples were tested with PCR, LAMP, and RPA with specific primer for bla_{NDM-1} gene and compared with universal primer for standard method (PCR) from EUCAST. The results were calculated in the sensitivity, specificity, and accuracy followed by WHO (Molecular Diagnostic Assay Validation). [58]

Table 6 Calculation of sensitivity, specificity, and positive and negative predictive.

TEST OUTCOME	CLINICAL CONDITION		
	POSITIVE	NEGATIVE	
POSITIVE	True Positive	False Positive	**Positive Predictive Value** TP/ (TP + FP)
NEGATIVE	False Negative	True Negative	**Negative Predictive Value** TN/ (TN + FN)
	Sensitivity TP/ (TP + FN)	**Specificity** TN/ (FP + TN)	

CHAPTER IV
RESULTS

1. Antimicrobial Susceptibility Testing

The inhibition zone was measured by comparing to CLSI 2018 (Clinical and Laboratory Standard Institute). The carbapenem resistance strain must show resistant at least one-third of the selected disc according to CLSI 28th ed.

These colonies can be susceptible, resistant, or intermediate in response to the antibiotics:

Susceptible means they can't grow if the drug is present. This means the antibiotic is effective against the bacteria. (Meropenem = ≥ 23, Imipenem = ≥ 23 and Ertapenem = ≥ 22 mm)

Intermediate means a higher dose of the antibiotic is needed to prevent growth. (Meropenem = 20-22, Imipenem = 20-22 and Ertapenem = 19-21 mm)

Resistant means the bacteria can grow even if the drug is present. This is a sign of an ineffective antibiotic. (Meropenem = ≤ 19, Imipenem = ≤ 19 and Ertapenem = ≤ 18 mm) (Figure 12).

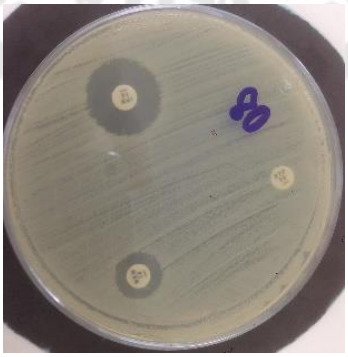


Figure 12 The disk diffusion method. Three types of carbapenem resistant disc consist of meropenem, imipenem, and ertapenem.

110 isolates were resistant to at least one of the tested carbapenem (imipenem, meropenem, and ertapenem) antibiotics, consisting of 97 *Klebsiella pneumoniae*, 10 *Escherichia coli*, 2 *Enterobacter cloacae*, and 1 *Citrobacter freundii*. The resistance rates to imipenem, meropenem, and ertapenem were 78%, 95%, and 97%, respectively (Figure 13)

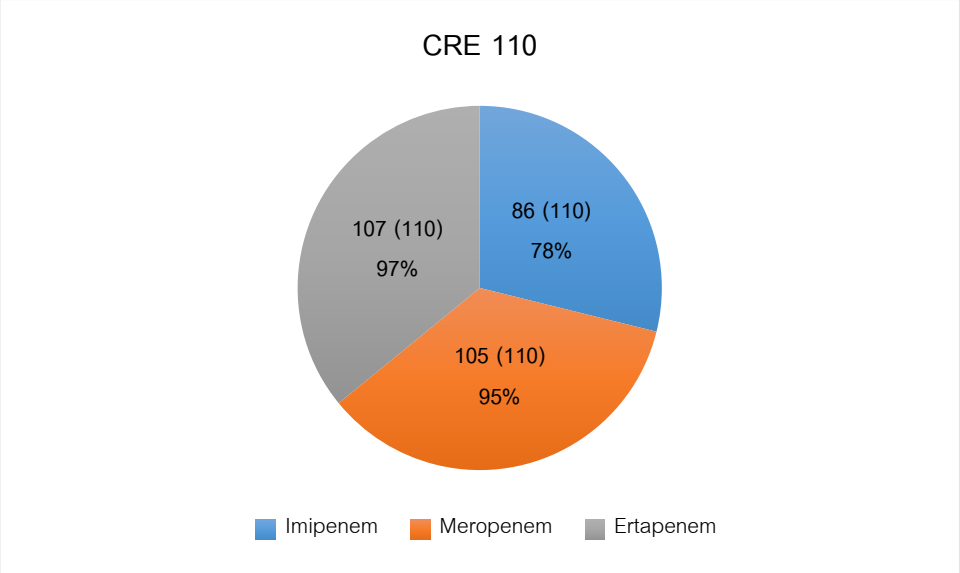


Figure 13 The resistance rates to imipenem, meropenem, and ertapenem were 78%, 95%, and 97%, respectively

2. **Detection of *NDM-1* gene by polymerase chain reaction (PCR) and sequencing**

Optimization of PCR of CRE isolates using specific primers for drug resistance were *NDM-1* gene revealed that appropriated of temperature 63 ^{o}C (Figure 14)

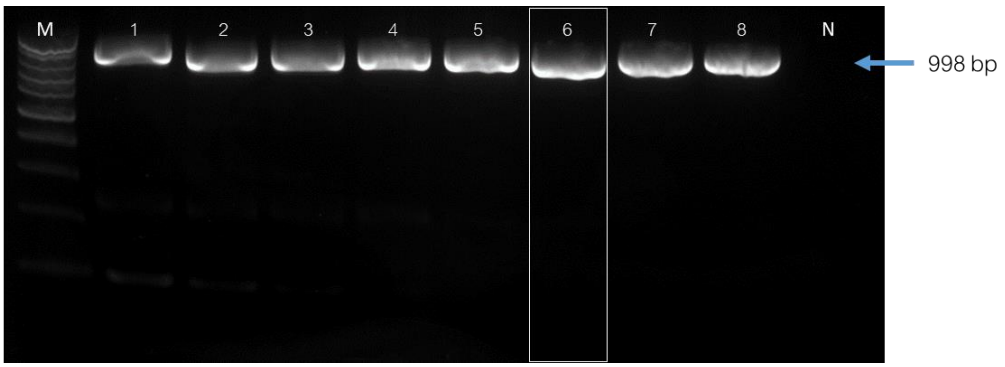


Figure 14 The optimized temperature analyzed by 0.8% gel-electrophoresis.

Lane M represent 100 bp λ ladder marker. Lane 1-8 represents optimized temperature were 55.0 °C, 55.7 °C, 57.0 °C, 59.0 °C, 61.4 °C, 63.3 °C, 64.5 °C, and 65.0 °C, respectively and Lane N represent negative control.

Polymerase Chain Reaction (PCR) of 110 isolates using specific primers for drug resistance revealed that 85 species carried a have New Delhi metallo-β-lactamase-1 (*NDM-1*) (Figure 15). In addition, the nucleotide sequences of NDM-1 genes found in *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae* and *Citrobacter freundii* were identical. Nucleotide sequence of the isolates was aligned with reference sequence.

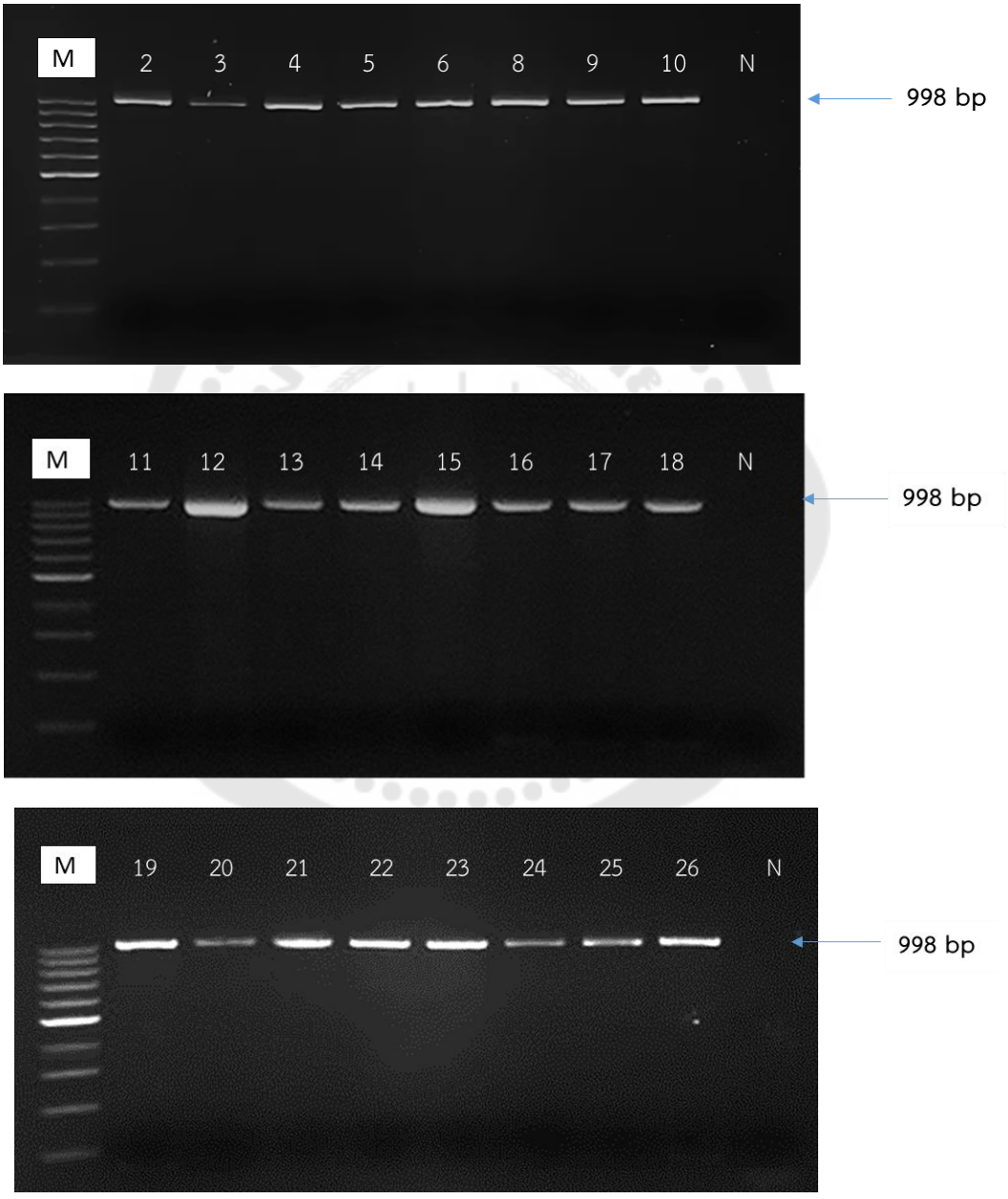
Reference of *E. coli*; AB604953, JN255860, and MG736960 compared sample 2, 3, 4, 5, 9, 12, 13, and 17 total **8** isolates.

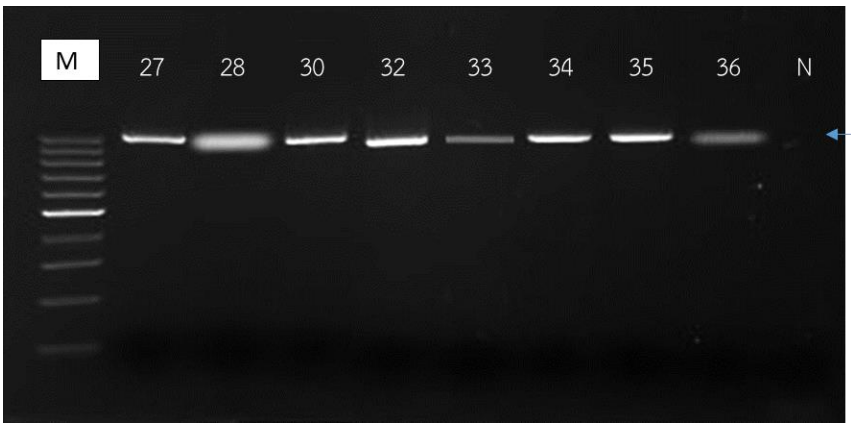
Reference of *K. pneumonia*; KY074638, HE866523, KT365396, MF038875, KT365395, KU162940, AB604954, JN255858, and JN255859 compared sample 6, 11, 14, 16, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 30, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52, 53, 54, 55, 57, 59, 61, 63, 64, 67, 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 82, 83, 84, 85, 87, 88, 89, 91, 92, 94, 96, 99, 100, 101, 102, 103, 106, 107, and 109 total **74** isolates.

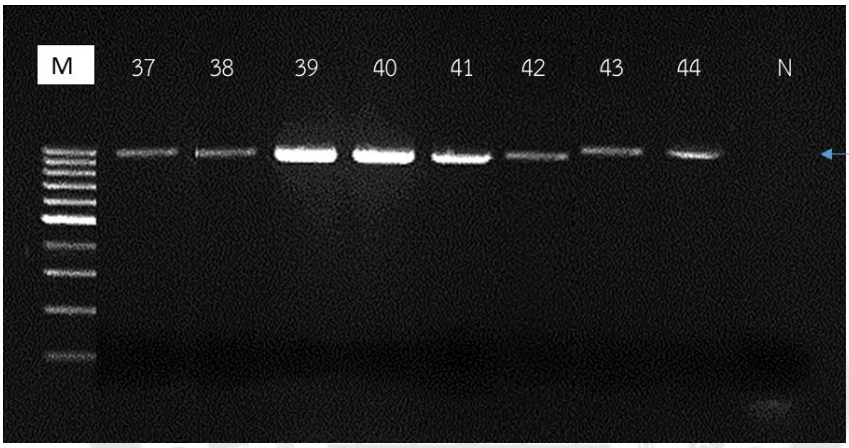
Reference of *E. cloacae*; KJ018855, KF699332, MH087224, and KJ558508 compared sample 8 and 10 total **2** isolates.

Reference of *C. freundii*; KJ558509, KF951469, KF951471, MK101346, and KR816561 compared sample 15 total **1** isolates.

*** The selected isolates showed 100 per cent homology with the reference strain.




M
27
28
30
32
33
34
35
36
N
998 bp


M
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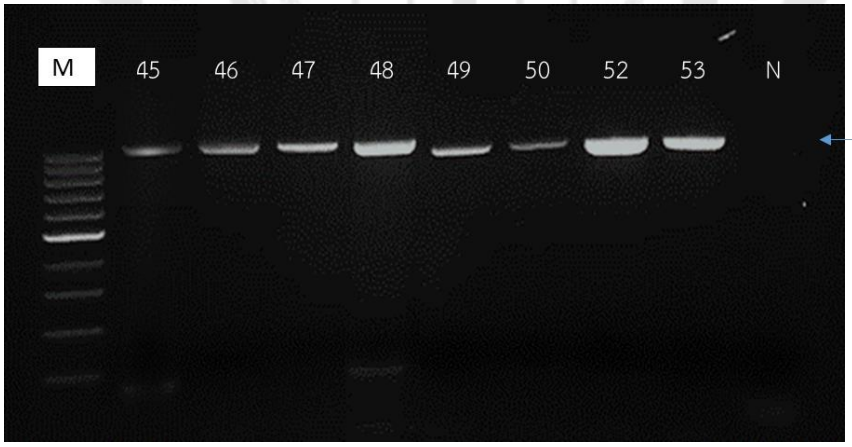
N
998 bp


M
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48
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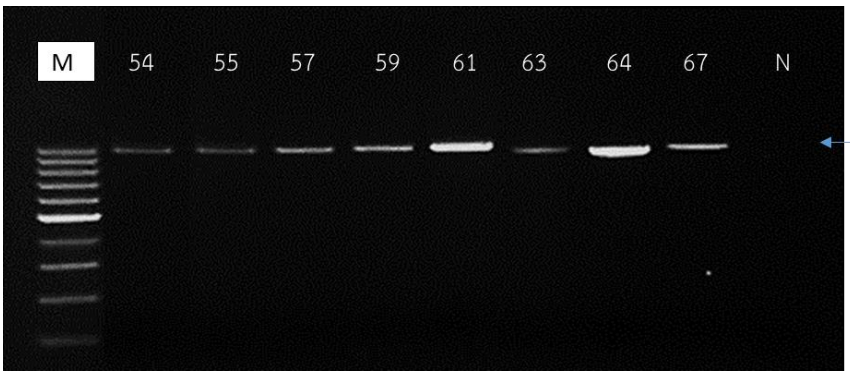
52
53
N
998 bp


M
54
55
57
59
61
63
64
67
N
998 bp

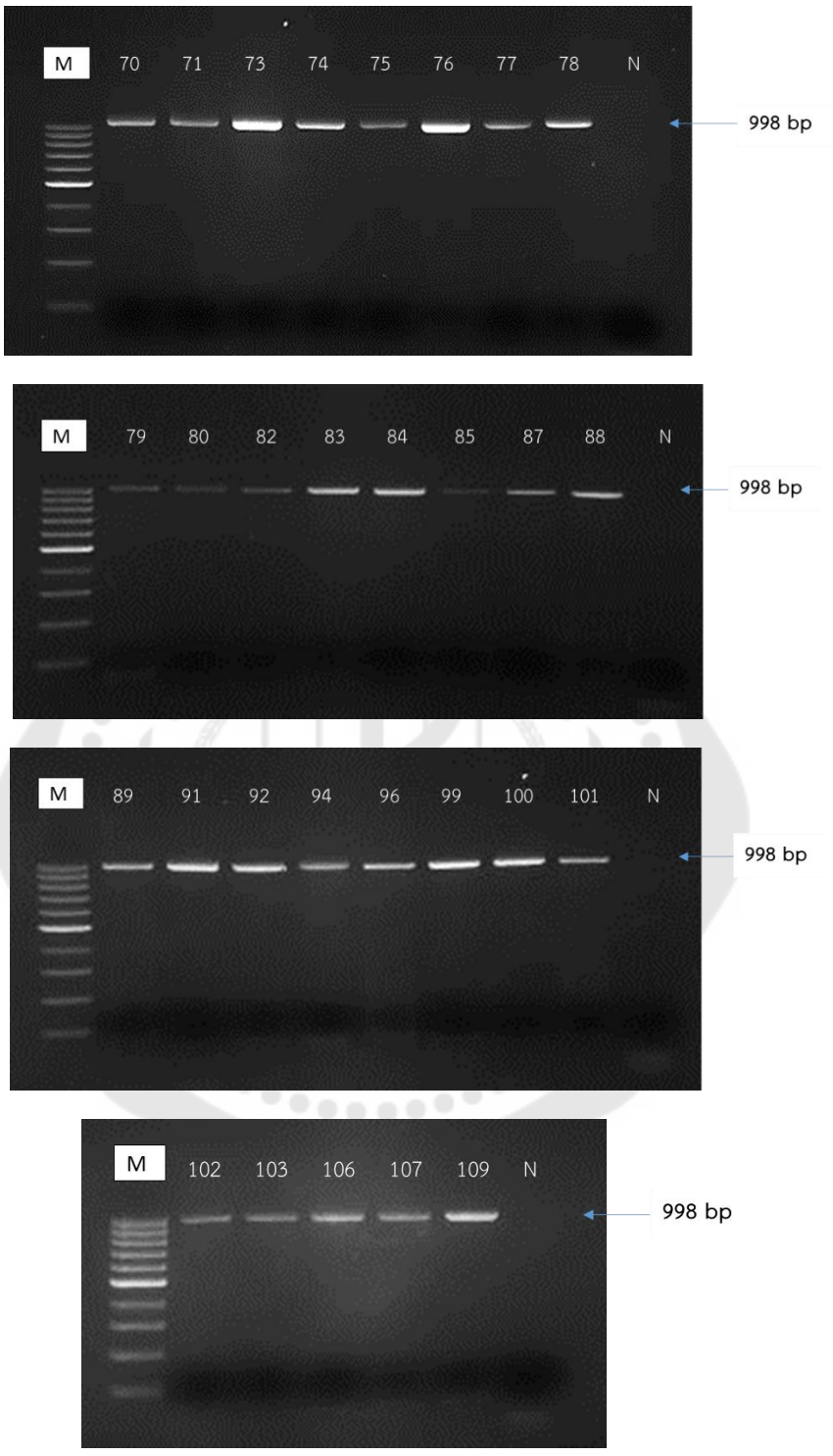


Figure 15 The represent NDM-1 gene by gel electrophoresis pattern of PCR amplification.

Lane M = 100 bp λ ladder marker, Lanes of number = positive CRE 85 strains (E. coli, E. cloacae, C. freudii, and K. pneumonia) of NDM-1 gene of 998 bp in size, and N = negative control.

Multiple sequence alignment

```
19NDM998        ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
5NDM998         ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
4NDM998         ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCA 60
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23NDM998       CC--CCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCC-CGATAGCCGCGCCGCAAT 776
AB604954       CC--CCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCC-CGATAGCCGCGCCGCAAT 776
37NDM998       CC--CCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCC-CGATAGCCGCGCCGCAAT 776
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8NDMF998       CC--CCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCC-CGATAGCCGCGCCGCAAT 776
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106NDM998       -CCCCAA-GGCCAGCATGATCGTGATGAGCCATTCCGCCCC-CGATAGCCGCGCCGCAAT 775
                 *  * * * *       **********************  ******************

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AB604953        CACTCATACGGCCCGCATGGCCGACAAGCTGCGCTGA---  813
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106NDM998       CACTCATACGGCCCGCATGGCCGACAAGCTGCGCTGA---  812
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Nucleotide sequence of the isolates was aligned with reference sequence. The selected isolates showed 100 per cent homology with the reference strain.

3. Optimization of *NDM-1* gene based on PCR, LAMP, and RPA amplification methods

3.1 PCR amplification

Optimization of PCR of CRE isolates using specific primers for drug resistance were *NDM-1* gene revealed that appropriated of temperature 58 °C (Figure 16)

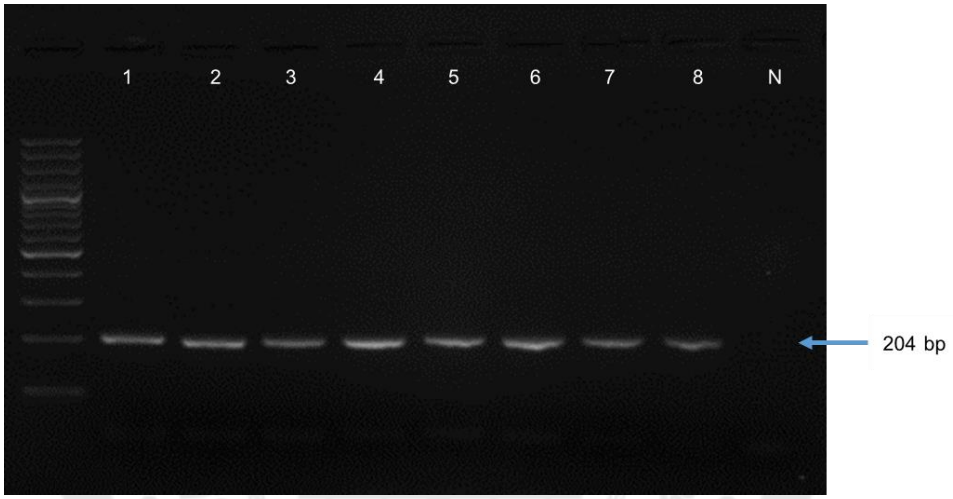

Figure 16 The optimized temperature analyzed by 2% gel-electrophoresis. Lane M represent 100 bp λ ladder marker.

Lane 1-8 represents optimized temperature were 62.0 °C, 61.2 °C, 60.0 °C, **58.1 °C**, 55.8 °C, 53.9 °C, 52.7 °C, and 52.0 °C, respectively and Lane N represent negative control.

The CRE strains (*E. coli*, *E. cloacae*, *C. freudii,* and *K. pneumonia)*, *NDM-1* gene of 204 bp in size and analyzed gel electrophoresis (Figure 17).

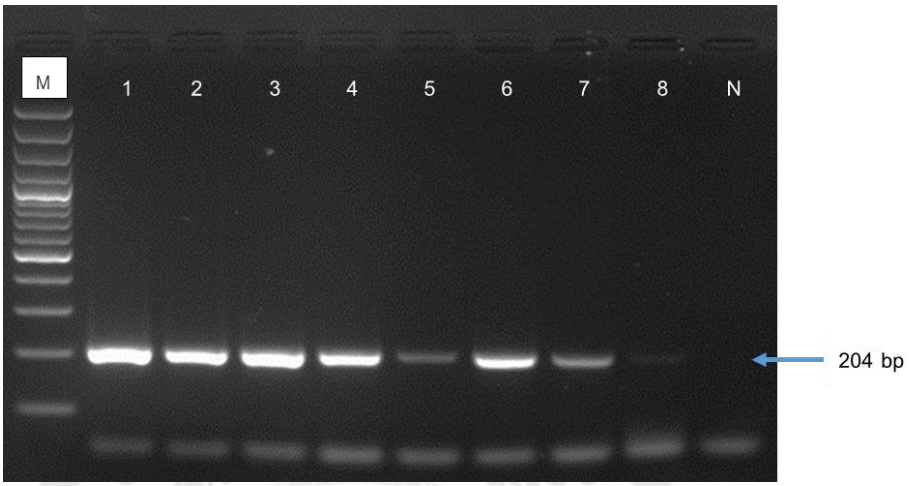

Figure 17 The represent NDM-1 gene by gel electrophoresis pattern of PCR amplification.

Lane M = 100 bp λ ladder marker, Lanes 1-8 = positive CRE strains (*E. coli, E. cloacae*, *C. freudii,* and *K. pneumonia*) of NDM-1 gene of 204 bp in size, and N = negative control

3.2 LAMP amplification

Optimization of LAMP of CRE isolates using specific primers for drug resistance were *NDM-1* gene revealed that the concentration of $MgSO_4$ were 1 mM and temperature 63 ^{o}C (Figure 18)

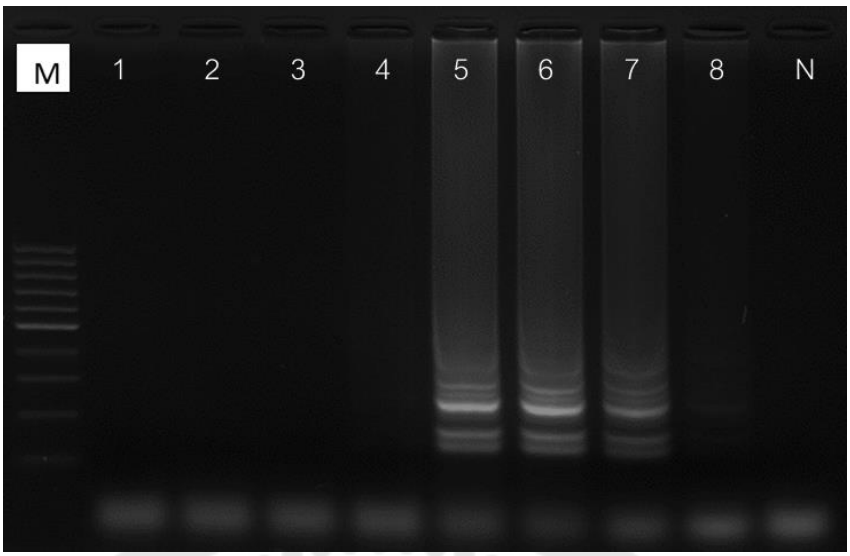

Figure 18 The optimized temperature analyzed by 2% gel-electrophoresis.

Lane M represent 100 bp λ ladder marker. Lane 1-8 represents optimized temperature were 57.0 °C, 57.7 °C, 58.9 °C, 60.8 °C, **63.1 °C**, 65 °C, 66.3 °C, and 67.0 °C, respectively and Lane N represent negative control.

The CRE strains (*E. coli*, *E. cloacae*, *C. freudii,* and *K. pneumonia)*, *NDM-1* gene by gel electrophoresis pattern of LAMP amplification (Figure 19).

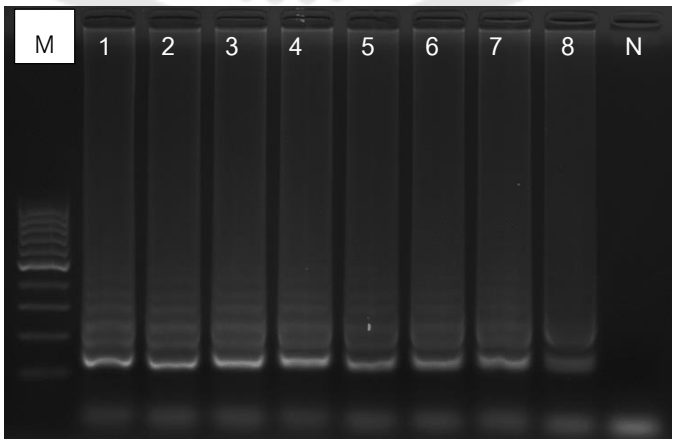

Figure 19 The represent NDM-1 gene by gel electrophoresis pattern of LAMP amplification.

Lane M = 100 bp λ ladder marker, Lanes 1-8 = positive CRE strains *(E. coli, E. cloacae, C. freudii,* and *K. pneumonia)* of *NDM-1* gene, and N = negative control.

3.3 RPA amplification

Optimization of RPA of *NDM-1* gene revealed that the final time and temperature of RPA amplification were 20 minutes and 33 °C, respectively (Figure 20 and 21)

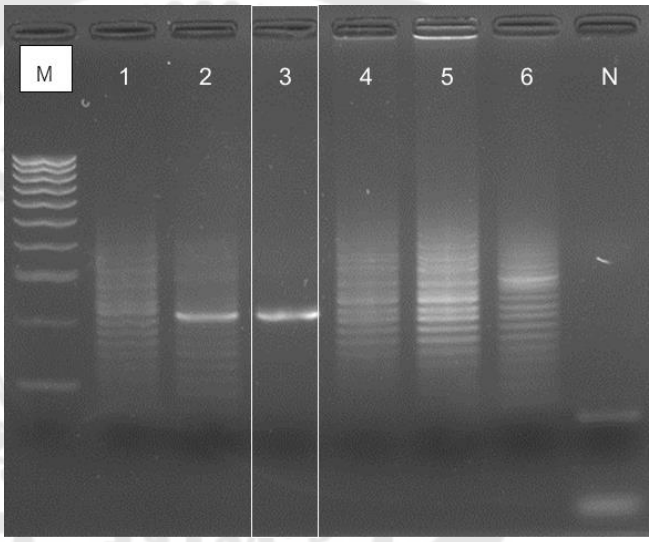

Figure 20 The final time of RPA amplification analyzed by 2% gel-electrophoresis.

Lane M represent 100 bp λ ladder marker. Lane 1-6 represents time of RPA amplification were 10 min, 15 min, 20 min, 25 min, 30 min, and 35 min respectively and Lane N represent negative control.

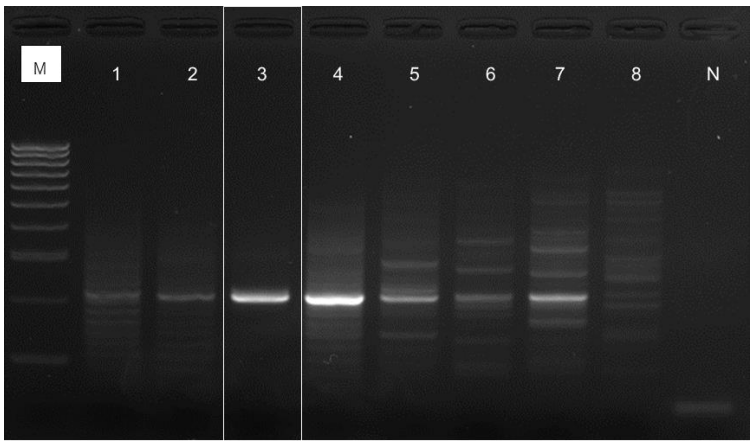

Figure 21The optimized temperature analyzed by 2% gel-electrophoresis.

Lane M represent 100 bp λ ladder marker. Lane 1-8 represents optimized temperature were 32.0 °C, 32.5 °C, 33.4 °C, 35.2 °C, 37.1 °C, 38.3, 39.3 °C, and 40.0 °C, respectively and Lane N represent negative control.

The CRE strains (*E. coli*, *E. cloacae*, *C. freudii*, and *K. pneumonia*), *NDM-1* gene of 204 bp in size and analyzed gel electrophoresis (Figure 22).

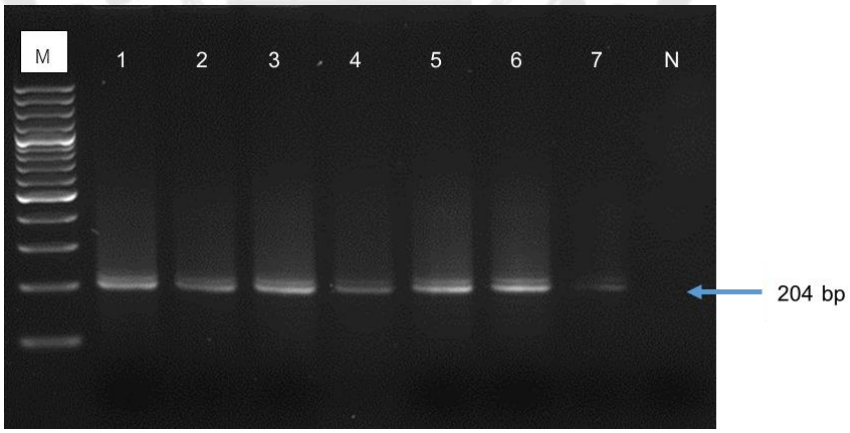

Figure 22 The represent NDM-1 gene by gel electrophoresis pattern of RPA amplification.

Lane M = 100 bp λ ladder marker, Lanes 1-7 = positive CRE strains (E. coli, E. cloacae, C. freudii, and K. pneumonia) of NDM-1 gene of 204 bp in size, and N = negative control

4. The limit of detection and specificity test of PCR, LAMP, and RPA assays

4.1 Sensitivity and Specificity of *NDM-1* gene by PCR-AGE

Sensitivity and Specificity of PCR were determined. The limit of detection using PCR was 0.74 ng/ µl (Figure 23). No cross-reaction to host DNA and other Microorganisms are use as negative control such as *Enterococcus faecalis* (ACTT 29212), *Pseudomonas aeruginosa* (ACTT 27853), *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ACTT 12386), *Klebsiella pneumonia* (ATCC 700603), *Streptococcus pneumonia* (ATCC 49619), *Shigella sonnei* (ATCC 25931), *Escherichia coli* (ATCC 35218), *Enterococcus casseliflavus* (ATCC 700327), *Proteus mirabilis* (ATCC 43071), *Acinetobacter baumannii* (ATCC 196606), *Staphylococcus aureus* (ATCC 29213), *Enterobacter hormaechei* (ATCC 700323), *Streptococcus pyogenes* (ATCC 19615), and *Stenotrophomonas maltophilia* (ATCC 17666) (Figure 24).

Figure 23 The represent sensitivity test of PCR amplification using NDM-1 as primer.

The limit of detection was 0.74 ng/ µl. Lane M = 100 bp λ ladder marker, Lane 1 = 74 ng/ µl, Lane 2 = 7.4 ng/ µl, Lane 3 = 0.74 ng/ µl, Lane 4 = 74 pg/ µl, Lane 5 = 7.4 pg/ µl, Lane 6 = 0.74 pg/ µl, Lane 7 = 74 fg/ µl, Lane 8 = 7.4 fg/ µl, Lane 9 = 0.74 fg/ µl, and N = negative control

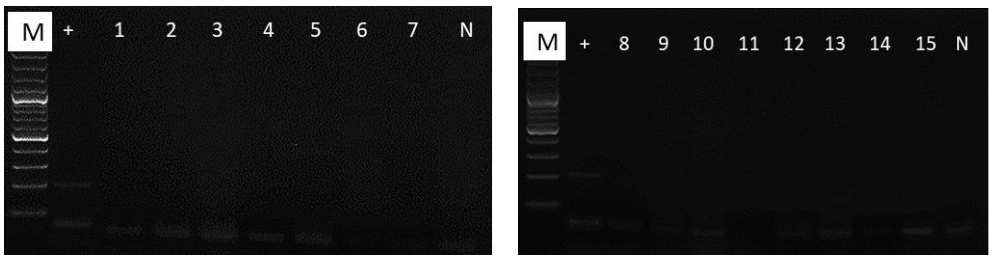

Figure 24 The represent specificity test of PCR amplification using NDM-1 as primer No cross-hybridization to host DNA and other microorganism bacteria were observed.

Lane M = 100 bp λ ladder marker, + = positive *NDM-1 gene* of 204 bp, Lane 1-15 = other microorganisms are use as negative*; Enterococcus faecalis (ACTT 29212), Pseudomonas aeruginosa (ACTT 27853), Staphylococcus aureus (ATCC 25923), Streptococcus agalactiae (ACTT 12386), Klebsiella pneumonia (ATCC 700603), Streptococcus pneumonia (ATCC 49619), Shigella sonnei (ATCC 25931), Escherichia coli (ATCC 35218), Enterococcus casseliflavus (ATCC 700327), Proteus mirabilis (ATCC 43071), Acinetobacter baumannii (ATCC 196606), Staphylococcus aureus (ATCC 29213), Enterobacter hormaechei (ATCC 700323), Streptococcus pyogenes (ATCC 19615), Stenotrophomonas maltophilia (ATCC 17666)* respectively, and N = negative control

4.2 Sensitivity and Specificity of *NDM-1* gene by LAMP-AGE

Sensitivity and Specificity of LAMP were determined. The limit of detection using LAMP was 7.4 pg/ µl (Figure 25). No cross-hybridization to host DNA and other Microorganisms are use as negative control such as *Enterococcus faecalis* (ACTT 29212), *Pseudomonas aeruginosa* (ACTT 27853), *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ACTT 12386), *Klebsiella pneumonia* (ATCC 700603), *Streptococcus pneumonia* (ATCC 49619), *Shigella sonnei* (ATCC 25931), *Escherichia coli* (ATCC 35218), *Enterococcus casseliflavus* (ATCC 700327), *Proteus mirabilis* (ATCC 43071), *Acinetobacter baumannii* (ATCC 196606), *Staphylococcus aureus* (ATCC 29213), *Enterobacter hormaechei* (ATCC 700323), *Streptococcus pyogenes* (ATCC 19615), and *Stenotrophomonas maltophilia* (ATCC 17666) (Figure 26).

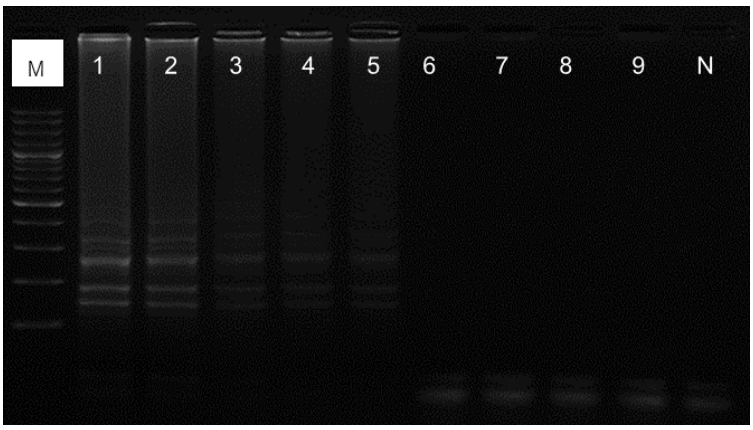

Figure 25 The represent sensitivity test of LAMP amplification using NDM-1 as primer.

The limit of detection was 7.4 pg/ µl. Lane M = 100 bp λ ladder marker, Lane 1 = 74 ng/ µl, Lane 2 = 7.4 ng/ µl, Lane 3 = 0.74 ng/ µl, Lane 4 = 74 pg/ µl, Lane 5 = 7.4 pg/ µl, Lane 6 = 0.74 pg/ µl, Lane 7 = 74 fg/ µl, Lane 8 = 7.4 fg/ µl, Lane 9 = 0.74 fg/ µl, and N = negative control

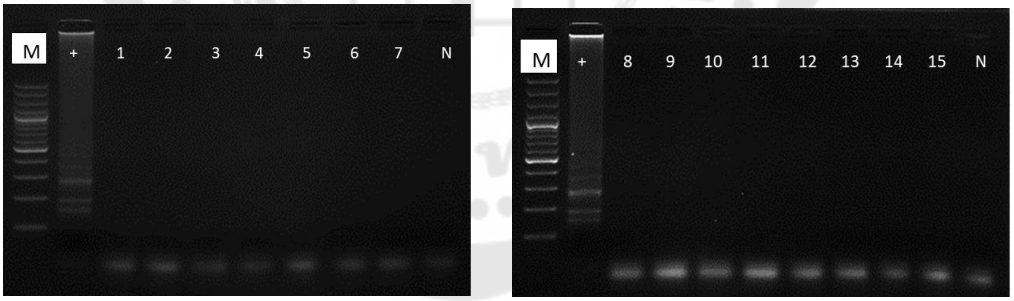

Figure 26 The represent specificity test of LAMP amplification using NDM-1 as primer No cross-hybridization to host DNA and other microorganism bacteria were observed.

Lane M = 100 bp λ ladder marker, + = positive *NDM-1* gene, Lane 1-15 = other microorganisms are use as negative*; Enterococcus faecalis (ACTT 29212), Pseudomonas aeruginosa (ACTT 27853), Staphylococcus aureus (ATCC 25923), Streptococcus agalactiae (ACTT 12386), Klebsiella pneumonia (ATCC 700603), Streptococcus pneumonia (ATCC 49619), Shigella sonnei (ATCC 25931), Escherichia*

coli (ATCC 35218), Enterococcus casseliflavus (ATCC 700327), Proteus mirabilis (ATCC 43071), Acinetobacter baumannii (ATCC 196606), Staphylococcus aureus (ATCC 29213), Enterobacter hormaechei (ATCC 700323), Streptococcus pyogenes (ATCC 19615), Stenotrophomonas maltophilia (ATCC 17666) respectively, and N = negative control

4.3 Sensitivity and Specificity of *NDM-1* gene by RPA-AGE

Sensitivity and Specificity of RPA were determined. The limit of detection using RPA was 0.74 ng/ µl (Figure 27). No cross-hybridization to host DNA and other Microorganisms are use as negative control such as *Enterococcus faecalis* (ACTT 29212), *Pseudomonas aeruginosa* (ACTT 27853), *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ACTT 12386), *Klebsiella pneumonia* (ATCC 700603), *Streptococcus pneumonia* (ATCC 49619), *Shigella sonnei* (ATCC 25931), *Escherichia coli* (ATCC 35218), *Enterococcus casseliflavus* (ATCC 700327), *Proteus mirabilis* (ATCC 43071), *Acinetobacter baumannii* (ATCC 196606), *Staphylococcus aureus* (ATCC 29213), *Enterobacter hormaechei* (ATCC 700323), *Streptococcus pyogenes* (ATCC 19615), and *Stenotrophomonas maltophilia* (ATCC 17666) (Figure 28).

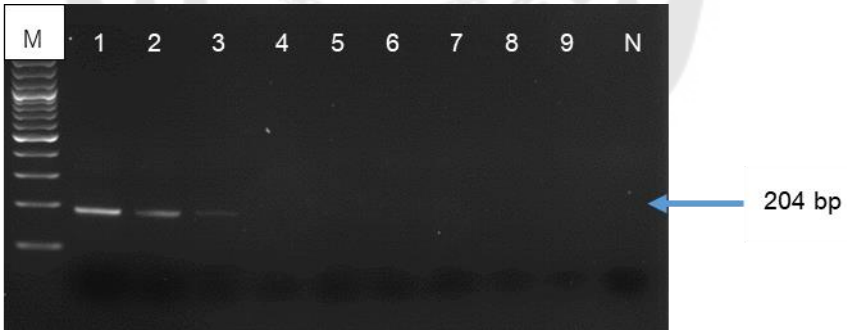

Figure 27 The represent sensitivity test of RPA amplification using NDM-1 as primer.

The limit of detection was 0.74 ng/ µl. Lane M = 100 bp λ ladder marker, Lane 1 = 74 ng/ µl, Lane 2 = 7.4 ng/ µl, Lane 3 = 0.74 ng/ µl, Lane 4 = 74 pg/ µl, Lane 5 = 7.4 pg/ µl, Lane 6 = 0.74 pg/ µl, Lane 7 = 74 fg/ µl, Lane 8 = 7.4 fg/ µl, Lane 9 = 0.74 fg/ µl, and N = negative control

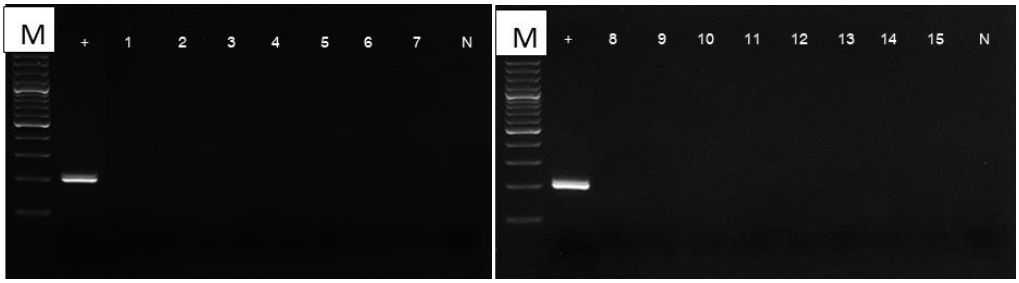

Figure 28 The represent specificity test of RPA amplification using NDM-1 as primer No cross-hybridization to host DNA and other microorganism bacteria were observed.

Lane M = 100 bp λ ladder marker, + = positive *NDM-1 gene* of 204 bp, Lane 1-15 = other microorganisms are use as negative; *Enterococcus faecalis (ACTT 29212), Pseudomonas aeruginosa (ACTT 27853), Staphylococcus aureus (ATCC 25923), Streptococcus agalactiae (ACTT 12386), Klebsiella pneumonia (ATCC 700603), Streptococcus pneumonia (ATCC 49619), Shigella sonnei (ATCC 25931), Escherichia coli (ATCC 35218), Enterococcus casseliflavus (ATCC 700327), Proteus mirabilis (ATCC 43071), Acinetobacter baumannii (ATCC 196606), Staphylococcus aureus (ATCC 29213), Enterobacter hormaechei (ATCC 700323), Streptococcus pyogenes (ATCC 19615), Stenotrophomonas maltophilia (ATCC 17666)* respectively, and N = negative control

5. Lateral Flow Dipstick (LFD) biosensor detection

5.1 Optimization of DNA probe concentration

The optimization of DNA probe concentration of PCR, LAMP, and RPA assays were 1.0 μM (Figure 29).

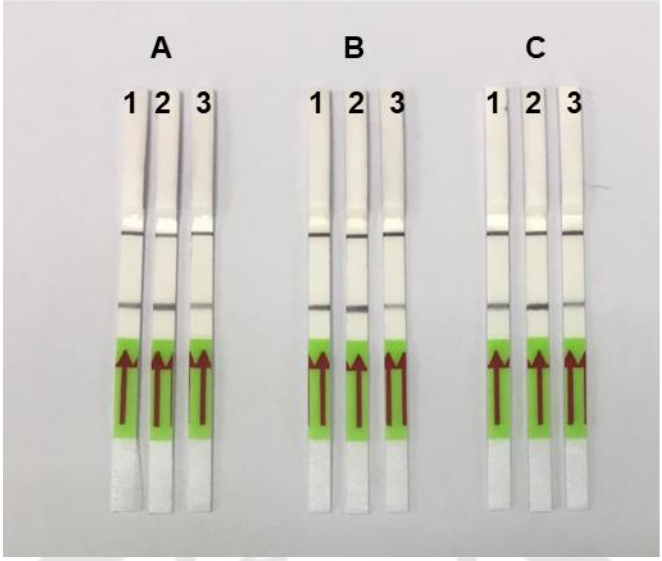

Figure 29 The optimization of DNA probe of PCR, LAMP and RPA assay.

ABC represents PCR, LAMP and RPA, respectively. 1, 2, 3 represents concentration of probe 0.1 µM, 1 µM and 10 µM, respectively.

5.2 Sensitivity and Specificity of *NDM-1* gene by PCR-LFD

Sensitivity and Specificity of PCR-LFD were determined. The limit of detection using PCR was 0.74 ng/ µl (Figure 30). No cross-hybridization to host DNA and other Microorganisms are use as negative control such as *Enterococcus faecalis* (ACTT 29212), *Pseudomonas aeruginosa* (ACTT 27853), *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ACTT 12386), *Klebsiella pneumonia* (ATCC 700603), *Streptococcus pneumonia* (ATCC 49619), *Shigella sonnei* (ATCC 25931), *Escherichia coli* (ATCC 35218), *Enterococcus casseliflavus* (ATCC 700327), *Proteus mirabilis* (ATCC 43071), *Acinetobacter baumannii* (ATCC 196606), *Staphylococcus aureus* (ATCC 29213), *Enterobacter hormaechei* (ATCC 700323), *Streptococcus pyogenes* (ATCC 19615), and *Stenotrophomonas maltophilia* (ATCC 17666) (Figure 31).

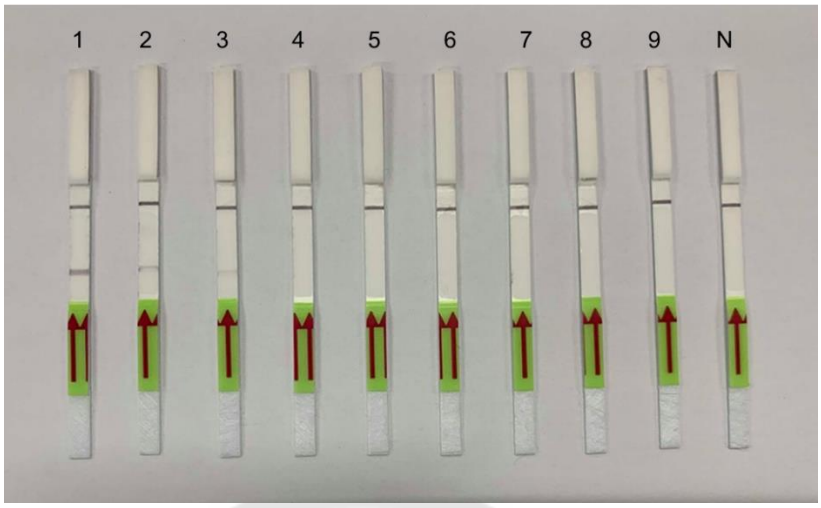

Figure 30 The represent sensitivity test of PCR-LFD.

The limit of detection was 0.74 ng/ µl. Lane 1 = 74 ng/ µl, Lane 2 = 7.4 ng/ µl, Lane 3 = 0.74 ng/ µl, Lane 4 = 74 pg/ µl, Lane 5 = 7.4 pg/ µl, Lane 6 = 0.74 pg/ µl, Lane 7 = 74 fg/ µl, Lane 8 = 7.4 fg/ µl, Lane 9 = 0.74 fg/ µl, and N = negative control

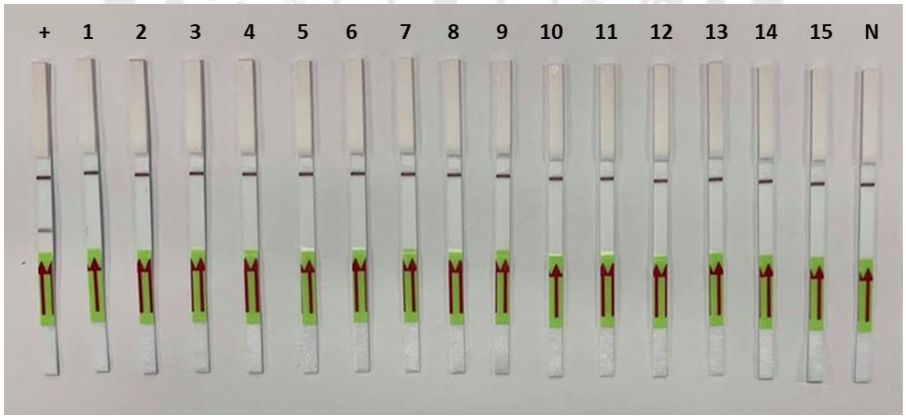

Figure 31 The represent specificity test of PCR-LFD No cross-hybridization to host DNA and other microorganism bacteria were observed.

+ = positive *NDM-1 gene,* Lane 1-15 = other microorganisms are use as negative*;* *Enterococcus faecalis (ACTT 29212), Pseudomonas aeruginosa (ACTT 27853), Staphylococcus aureus (ATCC 25923), Streptococcus agalactiae (ACTT 12386),*

Klebsiella pneumonia (ATCC 700603), Streptococcus pneumonia (ATCC 49619), Shigella sonnei (ATCC 25931), Escherichia coli (ATCC 35218), Enterococcus casseliflavus (ATCC 700327), Proteus mirabilis (ATCC 43071), Acinetobacter baumannii (ATCC 196606), Staphylococcus aureus (ATCC 29213), Enterobacter hormaechei (ATCC 700323), Streptococcus pyogenes (ATCC 19615), Stenotrophomonas maltophilia (ATCC 17666) respectively, and N = negative control

5.3 Sensitivity and Specificity of *NDM-1* gene by LAMP-LDF

Sensitivity and Specificity of LAMP-LFD were determined. The limit of detection using LAMP was 7.4 pg/ µl (Figure 32). No cross-hybridization to host DNA and other Microorganisms are use as negative control such as *Enterococcus faecalis* (ACTT 29212), *Pseudomonas aeruginosa* (ACTT 27853), *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ACTT 12386), *Klebsiella pneumonia* (ATCC 700603), *Streptococcus pneumonia* (ATCC 49619), *Shigella sonnei* (ATCC 25931), *Escherichia coli* (ATCC 35218), *Enterococcus casseliflavus* (ATCC 700327), *Proteus mirabilis* (ATCC 43071), *Acinetobacter baumannii* (ATCC 196606), *Staphylococcus aureus* (ATCC 29213), *Enterobacter hormaechei* (ATCC 700323), *Streptococcus pyogenes* (ATCC 19615), and *Stenotrophomonas maltophilia* (ATCC 17666) (Figure 33).

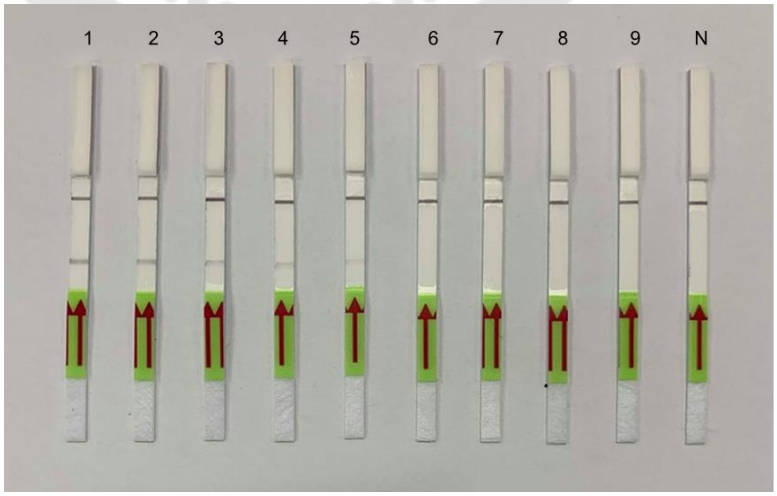

Figure 32 The represent sensitivity test of LAMP-LFD.

The limit of detection was 7.4 pg/ µl. Lane 1 = 74 ng/ µl, Lane 2 = 7.4 ng/ µl, Lane 3 = 0.74 ng/ µl, Lane 4 = 74 pg/ µl, Lane 5 = 7.4 pg/ µl, Lane 6 = 0.74 pg/ µl, Lane 7 = 74 fg/ µl, Lane 8 = 7.4 fg/ µl, Lane 9 = 0.74 fg/ µl, and N = negative control

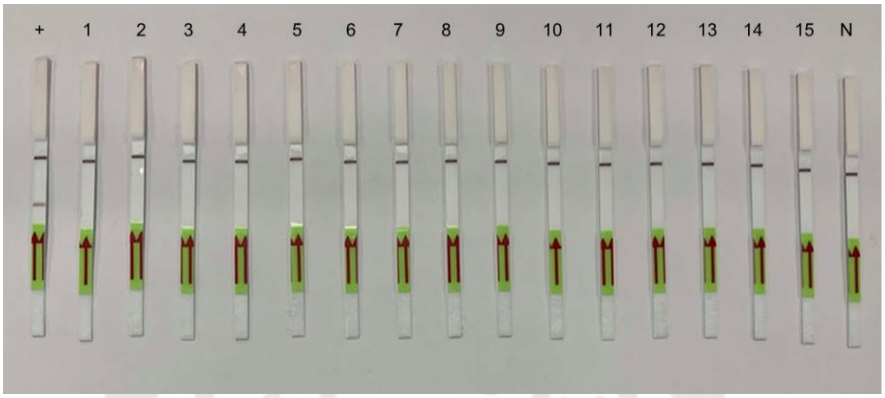

Figure 33 The represent specificity test of LAMP-LFD No cross-hybridization to host DNA and other microorganism bacteria were observed.

+ = positive *NDM-1 gene*, Lane 1-15 = other microorganisms are use as negative; *Enterococcus faecalis (ACTT 29212), Pseudomonas aeruginosa (ACTT 27853), Staphylococcus aureus (ATCC 25923), Streptococcus agalactiae (ACTT 12386), Klebsiella pneumonia (ATCC 700603), Streptococcus pneumonia (ATCC 49619), Shigella sonnei (ATCC 25931), Escherichia coli (ATCC 35218), Enterococcus casseliflavus (ATCC 700327), Proteus mirabilis (ATCC 43071), Acinetobacter baumannii (ATCC 196606), Staphylococcus aureus (ATCC 29213), Enterobacter hormaechei (ATCC 700323), Streptococcus pyogenes (ATCC 19615), Stenotrophomonas maltophilia (ATCC 17666)* respectively, and N = negative control

5.4 Sensitivity and Specificity of *NDM-1* gene by RPA-LFD

Sensitivity and Specificity of RPA-LFD were determined. The limit of detection using RPA was 0.74 ng/ µl (Figure 34). No cross-hybridization to host DNA and other Microorganisms are use as negative control such as *Enterococcus faecalis* (ACTT 29212), *Pseudomonas aeruginosa* (ACTT 27853), *Staphylococcus aureus* (ATCC

25923), *Streptococcus agalactiae* (ACTT 12386), *Klebsiella pneumonia* (ATCC 700603), *Streptococcus pneumonia* (ATCC 49619), *Shigella sonnei* (ATCC 25931), *Escherichia coli* (ATCC 35218), *Enterococcus casseliflavus* (ATCC 700327), *Proteus mirabilis* (ATCC 43071), *Acinetobacter baumannii* (ATCC 196606), *Staphylococcus aureus* (ATCC 29213), *Enterobacter hormaechei* (ATCC 700323), *Streptococcus pyogenes* (ATCC 19615), and *Stenotrophomonas maltophilia* (ATCC 17666) (Figure 35).

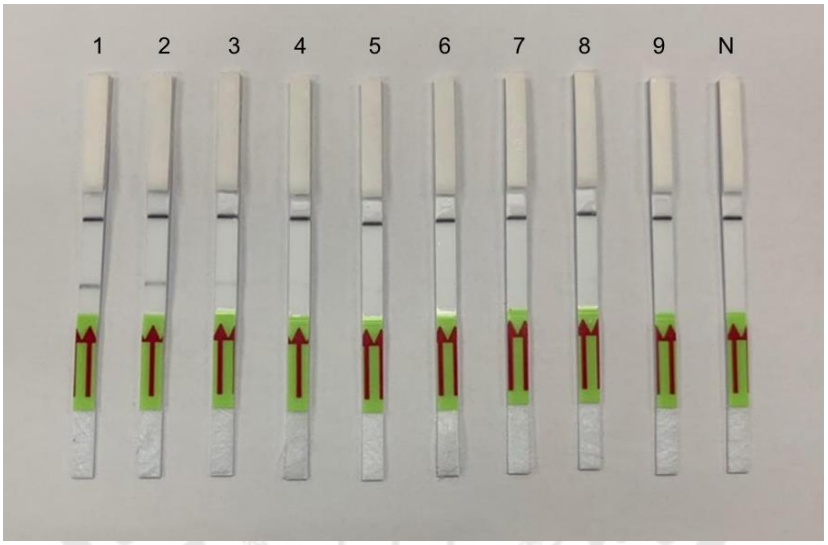

Figure 34 The represent sensitivity test of RPA-LFD.

The limit of detection was 0.74 ng/ µl. Lane 1 = 74 ng/ µl, Lane 2 = 7.4 ng/ µl, Lane 3 = 0.74 ng/ µl, Lane 4 = 74 pg/ µl, Lane 5 = 7.4 pg/ µl, Lane 6 = 0.74 pg/ µl, Lane 7 = 74 fg/ µl, Lane 8 = 7.4 fg/ µl, Lane 9 = 0.74 fg/ µl, and N = negative control

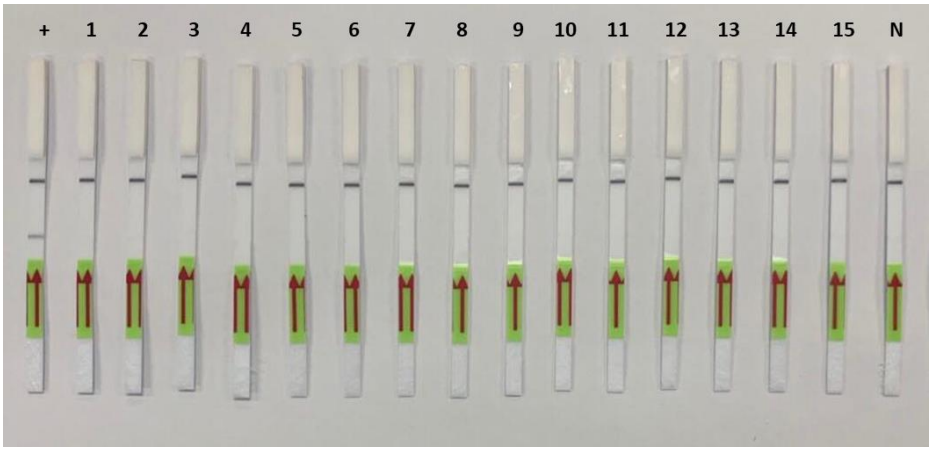

Figure 35 The represent specificity test of RPA-LFD No cross-hybridization to host DNA and other microorganism bacteria were observed.

+ = positive *NDM-1 gene*, Lane 1-15 = other microorganisms are use as negative; *Enterococcus faecalis (ACTT 29212), Pseudomonas aeruginosa (ACTT 27853), Staphylococcus aureus (ATCC 25923), Streptococcus agalactiae (ACTT 12386), Klebsiella pneumonia (ATCC 700603), Streptococcus pneumonia (ATCC 49619), Shigella sonnei (ATCC 25931), Escherichia coli (ATCC 35218), Enterococcus casseliflavus (ATCC 700327), Proteus mirabilis (ATCC 43071), Acinetobacter baumannii (ATCC 196606), Staphylococcus aureus (ATCC 29213), Enterobacter hormaechei (ATCC 700323), Streptococcus pyogenes (ATCC 19615), Stenotrophomonas maltophilia (ATCC 17666)* respectively, and N = negative control

CHAPTER V
DISCUSSION

From the past until now, the carbapenem-resistant *Enterobacteriaceae* are both effects for gram-negative bacteria and gram-positive bacteria. Although new antibiotics have been continuously developed for treatment, the bacteria have adapted themself to be new antibiotic resistance.

Enterobateriaceae are the most common human pathogens with cause many diseases including urinary tract infections (UTIs), peritonitis, meningitis, pneumonia, bloodstream infections, and skin and soft tissue infections, and they are also the source of nosocomial and community-acquired infections. [59,60]

Carbapenems [e.g. meropenem, imipenem, and ertapenem] with a broad spectrum of antibacterial activity are harmless and strong beta-lactam antibiotics that are normally productive therapeutic choice for treatment of severe gram-negative bacterial infections when resistance to other classes of antimicrobials is existent.[61]

Carbapenem resistance *Enterobacteriaceae* (CRE) is mostly correlated to the production of carbapenemases. These enzymes are capable to hydrolyze beta-lactam ring.[62] Resistant pathogens are associated with increased mortality, prolonged hospitalization and high medical costs.[63] The highest occurrence of CRE isolates was in urine specimen, in keeping with the findings of the reports of Bratu S [64] and Marchaim D. [65] The most common CRE species was *Klebsiella pneumonia*e (75%) in Thailand.[66] In a previous estimation, the prevalence of CRE in Vietnam and the Philippines was approximately 5-10%. The estimated prevalence in Thailand was approximately 1-5%. [67] The first proceed in the detection of carbapenemase is grounded on these methods for characterizing disc diffusion testing in clinical isolates. To specify the suitable antimicrobial treatment, phenotypic breakpoints recognized by Clinical and Laboratory Standards Institute (CLSI). The resistance rates to imipenem, meropenem, and ertapenem were 78%, 95%, and 97%, respectively in this study. Similar to previous study. [60] CRE strains could spread via physical contact with other people and have the propensity to acquire genetic materials mostly in the form of plasmids and transposons

through horizontal gene transfer.[68] *NDM-1* and its minor variants, a class B carbapenemase first clinically isolated from a patient at a hospital in New Delhi, India, has since been identified all over the world. [69] Among of them, *NDM-1*, a member of class B is the most common types of CRE found in Asia.[70] For these reasons, the present study was interested in developing the rapid method instead of conventional method (disc sensitivity) for detecting *NDM-1* in clinical field.

Molecular techniques are as well required for the fast, sensitive and accurate detection of carbapenemase genes. The standard methods for the detection of carbapenemases are established molecular techniques, PCR, such as conservative PCR with sequencing, multiplex PCRs and DNA hybridization, and others. The molecular techniques were detected of carbapenemase genes, have advantages more than culture-based methods, such as fast, sensitive, specific and recognized and identification of these important resistance genes.[61] Loop-mediated isothermal amplification (LAMP) is simple and field-adaptable method, these technique used widely for detect of epidemic bacteria, virus and parasites. [71] Currently, the Recombinase Polymerase Amplification (RPA) is now being evaluated as a diagnostic tool for a variety of pathogenic microorganisms including protozoa infection.[72] and other infectious diseases. RPA technique was carried out for only 20 minutes which was less than LAMP and PCR. However, the over protein in the reaction may interfere the detection can cause false positive. Therefore, RPA product were clean up before detection by agarose gel electrophoresis analysis was necessary. Amplification product could be achieved using nonfluorescence formats such as lateral flow assays or lateral flow strips.[73]

At present, reference methods for detection of $bla_{\text{NDM-1}}$ gene was based on molecular techniques due to their excellent sensitivity and specificity and robust performance. [74],[75] Basically, the conventional Polymerase Chain Reaction (PCR) is the employed as the reference molecular technique. Alternatively, LAMP has been introduced for the rapid and sensitive detection of $bla_{\text{NDM-1}}$ since it relied on isothermal amplification which does not require expensive thermocyclers. In addition, LAMP has

been shown to exhibit higher sensitivity for detecting *bla*$_{NDM-1}$ than conventional PCR. [76-78i]

To develop an effective screening test, the factors of timesaving and its convenience should be included. Among three DNA amplification assays, RPA is the most rapid when compares to PCR and LAMP. However, the assay is still costly due to the patented recombinase polymerase. In similarity to LAMP, RPA is the isothermal amplification that can be conducted at 37 °C for 20 minutes which is convenient to perform the reaction while LAMP can be completed at 60-65 °C for 60 minutes. Both LAMP and RPA assays does not require the complicated and expensive equipment since the only a simple heating box is necessary for LAMP and RPA amplification process while PCR is basically relied on expensive PCR Thermal cycler.

Concerning limit of detection (LOD) of PCR, LAMP and RPA assays, the 0.74 ng/ µl, 7.4 pg/ µl and 0.74 ng/ µl respectively were observed. It is noted that LAMP showed more sensitive than PCR and RPA about 100 times. (Table 7) Hence, it is appropriate for the use as frist detection test for CRE strains containing *bla*$_{NDM-1}$ gene. Furthermore, all DNA amplification assays (PCR, LAMP, and RPA) showed no cross amplification to a target isolate and non-target other 15 related bacterial isolates, indicating that all assay primer are specific for the *bla*$_{NDM-1}$ gene. Moreover, 60 isolates from clinical samples tested carbapenem-resistance *Enterobacteriaceae*, with 30 isolates detected as negative and 30 isolates detected as positive *bla*$_{NDM-1}$ gene on PCR, LAMP, and RPA with specific primer for *bla*$_{NDM-1}$ gene and compared with universal primer for standard method (PCR) from EUCAST as shown in (Table 8).

Table 7 Comparison of methods in this study.

Methods	Limited of detection	Cross-hybridization
PCR-Gel electrophoresis	0.74 ng/ µl	No
PCR-LFD	0.74 ng/ µl	No
LAMP-Gel electrophoresis	7.4 pg/ µl	No
LAMP-LFD	7.4 pg/ µl	No
RPA-Gel electrophoresis	0.74 ng/ µl	No
RPA-LFD	0.74 ng/ µl	No

Table 8 Validation clinical samples are examined using to detect the bla_{NDM-1} gene.

DNA amplification methods	No. Positive bla_{NDM-1}/ number of total sample	No. Negative bla_{NDM-1}/ number of total sample	Sensitivity (%)	Specificity (%)	Accuracy (%)	Process time (minutes)
Culture	30/ 30	0/ 30	100	100	100	24 h
PCR primer standard	30/ 30	0/ 30	100	100	100	90
PCR-LFD	30/ 30	0/ 30	100	100	100	100
LAMP-LFD	30/ 30	0/ 30	100	100	100	70
RPA-LFD	30/ 30	0/ 30	100	100	100	30

Conclusion: The LAMP-LFD has been developed for the detection of *NDM-1* with efficient, rapid and easy to implement in the routine. The highly sensitive and early screening detection can help the physician to choose the appropriate antibiotic to treat the infection by CRE bacteria and can decrease morbidity and mortality rates.

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APPENDIX

Materials

100 bp DNA marker	Biolabs
100 bp DNA marker plus	Biolabs
Agarose	Vivantis
Betaine	Sigma
Bst DNA polymerase	Biolabs
Boric acid	Bio Basic Canada Inc.
Deoxynucleotide triphosphate (dNTP)	Biolabs
Ethylenediaminetetraacetic acid (EDTA)	Bio Basic Canada Inc.
GenepHlow™ Gel/ PCR Kit	Geneaid
Hydrochloric (HCl)	Mark
LFD strip kit	Milenia biotec
Magnesium sulfate ($MgSO_4$)	Biolabs
Magnesium chloride ($MgCl_2$)	Vivantis
PrestoTM Mini Plasmid Kit	Geneaid
Sodium chloride (NaCl)	Vivantis
Taq DNA polymerase	Invitrogen®
Taq *Pfu* polymerase	Promega
Tris-HCl	Amaesco®

Material

TwistAmp® Basic Kit Quick Guide Intabas

1. Buffer preparation

1.1 10X Tris Borate EDTA (TBE) buffer (pH8)

Tris-HCl	108	g
Boric acid	55	g
EDTA	9.5	g

Dissolve the following reagents in 900 ml of distilled water. Then the solution was adjusted to pH8 with HCl. Bring volume to 1 liter, autoclave or sterilize by filtration.

1.2 0.5X Tris Borate EDTA (TBE) buffer (pH8)

10X TBE	50	ml
dH_2O	950	ml

The 0.5X TBE buffer was prepared by adding 0.5 volume of 10X TBE buffer to 9.5 volume of sterilized distilled water.

Table 1 Paper submission

No.	Original Article	Status
1	Comparative Study on DNA Amplification Methods for Detection of Carbapenem-Resistant Enterobateriaceae (CRE)	Accepted 17 June 2021, J Med Assoc Thai 2021; 104(8): 1-8

Table 2 Patent of primer and DNA probe for detection of *NDM-1*

No.	Patent	Status
1	Primers and DNA probe for detection of *NDM-1* using Loop-mediated isothermal amplification combined with lateral flow dipstick	Patent submission number: 2103000755 Since March 12, 2021

VITA

NAME	Mr. Supparit Pakdeethai
DATE OF BIRTH	7 May 1987
PLACE OF BIRTH	Suratthani, Thailand
INSTITUTIONS ATTENDED	2009: B.Sc. (Biomedical Science), Rangsit University 2012: M.Sc. Medical Science (Pathology), Chulalongkorn University 2021: Ph.D. (Molecular Biology), Srinakharinwirot University
HOME ADDRESS	88/130 The Trust Ville, Lat Sawai, Lam Luk Ka, Pathum Thani: 12150