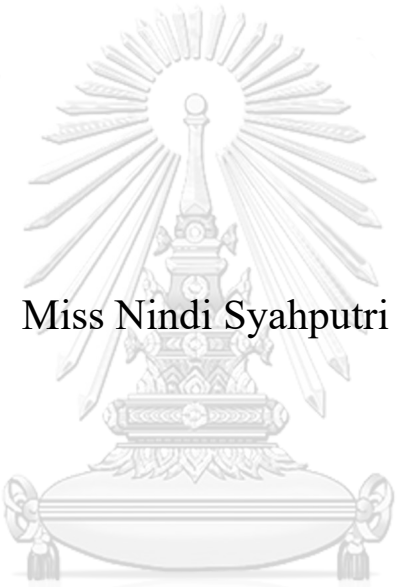
Development of duplex loop-mediated isothermal amplification (dLAMP) for detection of carbapenem antibiotic-resistant genes *KPC* and *NDM*

Miss Nindi Syahputri Lubis



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การพัฒนาลูปเมดิเอเตดไอโซเทอร์มอลแอมพลิฟิเคชันแบบคู่ (dLAMP) สาหรับการตรวจหายีน KPC และ NDM ที่ดื้อต่อยาคาร์บาเพเนม

น.ส.นินดิ ซยาปูตริ ลูบิส

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	Development of duplex loop-mediated isothermal amplification (dLAMP) for detection of carbapenem antibiotic-resistant genes *KPC* and *NDM*
By	Miss Nindi Syahputri Lubis
Field of Study	Microbiology and Microbial Technology
Thesis Advisor	Associate Professor NARAPORN SOMBOONNA, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

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นินดิ ซยาปูตริ ลูบิส : การพัฒนาลูปเมดิเอเตดไอโซเทอร์มอลแอมพลิฟิเคชันแบบคู่ (dLAMP) สาหรับการตรวจหายีน KPC และ NDM ที่ดื้อต่อยาคาร์บาเพเนม. (Development of duplex loop-mediated isothermal amplification (dLAMP) for detection of carbapenem antibiotic-resistant genes *KPC* and *NDM*) อ.ที่ปรึกษาหลัก : นราพร สมบูรณ์นะ

การใช้ยาปฏิชีวนะอย่าง Carbapenems ถือเป็นทางเลือกสุดท้ายในการรักษาการติดเชื้อจากแบคทีเรียทั้งแกรมบวกและแกรมลบ ในปัจจุบันพบยีนที่ต้านการใช้ Carbapenems เพิ่มขึ้นในผู้ป่วย พบว่ามีหลายปัจจัยที่เกี่ยวข้อง เช่น การพักรักษาที่โรงพยาบาลเป็นเวลานาน, มีประวัติการใช้ยาปฏิชีวนะ, การรักษาด้วยยาปฏิชีวนะที่ไม่เหมาะสมหรือไม่เพียงพอ, และการปนเปื้อนจากการผ่าตัดหรือบาดแผลจากการผ่าตัด ในปี 2005 carbapenem-resistant genes (CR genes) ได้รับความสนใจเป็นอย่างมาก เนื่องจากยีนเหล่านี้มี plasmid หรือ transposons ที่สามารถถูกแพร่ไปยังเชื้อแบคทีเรียอื่นๆได้ *Klebsiella pneumoniae carbapenemase* (*KPC*) and *New delhi metallo-β-lactamase* (*NDM*) เป็นยีนในกลุ่ม CR ที่พบมากที่สุดทั้งในประเทศแถบเอเชียตะวันออกเฉียงใต้และทั่วโลก ยีนเหล่านี้มี 123 และ 43 ชนิดย่อย สำหรับ *KPC* และ *NDM* ตามลำดับ ดังนั้น ในงานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาเทคนิคลูปเมดิเอเตดไอโซเทอร์มอลแบบยีนเดียว (LAMP) และสองยีน (dLAMP) ร่วมกับสีย้อม HNB ที่มีความจำเพาะต่อยีน *KPC* และ *NDM* รวมถึงชนิดย่อยๆของสองยีน ที่มีการแพร่กระจายไปทั่วโลกมากที่สุดในกลุ่ม CR การตรวจจับสำหรับยีนเดียวสามารถทำได้ที่สภาวะที่เหมาะสม นั่นคือ 65 องศาเซลเซียส เป็นเวลา 55 นาที และเมื่อใช้ร่วมกับสีย้อม HNB สังเกตการเปลี่ยนแปลงของสีจากสีม่วง (ผลลบ) เป็นสีฟ้า (ผลบวก) ด้วยความเข้มข้นของ $MgSO_4$ และ HNB ที่เหมาะสม นั่นคือ 6.5 มิลลิโมลาร์ และ 180 มิลลิโมลาร์ ตามลำดับ ซึ่งยืนยันผลด้วยการสังเกตการดูดกลืนของแสงที่ความยาวคลื่น 650 นาโนเมตร นอกจากนี้เมื่อตรวจจับด้วยเทคนิค PCR พบว่าไพรเมอร์ที่ออกแบบมีความจำเพาะต่อยีน *KPC* และ *NDM* และความไวของการทดสอบที่พัฒนาขึ้นในการตรวจจับทั้งสองยีนมีความไวสูงถึงสิบเท่าเมื่อเทียบกับ PCR สามารถอ่านผลได้ภายในเวลาไม่ถึงหนึ่งชั่วโมง

สาขาวิชา	จุลชีววิทยาและเทคโนโลยีจุลินทรีย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2566	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6478005223 : MAJOR MICROBIOLOGY AND MICROBIAL TECHNOLOGY
KEYWORD: Carbapenems, Carbapenem-resistant genes, Klebsiella pneumoniae Carbapenemase (KPC), New Delhi Metallo β-lactamase (NDM)

Nindi Syahputri Lubis : Development of duplex loop-mediated isothermal amplification (dLAMP) for detection of carbapenem antibiotic-resistant genes *KPC* and *NDM*. Advisor: Assoc. Prof. Dr. NARAPORN SOMBOONNA, Ph.D.

Carbapenems are regarded as a last-resort option for treating a wide range of Gram-positive and Gram-negative bacterial infections. Unfortunately, the prevalence of carbapenem-resistant genes has been on the rise among patients. Some factors contribute to this trend, including prolonged hospital stays, prior antibiotic usage, inappropriate or insufficient antibiotic treatment, and contamination through wounds or feces. The emergence of carbapenem-resistant (CR) genes gained significant attention, particularly after 2005. These CR genes are frequently carried on mobile genetic elements such as plasmids or transposons, enabling their transmission to other bacteria. *Klebsiella pneumoniae* carbapenemase (*KPC*) and *New delhi metallo-β-lactamase* (*NDM*) stand out as the most prevalent CR genes, both in Southeast Asian countries and worldwide. These genes exhibit numerous subtypes, with 123 and 43 subtypes for *KPC* and *NDM*, respectively. Hence, in this study, our focus was on *KPC* and *NDM* as the most widespread CR genes that have been responsible for numerous global outbreaks in recent times. We developed universal primers for *KPC* and *NDM* genes, aiming to detect the subtypes using loop-mediated isothermal amplification (LAMP) techniques. Additionally, we developed a duplex-LAMP assay which is capable of simultaneously detecting both genes in a single reaction. As a result, the developed dLAMP can detect *KPC* and *NDM* in a single reaction using an optimum temperature and incubation time of 55 minutes at 65°C temperature. For visualization, using hydroxyl naphthol blue (HNB) which changed from violet (negative) to blue (positive). With optimum $MgSO_4$ and HNB concentration, 6.5 mM, and 180 mM, showed the highest absorbance at 650 nm. The developed universal primers *KPC* and *NDM* proved to be specific only for detecting both genes using PCR. Furthermore, the sensitivity of developed dLAMP in the detection of both genes was ten times higher compared to traditional PCR with approximately 1 hour to determine the positive results of dLAMP.

Field of Study:	Microbiology and Microbial Technology	Student's Signature .....
Academic Year:	2023	Advisor's Signature .....

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Nindi Syahputri Lubis

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CR	Carbapenem-Resistant
CR genes	Carbapenem-Resistant Genes
KPC	*Klebsiella pneumoniae* Carbapenemase
NDM	New delhi metallo-β-lactamase
LAMP	Loop-mediated Isothermal Amplification
dLAMP	Duplex Loop-mediated Isothermal Amplification
HNB	Hydroxyl Naphthol Blue
PCR	Polymerase Chain Reaction
$MgSO_4$	Magnesium Sulfate
HNB	Hydroxyl Naphthol Blue
UV-Vis	Ultraviolet-Visible (spectrophotometry)
mm	Millimeter
mM	Millimolar
°C:	Degrees Celsius
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
dNTPs	Deoxynucleotide Triphosphates

CHAPTER I
INTRODUCTION

1.1 Background

Antibiotic consumption and usage are increasing annually, provided that unnecessary consumption promotes the increase of antibiotic resistance (Brink, 2019; Browne et al., 2021; WHO, 2019). Carbapenems are derived from beta-lactam antibiotics. This antibiotic is highly effective against aerobic-anaerobic, Gram-positive (GPB), and Gram-negative bacteria (GNB) yet is respected as the last antibiotic treatment choice for severe infections (Falagas et al., 2014). Carbapenems are primarily resistant to β-lactamase hydrolysis, which shows as a "slow substrate" or act as β-lactamase inhibitors yet still target the penicillin-binding proteins (PBPs) of the bacterial cell wall (K. M. Papp-Wallace et al., 2011). Additionally, Carbapenem has a unique molecular structure of beta-lactam ring that provides high stability against β-lactamase, such as ampicillin, carbenicillin (*AmpC*), and extended-spectrum beta-lactamase (Meletis, 2016). The emergence of carbapenem-resistant (CR) genes has been reported, especially after 2005. The reports were particularly on GNB because of the ability of the outer layer structure of GNB pathogens to sense and repair yet protect their cells from being damaged by antibiotic exposure.

Moreover, these CR genes are commonly carried on mobile genetic elements like plasmid or transposon that can be transmitted to other bacteria (Breijyeh et al., 2020; Miller, 2016). For instance, a Carbapenem-resistant Enterobacteriaceae (CRE) outbreak in ICU patients in the USA cost approximately $275 million, with a 25% attributable mortality in hospitals with a loss of 8841 lives and severe ill combined (Bartsch et al., 2017). General factors that promote CR genes include prolonged hospitalization, prior antibiotic use, inappropriate or inadequate antibiotic therapy,

and contact or object contamination with wounds or stools (Meletis, 2016). Note that the primary factors confer public pressure on CR gene evolution and spread, and the latter factor confers contact with CR gene source bacteria.

Effective mechanisms for CR include enzyme production, efflux pumps, and porin mutations. *Carbapenemase* is the enzyme produced by the pathogen after acquiring CR genes. This enzyme can hydrolyze Carbapenem antibiotics by breaking the β-lactam ring that makes Carbapenem antibiotics lose the ability to degrade the PBP of the bacteria cell wall. The efflux pump is a transporter that allows the bacteria to pump out the Carbapenem antibiotics from their cellular environment. The porin mutation, such as diminished porins, prevents the antibiotic from reaching the cellular environment. Other mechanisms include producing low-affinity PBPs (Armstrong et al., 2021). A high correlation between CR and multidrug (e.g., imipenem) resistance was also reported (Micek et al., 2015; Sader et al., 2019). The reason could be the assembly of resistance genes accumulation on a single plasmid that can be transmitted to other bacteria (Hiroshi Nikaido, 2009). To date, the three most prevalent CR genes that have been reported worldwide distribution are *Klebsiella pneumoniae* carbapenemase (*KPC*), New delhi metallo-β-lactamase (*NDM*), and oxacillinase (*OXA*) (Brink, 2019). Of these, *KPCs* represent the most found CR gene cases, mainly in China, Vietnam, Thailand, the United States, Italy, and most regions of South America, with *KPC-2* being the most common gene type (Hernández-García et al., 2022). *NDMs* have been reported to cause outbreaks globally, including in Thailand, China, Australia, European countries, and Middle East countries, with more cases in Southeast Asian countries (Bonomo et al., 2018). *OXAs* have been reported to spread sporadically in China, Australia, American

regions, and Middle Eastern countries, but currently, the outbreaks registered mainly in European countries and narrower compared to *NDMs* and *KPCs* that show caused outbreaks and sporadically spread worldwide, particularly in Asian countries (Brink, 2019). Thus, we choose *KPCs* and *NDMs* in this research as the most prevalent CR genes that the spread and outbreaks happen worldwide. These three genes are known for their high genetic mobility (i.e., in plasmid). A single mutation can cause resistance to the Carbapenem antibiotic; for example, porin mutation, which can be lost or diminished, makes the antibiotics cannot enter the cell environment or production of Carbapenemase that can hydrolyze the carbapenem antibiotic (Armstrong et al., 2021; Bojer et al., 2012). The high mortality rates in CR cases in hospitals are due to the difficulty and rapidness of accurately detecting CR genes in patients to allow a successful choice of antibiotic therapy (Mangold et al., 2011).

Traditional CR gene detection will require bacterial culture such as the modified Hodge test (Bartolini et al., 2014), followed by the disc diffusion test (Sood, 2014). This method depends on appropriate bacterial culture media and conditions, which require labor and time, approximately 18-24 hours. Another CR assay is by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Ghebremedhin et al., 2016) and Carbapenem inhibition test (Kuchibiro et al., 2018). However, these methods are not possible for local application (requires expensive instrument) or still requires > 8 hours of assay time (Nordmann et al., 2011). For the carba NP test, the assay has disadvantages in the high cost of reference standard imipenem powder (> 317 USD for 100 mg), fresh preparation of reagents each time, and relatively poorer assay accuracy compared

with molecular genetic amplification detection methods like polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) (Nordmann & Poirel, 2019). However, PCR is not rapid and may not be appropriate for resource-restricted and local settings (Carmeli et al., 2010).

Therefore, this study aimed to develop LAMP and demonstrate its effectiveness in possible local uses, which include simple, rapid, low price and accurate (Nakano et al., 2015; Poirier et al., 2021; Solanki et al., 2013; Yamamoto et al., 2015). Local rapid CR gene detection assay would help to select appropriate antibiotics for patients, reducing mortality rates and limiting the spread of further antimicrobial resistance (Brogan & Mossialos, 2016). LAMP utilizes a unique strand displacing *Bst* DNA polymerase enzyme and specially designed self-loop primers to allow a copy of target DNA to amplify to billions of copies within 1 hour at a constant temperature. The final product can be detected by turbidity, agarose gel electrophoresis, or combined with specific dyes or probes for visual or fluorescent color detection (Notomi et al., 2000). This technique has been used successfully to detect a variety of viral, bacterial, and fungal pathogens (Fan et al., 2022; Khan et al., 2018; Osterdahl et al., 2020; Rohatensky et al., 2018). Additionally, multiplex LAMPs in a single reaction have been demonstrated since 2015: (Nyan & Swinson, 2015) identifying six viruses in blood plasma. This study thereby designed universal *KPC* and *NDM* primers for dual LAMP (dLAMP) assay, will find optimal reaction recipe and conditions (incubation temperature and time for the maximum limit of detection) and determine the limit of detection and specificity of the developed dLAMP assay in laboratory references and infected mock samples. As too many primers could affect the assay sensitivity and specificity (Liu et al., 2017), our

developed dLAMP preliminary focus on the prevalent CR genes in Thailand and Southeast Asian countries and hopes our assay will offer an effective rapid, and inexpensive detection for outbreaking CR genes in this region and supports local and resource-restricted setting diagnoses. Moreover, as our developed dLAMP targets the presence of CR genes, the assay is not limited to specific microbial species. It can detect samples of any type (e.g., clinical samples and medical devices).

1.2 Study objectives

The objectives of this study are mentioned as follows:

1. To develop a universal *KPC* and *NDM* carbapenem-resistant (CR) gene detection assay using a duplex loop-mediated isothermal amplification (dLAMP) technique.
2. To determine the limit of detection and specificity of the developed dLAMP assay compared to PCR assay.

1.3 Hypothesis

The hypotheses of this study are mentioned as follows:

1. The developed dLAMP technique can detect the universal *KPC* and *NDM* CR genes.
2. The limit of detection (LOD) and specificity of the developed dLAMP assay have a high sensitivity and specificity compared to PCR method.

CHAPTER II
LITERATURE REVIEW

2.1 Overview of antibiotic resistance and its global impact

2.1.1 Definition of antibiotic resistance and its mechanisms

Antibiotic resistance refers to the capability of microorganisms such as bacteria to withstand antibiotic actions that lead to reduced effectiveness or complete ineffectiveness in treating infections caused by these resistant strains (Davies & Davies, 2010). This phenomenon then poses a significant public health challenge as it shows the persistence and dissemination of challenging-to-treat conditions, resulting in increasing morbidity, mortality, and healthcare costs (Bush et al., 2011).

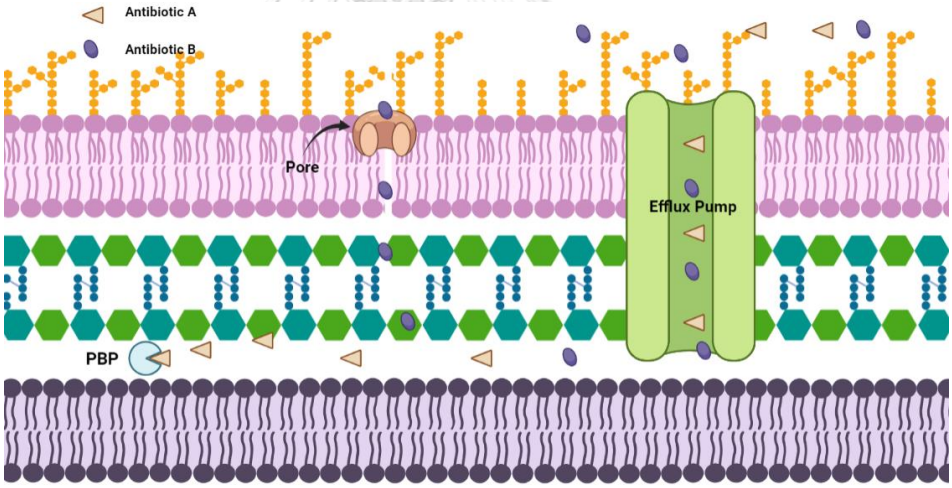

Figure 1 Intrinsic mechanisms of resistance

Antibiotic resistance in bacteria can occur intrinsically (Figure 1) to certain antibiotics and also can be acquired via mutations in chromosomal genes and horizontal gene transfer (Blair et al., 2015). The intrinsic resistance in bacteria to a specific antibiotic is its ability to resist the effect of that antibiotic as the result of inherent structural or functional traits. One of the intrinsic-resistance examples in bacteria such as the absence of a susceptible target of specific antibiotics which then

supports the bacteria to inhibit the antibiotic effect such as the production of enoyl-ACP reductase enzyme by *fabI* allele in *Pseudomonas* that can inhibit triclosan effect in their cell environment. Recent studies also led to the discovery of many genes that play a role in inherent resistance in bacteria to various antibiotics, such as β-lactams, fluoroquinolones, and aminoglycosides. Additionally, intrinsic resistance in bacteria can be acquired or developed to resistance to antibiotics, which is mediated by several mechanisms; first, minimize the intracellular concentrations of the antibiotic because of poor penetration into the bacterium or of antibiotic efflux (Fernandez & Hancock, 2012); second, modify the antibiotic target by genetic mutation of the target (H. Nikaido, 2009); third, inactivate the antibiotic by hydrolysis or modification (Wright, 2011).

2.1.2 Prevalence and consequences of antibiotic-resistant infections worldwide

The antibiotic-resistant infections have already been a challenge worldwide due to the increasing over and misuse of antibiotics in human medicine and agriculture which have contributed to the emergence and spread of antibiotic-resistant bacteria (McKernan et al., 2021). The World Health Organization (WHO) stated that antimicrobial resistance (AMR) is one of the top 10 global public health threats, which require urgent multisectoral action. The misuse and overuse of antimicrobials are the main factors in the increasing AMR cases. Additionally, lack of clean water, knowledge of sanitation, and inadequate infection prevention and control also promote the spread of microbes, some of which can be resistant to antimicrobial treatment (WHO, 2021). Thus, the prevalence of AMR transmission is urgently needed.

As of September 2017, AMR infections continue to be a significant global health issue with a rising prevalence worldwide (CDC, 2021; Coque et al., 2023). The prevalence of AMR varies across regions and countries, but it affects all parts of the world. As a global health concern, AMR is a major public health concern in both developed and developing countries. It affects people of all ages and can occur in various settings, including healthcare facilities, communities, and agricultural settings. In hospital and healthcare settings, AMR infections are a common problem (CDC, 2021). Healthcare-associated infections (HAI) are caused by multidrug-resistant organisms (MDROs) which can lead to increased morbidity, mortality, and healthcare costs (Al-Tawfiq & Tambyah, 2014).

Furthermore, the rise of multidrug-resistant organisms has severely limited the treatment options available to physicians, leading to a rise in treatment failures and a resurgence of once-controlled infectious diseases. The spread of these resistant strains transcends national borders, facilitated by international travel and trade, necessitating global cooperation and surveillance (Laxminarayan et al., 2013). Consequently, the AMR extends beyond healthcare settings, affecting agriculture, food safety, and the environment. The widespread use of antibiotics in agriculture contributes to the development of resistant strains, which can then spread to humans through the food chain (McKernan et al., 2021). Additionally, antibiotic residues in the environment can further foster resistance development.

The prevalence and consequences of AMR infections demand a multifaceted approach. This includes promoting prudent and responsible antibiotic use, implementing infection prevention and control measures, enhancing surveillance systems, fostering research and development of new antibiotics and alternative

treatments, and advocating for global collaboration to combat this urgent public health threat (O'Neill, 2016). In conclusion, the global prevalence of antibiotic-resistant infections and their consequences underscore the need for immediate and concerted action. Effective strategies are essential to preserve the efficacy of existing antibiotics and ensure that future generations can rely on these life-saving medications in the face of evolving microbial challenges.

2.1.3 The Economic and healthcare burden of antibiotic resistance

Nowadays, AMR infections have become a burden on the economy and healthcare because it is difficult to treat and have already contributed to the increase of morbidity and mortality, they are also simultaneously adding high costs to the health systems. On the other hand, some reports through death analysis that are associated with AMR show unstraightforward information (Cassini et al., 2019). The effects of AMR pathogens can be manifested in different AMR infections is the reason for this phenomenon. For example such as methicillin-resistant *Staphylococcus aureus* (MRSA) commonly causes infection on the skin, wound, pneumonia, and bloodstream infection (Garoy et al., 2019), though other pathogens harboring other AMR might cause the same infections. Due to this reason, the actual impact of resistant infections on public health has been unfocused and underestimated in the population (CDC, 2019b).

The attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015 showed the health burden of five types of antibiotic-resistant bacteria. The report in 2015 estimated 671.689 infections with antibiotic-resistant bacteria, with 63%

associated with health care. The estimation for attributable death is approximately 33.110 cases, which is highest in infants and people aged 65 years older which has been increasing since 2007 in Italy and Greece (Cassini et al., 2019).

The intergovernmental economic organization, OECD has been working on reports for AMR since 2015 collaborating with CDC. The OECD estimated about 60.000 deaths from resistant infection every year in the USA and Europe. By 2050, the OECD estimated the AMR will cause 2.4 deaths in the same countries (OECD, 2018). Additionally, the long-term public health impact of this increment of morbidity and mortality of AMR is the rise of antibiotic resistance jeopardizes our ability to control infectious diseases and manage common medical procedures like surgeries, chemotherapy, and organ transplantation, as they heavily rely on effective antibiotics to prevent or treat infections (CDC, 2019a).

AMR infections require more extensive and expensive treatments, including longer hospital stays, additional diagnostic tests, and the use of costly second-line or last-resort antibiotics, which contribute to the escalation of healthcare expenditure (O'Neill, 2014). Moreover, the productivity loss might be the consequence of longer periods of illness, reduced workforce productivity, and missed days of work or school, which leads to economic losses for individuals and business (CDC, 2019a).

2.2 Carbapenem antibiotics and their significance in treating infections

2.2.1 Introduction to carbapenems and their mode of action

Carbapenems are a class of broad-spectrum antibiotics that belong to the beta-lactam group (K. M. Papp-Wallace et al., 2011). The carbapenems consist of β-lactams antibiotics with a unique structure that makes it different from penicillin by

having a carbon atom that replaces Sulphur at position I and by the presence of unsaturated bond between carbon atoms 2 and 3 in the 5-membered ring (Figure 2) (Moellering & Sentochnik).

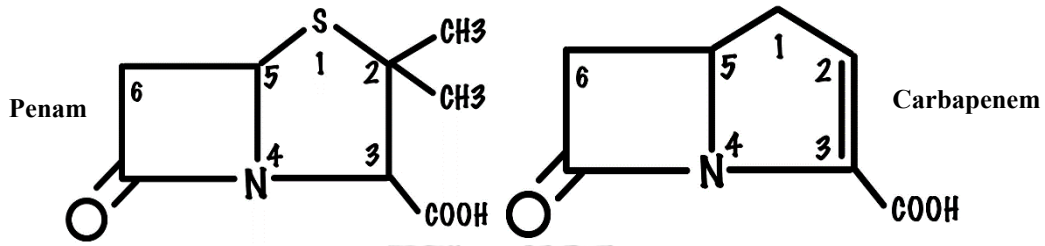

Figure 2 Chemical structure of Penam and Carbapenem

Among many types of carbapenem antibiotics, imipenem is the representative of carbapenem and is known to be able to its ability to penetrate the cell envelope of Gram-negative bacilli and its high affinity for certain penicillin-binding protein (PBP) targets (Kahan et al., 1983). Additionally, imipenem also inhibits the synthesis of bacterial cell walls which is a vital component for the structural integrity and survival of bacteria. Furthermore, this mechanism will disrupt the growth and replication of a diverse array of pathogens. If imipenem is combined with cilastatin, a compound that prevents the carbapenem antibiotic breakdown by renal enzymes, will allow for extended dosing intervals and improved therapeutic outcomes (Drawz & Bonomo, 2010; Livermore, 1995).

Carbapenem antibiotics including imipenem have mode of action that makes them highly effective against the wide range of bacterial infections. As they belong to the beta-lactam class of antibiotics, their mechanism of action involves interfering with the synthesis of bacterial cell walls. By targeting an essential component of

bacterial cell walls called peptidoglycan, carbapenem antibiotic will inhibit the activity of enzymes known as penicillin-binding protein (PBPs), which are responsible for cross-linking the peptidoglycan chains, a process vital for maintaining the structural integrity of bacterial cell walls, these whole mechanisms known as inhibition of the bacterial cell wall synthesis. By inhibiting the PBPs, carbapenem prevents the proper formation of peptidoglycan, leading to the weakening pressure changes and ultimately burst due to the inability to maintain cell wall integrity. Furthermore, carbapenem also exhibit bactericidal or killing activity against broad spectrum of bacteria, including Gram-positive and Gram-negative species. This characteristic makes them particularly effective against severe and life-threatening infections (Brown & Wright, 2016; Fisher et al., 2005; Wright, 2016).

2.2.2 Clinical importance and spectrum of activity of carbapenem antibiotics

Carbapenem antibiotics hold significant various clinical importance due to their broad-spectrum activity and effectiveness against various bacterial infections. They are often considered critical antibiotics for treating severe or life-threatening infections when other antibiotics have failed or when the precise infecting organism is unknown. As carbapenem antibiotics have a broad-spectrum activity, it makes them effective against a wide range of bacteria, including Gram-positive and negative organisms. This versatility is especially valuable when treating severe infections where the causative bacteria may not be identified immediately (Bassetti et al., 2018; Papp-wallace et al., 2011; Patel & Bonomo, 2011).

Carbapenems are often reserved for specific clinical situations, such as hospital-acquired infections, multi-drug resistant infections, or immunocompromised

patients, thus they are called as reserve antibiotics. Their use is crucial in combating infections caused by resistant bacterial strains. Furthermore, due to their potency and ability to penetrate various tissues, carbapenems are indispensable for treating life-threatening infections like sepsis, pneumonia, complicated urinary tract infections, and intra-abdominal infections (Papp-wallace et al., 2011; Tamma et al., 2017).

2.2.3 Challenges in using carbapenems due to the emergence of resistant strains

The emergence of resistance to carbapenem antibiotics poses significant challenges in the field of healthcare and antimicrobial therapy. Carbapenems, once considered reliable agents against a wide range of bacterial infections, are increasingly facing resistance, limiting their effectiveness. The high mortality rates caused by carbapenem resistant bacteria are associated with higher mortality rates compared to infections caused by susceptible strains. Patients with limited treatment options due to carbapenem resistance face a greater risk of poor outcomes (Tzouvelekis et al., 2012). The limited treatment options are another challenge. Since carbapenem-resistant bacteria often exhibit resistance to multiple classes of antibiotics, leaving healthcare providers with limited or no effective treatment options. This can lead to the use of less effective or more toxic antibiotics (Patrice Nordmann et al., 2012).

Carbapenem-resistant bacteria are frequently associated with healthcare-associate infections, including those acquired in hospitals and long-term care facilities. These infections are often challenging to control and contain within healthcare settings (De Oliveira et al., 2020). Furthermore, the carbapenem-resistant bacteria can rapidly spread across geographic regions, creating a global health threat.

The international dissemination of resistant strains make containment and control efforts more complex (van Duin & Doi, 2018). Additionally, the overuse and misuse of carbapenem antibiotics in healthcare settings can contribute to the development and spread of resistance. Inappropriate prescribing practices can exert selective pressure on bacterial populations (Boucher et al., 2009). Finally, the carbapenem resistance genes can be transmitted between different species of bacteria through horizonal gene transfer mechanisms. This facilitates the rapid dissemination of resistance (Walsh & Toleman, 2012).

2.3 Carbapenem-resistant bacteria and the role of *KPC* and *NDM* genes

2.3.1 Overview of carbapenem-resistant bacteria and their clinical impact

Carbapenem-resistant bacteria represent a growing global health concern due to their ability to resist treatment with carbapenem antibiotics, which are often considered the last line of defense against drug-resistant infections. These bacteria pose a substantial clinical impact by limiting treatment options and increasing the risk of healthcare-associated infections.

Carbapenem-resistant bacteria are microorganisms that have acquired mechanisms to resist the action of carbapenem antibiotics, which are a class of beta-lactam antibiotics known for their broad-spectrum activity. These bacteria can exhibit resistance through various mechanisms, including the production of Carbapenemases (enzymes that degrade carbapenems), efflux pumps, and alteration in cell wall permeability. Carbapenem resistance has been observed in a wide range of bacterial pathogens, including Enterobacteriaceae (e.g., *K. pneumoniae, Escherichia coli) Pseudomonas aeruginosa,* and *Acinetobacter baumannii.*

The limited treatment options are one of the clinical impacts of carbapenem resistant emergence. Carbapenems are often considered as last-resort antibiotics, and the emergence of resistance reduces the effectiveness of these critical drugs. This limitation in treatment options can lead to prolonged and more complicated infections (Pitout et al., 2015). Furthermore, the infections caused by carbapenem resistant bacteria are associated with higher mortality rates compared to infections caused by susceptible strains. Delayed or inadequate treatment can contribute to poor patient outcomes (Tzouvelekis et al., 2014). Additionally, carbapenem-resistant bacteria are often linked to healthcare-associated infections, particularly in intensive care units and long-term care facilities. Their ability to persist in healthcare environments poses a significant challenge (Bassetti et al., 2019). The global spread of carbapenem-resistant carrying bacteria especially those carrying Carbapenemase genes have rapidly spread across countries and continents. This global dissemination complicates infection control efforts and surveillance (Davey et al., 2017). Finally, the emergence of carbapenem resistance underscores the importance of antibiotic stewardship programs to optimize antibiotic use, prevent the development of resistance, and preserve the effectiveness of existing antibiotics.

2.3.2 Mechanisms of carbapenem resistance, with a focus on *KPC* and *NDM* genes

Carbapenem resistance can occur through various mechanisms, but two prominent mechanisms involve the presence of Carbapenemase genes, specifically *KPC* and *NDM*. These genes encode enzymes that can hydrolyze carbapenem antibiotics, rendering them ineffective. The *KPC* or *K. pneumoniae* Carbapenemase is a class A Carbapenemase that confers resistance to carbapenem antibiotics, such as

imipenem and meropenem. *KPC* enzymes hydrolyze the beta-lactam ring of carbapenems then makes them inactivated. The *KPC*-producing bacteria, particularly *K. pneumoniae*, are associated with healthcare-associated infections and have spread globally, posing a significant clinical challenge (Yigit et al.). The *NDM* or New Delhi Metallo-beta-lactamase is a class B metallo-beta-lactamase that confers resistance to carbapenem antibiotics by binding and hydrolyzing them. *NDM* enzymes require metal ions (usually zinc) for their catalytic activity. *NDM*-producing bacteria have been identified in various species, including *Enterobacteriaceae* and *Pseudomonas aeruginosa,* and are associated with healthcare-associated infections and they have spread globally and are challenging to treat (Yigit et al.).

Besides Carbapenemase production, carbapenem resistance can also arise from other mechanisms including efflux pumps, porin loss or alterations, AmpC β-Lactamase, and mutation in penicillin-binding protein (PBPs) (Patrice Nordmann et al., 2012). Some bacteria may overexpress efflux pumps that actively remove carbapenems from the bacterial cell, reducing intracellular drug concentrations, others can modify or lose outer membrane porins, which serve as channels for antibiotic entry, and this will reduce carbapenem uptake intro the bacterial cell. Furthermore, certain bacteria produce AmpC beta-lactamases, which can hydrolyze carbapenems to varying degrees. The mutations in PBPs can reduce the affinity of carbapenems for their target sites in the bacterial cell wall.

2.3.3 Epidemiology and prevalence of *KPC*- and *NDM*-producing bacteria

KPC and *NDM* are two prominent Carbapenemase enzymes that confer resistance to carbapenem antibiotics. The epidemiology and prevalence of *KPC*- and

NDM-producing bacteria have been a growing concern in recent years due to their global spread. The *KPC*-producing bacteria were initially identified in the United States in the early 2000s, primarily in *K. pneumoniae* strains. They have spread worldwide with significant outbreaks reported in various countries. *KPC*-producing bacteria are frequently associated with healthcare-associated infections, particularly in intensive care units and long-term facilities. Patients with prolonged hospitalization, exposure to broad-spectrum antibiotics, and invasive medical procedures are at higher risk of this resistant bacteria (Patrice Nordmann et al., 2012). The prevalence of *KPC*-producing bacteria varies by region. In some areas, the prevalence remains relatively low, while in others, it has become a significant concern. Surveillance and monitoring programs are essential to track the prevalence and spread of *KPC*-producing strains.

NDM-producing bacteria were first identified in New Delhi, India, in 2008. They belong to the class of Metallo-beta-lactamases and have rapidly spread globally. These bacteria have been found in both healthcare and community settings, making them a versatile and concerning threat (Walsh et al., 2011). The presence of *NDM* genes on mobile genetic elements facilitates their rapid dissemination. The prevalence of *NDM*-producing bacteria varies by region and as well influenced by factors such as antimicrobial use, infection control practices, and local epidemiology. These bacteria often coexist with other resistance mechanisms, making treatment challenging (Patel & Bonomo, 2011).

Both *KPC* and *NDM*-producing bacteria are associated with multidrug-resistant phenotypes, limiting treatment options, and increasing the risk of healthcare-associated infections. the global spread of these resistance mechanisms highlights the

importance of international collaboration in surveillance, infection control, and antimicrobial stewardship efforts to mitigate their impact (Patel & Bonomo, 2011).

2.4 Current diagnostic methods for detecting carbapenem-resistant genes

2.4.1 Conventional phenotypic methods for carbapenem resistance detection

Conventional phenotypic methods for detecting carbapenem resistance in bacteria involve various laboratory techniques that assess a bacterium's ability to resist the action of carbapenem antibiotics. These methods are essential for identifying resistance patterns in clinical isolates. The conventional phenotyping methods such as:

a. Disk diffusion method

This method involves testing the susceptibility of bacteria to carbapenem by placing antibiotic disks containing carbapenem drugs (e.g., imipenem or meropenem) onto an agar plate inoculated with the bacterial isolate. Zones or inhibition are measured to determine resistance or susceptibility (Fr, 2010).

b. Broth microdilution method

In this method, a series of twofold dilutions of carbapenem antibiotics are prepared in a liquid growth medium. Bacterial isolates are then exposed to these dilutions, and the minimum inhibitory concentration (MIC) is determined as the lowest concentration of antibiotic that inhibits visible growth (Fr, 2010).

c. Etest method

The Etest involves a plastic strip impregnated with a gradient of antibiotic concentrations. This strip is placed on an agar plate inoculated with

the test bacteria, and the intersection point of growth inhibition with the strip is used to determine the MIC (Kulengowski et al., 2019).

d. Modified hodge test (MHT)

The MHT is a qualitative test used to detect the production of Carbapenemases by Enterobacteriaceae. A carbapenem-susceptible *E. coli* strain is streaked across a streak of the test organism on agar plate, and the growth pattern is observed for an "enhanced" cloverleaf appearance (Fr, 2010).

e. Carba NP test

This test detects Carbapenemase activity by monitoring the hydrolysis of imipenem in the presence of a bacterial isolate. A color change due to the pH increase is indicative of Carbapenemase production (Patrice Nordmann et al., 2012).

2.4.2 Molecular techniques, such as PCR and real-time PCR, for identifying *KPC* and *NDM* genes

Molecular techniques like PCR (Polymerase-chained reaction) and real-time PCR (qPCR) are widely used for the detection and identification of specific resistance genes, including *KPC* and *NDM* genes in bacteria. These techniques provide highly sensitive and specific methods for identifying the presence of these genes. PCR-based detection such as PCR will amplify specific DNA sequences. For detection of both *KPC* and *NDM*, primers targeting both gene are used to amplify and confirm its presence in bacterial DNA (Cuzon et al., 2010; P. Nordmann et al., 2012; Yong et al., 2009). The real-time PCR (qPCR) allows the real-time monitoring of DNA

amplification. It uses specific primers and fluorescent probes to quantify the amount of *KPC* and *NDM* DNA in a sample (Kitchel et al., 2009).

2.4.3 Limitations and challenges of current diagnostic approaches

While diagnostic approaches for the detection of carbapenem-resistant genes have advanced significantly, several limitations and challenges still exist in the current methods. These limitations can impact the accuracy, timeliness, and effectiveness of detecting carbapenem resistance genes in clinical and laboratory settings. These are some key limitation and challenges of the current diagnostic in detection of carbapenem resistant genes:

a) Limited specificity and sensitivity

Some molecular techniques may lack of specificity or sensitivity when detecting Carbapenemase genes, leading to false positive or false negative results (Ramirez et al., 2020).

b) Diverse resistance mechanisms

Carbapenem resistance can result from various mechanisms, inkling Carbapenemase production, porin loss, and efflux pump overexpression. Current diagnostic tests may not capture all mechanisms simultaneously (Patrice Nordmann et al., 2012).

c) Emerging resistance genes

New Carbapenemase genes continue to emerge, necessitating regular updates of diagnostic assays to include these variants (Kitchel et al., 2009).

d) Time-consuming methods

Traditional molecular methods can be time-consuming, delaying the reporting of resistant results (Kitchel et al., 2009).

e) Need for infrastructure and expertise

Advanced molecular techniques require specialized equipment and trained personnel, which may not be readily available on all healthcare settings (Patel & Bonomo, 2011).

f) Const and resource constraints

The cost associated with implementing and maintaining molecular diagnostic tests may be a barrier, particularly in resource-limited settings (Bassetti et al., 2013).

g) Antibiotic stewardship implications

Rapid molecular diagnostic may lead to overuse or misuse of antibiotics if results are not interpreted and acted upon judiciously (Davey et al.).

Addressing these limitations and challenges in carbapenem-resistant gene detection is critical for the effective management of antibiotic resistance. Continued research and development of diagnostic assays that improve sensitivity, specificity, and turnaround time are essential to combat the global threat of carbapenem-resistant bacteria. Thus, in this research we plan to use loop mediated isothermal amplification (LAMP) method in solving these problems in identification of CR genes, *KPC* and *NDM*.

2.5 Loop-mediated isothermal amplification (LAMP) as a molecular diagnostic tool

2.5.1 Introduction to LAMP and its principles of operation

Loop-mediated isothermal amplification (LAMP) is a powerful molecular biology technique used for the rapid and specific amplification of DNA under isothermal conditions. Developed in the late 1990s, LAMP has gained popularity due to its simplicity, speed, and versatility in applications such as molecular diagnostics and pathogen detection. LAMP is a nucleic acid amplification method that was invented by Dr. Notomi and his colleagues in 2000 (Notomi et al., 2000) (figure 3). LAMP is designed to efficiently amplify a target DNA sequence with high specificity under isothermal conditions, typically at a single, constant temperature (usually around 60-65°C). This isothermal nature eliminates the need for a thermal cycler, making LAMP an attractive option for point-of-care testing and field applications.

LAMP amplifies DNA through a strand displacement mechanism and involves the use of four to six primers that specifically target different regions of the target DNA sequence. The LAMP reaction typically includes the following components (Goto et al., 2009; Mori & Notomi, 2009):

1. Target DNA: the DNA sample containing the target sequence to be amplified.
2. Forward and Backward Inner primers (FIP and BIP): these primes initiate DNA synthesis from the target DNA and create a stem-loop structure.
3. Forward and backward outer primers (F3 and B3): these primers further extend the DNA synthesis and assist in the formation of the stem-loop structure.
4. Loop primers (LF and LB, optional): these primers accelerate the amplification process by targeting loop regions within the stem-loop structure.

5. DNA polymerase with strand displacement activity: A DNA polymerase enzyme capable of strand displacement, such as Bst polymerase, is used to initiate and extend DNA synthesis within the stem-loop structure.

LAMP offers several advantages, including high specificity, rapid amplification (typically within 30-60 minutes), robustness against inhibitors, and the ability to detect low copy numbers of target DNA (Wong et al., 2018).

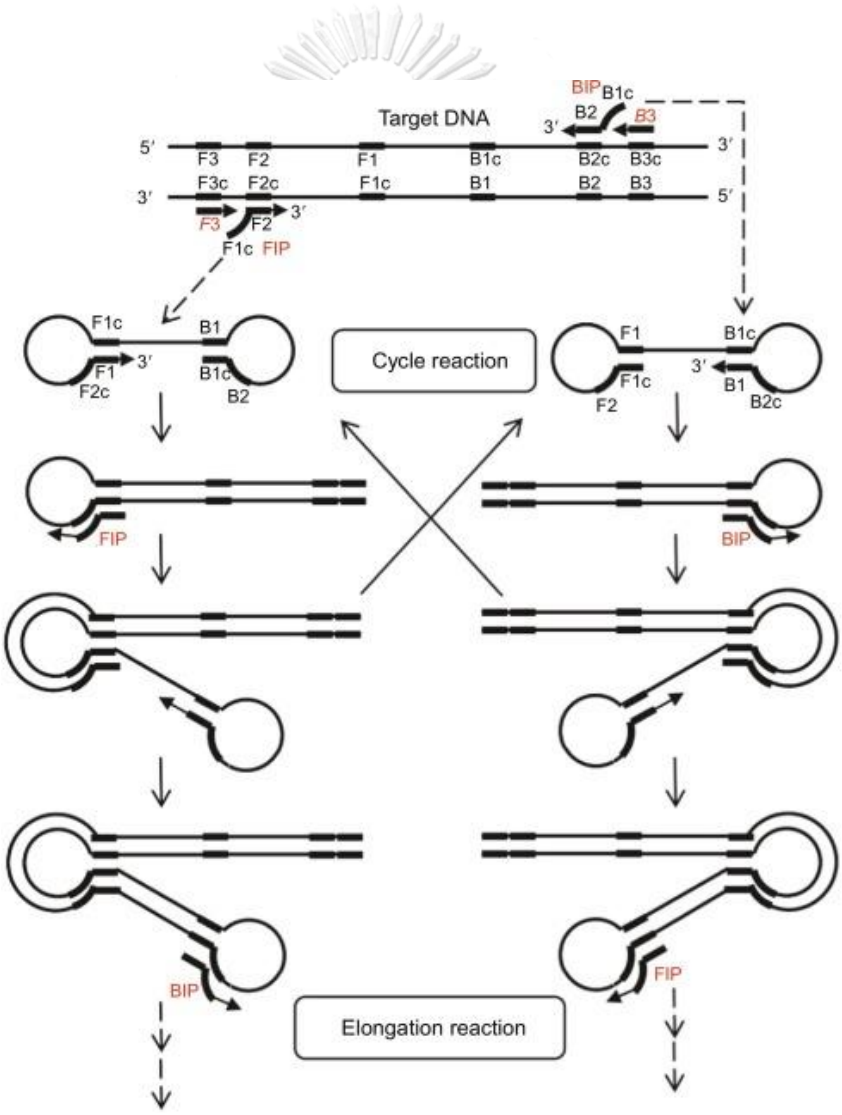

Figure 3 The schematic pathway of loop-mediated isothermal amplification (LAMP)

2.5.2 Advantages of LAMP over traditional PCR-based methods

Loop-mediated isothermal amplification (LAMP) offers several advantages over traditional PCR-based methods. These advantages have contributed to the growing popularity of LAMP in various applications, including molecular diagnostics, pathogen detection, and environmental testing. Here are some key advantages of LAMP over traditional PCR-based methods:

1) Isothermal amplification: LAMP operates at a constant temperature, typically around 60-65°C, eliminating the need for a thermal cycler. This simplify instrument requirement and reduces energy consumption (Notomi et al., 2000).
2) Speed: LAMP can amplify DNA rapidly, typically within 30-60 minutes, compared to traditional PCR, which requires multiple temperature cycling steps and may take several hours (Parida et al., 2008).
3) Simplicity: LAMP uses a set of four to six primers to target multiple regions of the DNA, simplifying primer design compared to traditional PCR, which often requires optimization (Mori & Notomi, 2009).
4) Robustness: LAMP is more tolerant of inhibitory substances and can be used with complex sample matrices, making it suitable for point-of-care and field application (Poon et al., 2004).
5) High specificity: LAMP's use of multiple primers targeting different regions enhances specificity, reducing the likelihood of non-specific amplification (Goto et al., 2009).

6) Visual detection: LAMP results can be visually assessed by turbidity or color change without the need for specialized equipment, enhancing its applicability in resource-limited settings (Tomita et al., 2008).
7) Less prone to contamination: LAMP reaction are less prone to contamination because they are performed in closed tubes, reducing the risk of false-positive results (Nagamine et al., 2002).

These advantages have made LAMP a valuable tool in various fields, including infectious disease diagnosis, food safety testing, and environmental monitoring, where speed, simplicity, and robustness are essential for reliable results.

2.5.3 Previous applications of LAMP in detecting antibiotic-resistant genes and its limitation

LAMP has been utilized in various studies to detect antibiotic-resistant genes, including *KPC* and *NDM* genes. Its speed, simplicity, and high specificity make LAMP a valuable tool for the rapid detection of these resistance genes. The detection of *KPC* and *NDM* genes using LAMP in clinical isolates of *K. pneumoniae* showed that the LAMP assay demonstrated high sensitivity and specificity (Iwamoto et al., 2003; Poirier et al., 2021). Not only *KPC* and *NDM* genes, LAMP also been known to be able to detect other resistant genes such as blaCTX-M genes in Extended-spectrum beta-lactamase (ESBL)-Producing bacteria (Parida et al., 2008), identification of mecA gene in Methicillin-Resistant Staphylococcus aureus (MRSA) (Iwamoto et al., 2003), detection of vanA and vanB genes in Vancomycin-resistant Enterococci (VRE) (Kim et al., 2014), and identification of 16S rRNA Methyltransferase genes in aminoglycoside-resistant bacteria (Wu et al., 2009).

While LAMP is a powerful tool for detection of various genetic targets, including antibiotic-resistant genes like *KPC* and *NDM*, it has some limitations. To design a specific and efficient LAMP primers for some target genes, especially with high sequence variability might be challenging (Goto et al., 2009), risk of false positive due to primer dimer formation or nonspecific amplification particularly in sample matrices (Nagamine et al., 2002), the need for proper positive and negative controls with may not always be readily available or feasible to use in all settings (Mori & Notomi, 2009), sensitivity to inhibitors which commonly found in clinical or environmental samples that might lead to false negative results (Nagamine et al., 2002), limited multiplexing especially when designing primers for multiple genes (Cheng et al., 2014), and complexity of interpretation that might be subjective particularly when relying on visual inspection of color changes that lead to variability in result interpretation (Tomita et al., 2008).

Thus, to overcome these limitations, it is crucial to carefully design LAMP assays, incorporate appropriate controls, and validate the result using complementary methods when necessary. LAMP's advantages in terms of speed, simplicity, and isothermal operation make it a valuable tool but understanding its limitations is essential for reliable and accurate molecular diagnostic. In terms of overcoming the limited multiplexing in LAMP, in this research we design specific primers for 123 subtypes of *KPC* and 43 types of *NDM* based on conserved region from multiple sequence alignment (MSA) of each DNA sequence. Thus, we plan to use dLAMP or duplex loop-mediated isothermal amplification (dLAMP) in detection of *KPCs* and *NDMs* genes.

2.6 Duplex LAMP (dLAMP) for simultaneous detection of multiple genes

2.6.1 Explanation of duplex LAMP and its capacity to detect two target genes

Duplex loop-mediated isothermal amplification (dLAMP) is an advanced application of the LAMP technique that enables the simultaneous detection of two target genes in a single reaction. This approach is particularly valuable in various molecular biology and diagnostic applications where it is necessary to assess the presence of multiple genetic targets concurrently. dLAMP involves the design of specific primers for two different target genes of interest. These primers are included in the same LAMP reaction mixture. Each set of primers is designed to recognize and amplify a unique target gene, enabling the simultaneous amplification and detection of both genes in a single reaction tube (Hong et al., 2023; Parida et al., 2005).

The key component of dLAMP include two sets of specific primers which one set of primers is designed for the first target genes, and another set is designed for the second target gene. Each set consists of four to six primers that recognize distinct regions within the respective target genes. isothermal amplification reaction is conducted at a constant temperature, typically around 60-65°C. Primers initiate DNA synthesis and create loop structures for each target gene, allowing for exponential amplification (Notomi et al., 2000). Detection of two target genes can be achieved through various means, including color changes, turbidity, or fluorescence, depending on the specific detection method used (Goto et al., 2009; Nyan & Swinson, 2015).

dLAMP offers several advantages when it comes to the simultaneous detection of two targets. It allows for the efficient and specific amplification of two different genetic targets within a single reaction tube. It simplifies the experimental setup by eliminating the need for running two separate reactions, reducing the time and

resources required. Conducting a single dLAMP reaction is often more cost-effective than running two separate reactions, moreover it saves time, making it suitable for applications where rapid detection of multiple genes is essential. Finally, dLAMP can conserve limited or precious sample material because it requires only one sample for testing (Gong et al., 2018; Jang, 2021; Jang et al., 2021; Jang et al., 2022; Kim et al., 2021; Osterdahl et al., 2020; Sattabongkot et al., 2014; Shao et al., 2011; Sharma et al., 2021; Sonaty, 2015; Tanner et al., 2012; Zhong et al., 2019).

2.6.2 Review of studies using dLAMP in various molecular diagnostic applications

The dLAMP technique allows the simultaneous detection and amplification of multiple target DNA sequences in a single reaction. This approach is valuable in various molecular diagnostic applications for the detection of multiple pathogens or genetic markers. The application of dLAMP has been applied for simultaneous detection of multiple pathogenic microorganisms, such as bacteria and virus in clinical samples (Li & Macdonald, 2015), to detect and differentiate between different serotypes of dengue virus in clinical samples (Parida et al., 2005), simultaneous detection of bacterial pathogen causing diarrhea and offering a rapid results (Phaneuf et al., 2018), for the detection of multiple genes in environmental samples including antibiotic resistance gens in wastewater (Miłobedzka et al., 2022), and detection of multiple plasmodium species (Selvarajah et al., 2020).

2.6.3 Potential benefits of using dLAMP for simultaneous detection of *KPC* and *NDM* genes

dLAMP has been developed as a technique for the simultaneous detection of two specific genes. and it is also potential in detection of *KPC* and *NDM* resistant

genes which may offer several potential benefits in molecular diagnostic. The concurrent detection of two specific resistance genes in a single reaction, providing a comprehensive assessment of antibiotic resistance (Yang et al., 2018) represent a simultaneous detection of multiple resistance genes. It also will give a rapid results because dLAMP typically offers faster results than traditional PCR-based methods, enabling quicker decision-making in clinical or epidemiological settings (Parida et al., 2008). This technique also offer high sensitivity and specificity which reducing the risk of false-positive or false-negative results (Mori & Notomi, 2009), additionally dLAMP can conserve resource as it requires only one reaction for the detection of multiple genes and minimizing reagent and sample consumption (Tomita et al., 2008). Other benefits such as dLAMP' s isothermal nature and simplicity make it suitable for field applications where access to sophisticated laboratory equipment is limited (Nagamine et al., 2002) and simultaneous detection of multiple resistance genes using dLAMP can enhance diagnostic accuracy for effective patient management and infection control (Goto et al., 2009).

2.7 Challenges and limitations of dLAMP in antibiotic-resistant gene detection

2.7.1 Factors affecting the specificity and sensitivity of dLAMP assays

The specificity and sensitivity of dLAMP assays can be influenced by several factors. The specificity of dLAMP assays depends on the design of primers for the target genes. Proper primer design, including sequence selection and optimization is critical. Well-design primers that efficiently anneal to the target sequences can enhance sensitivity (Li & Macdonald, 2015). The primer concentration in dLAMP reactions can affect specificity. An optimal primer concentration ensures specific

amplification and also contributes to sensitivity and preventing non-specific amplification (Goto et al., 2009). The reaction temperature in dLAMP should be carefully controlled to ensure specific amplification of target genes, and optimizing the reaction temperature can impact sensitivity by influencing the efficiency of DNA amplification (Mori et al., 2001).

Another factors that affect the specificity and sensitivity of dLAMP assay is primer cross-reactivity which can occur if primers have unintended interactions with non-target sequences, furthermore minimizing cross-reactivity improves sensitivity by reducing false-positive results (Parida et al., 2008). Proper sample preparation techniques such as DNA extraction can enhance specificity by reducing the risk of sample contaminants interfering with assay and efficient sample preparation methods can maximize DNA recovery and consequently the sensitivity (Cheng et al., 2014). The prolonged reaction times may increase the risk of non-specific amplification thus it is essential to optimize the reaction time, and adequate reaction times unsure sufficient amplification, contributing to sensitivity (Nagamine et al., 2002). Finally, the contamination from previously amplified product or environmental sources can compromise specificity thus to stringent contamination control measures is important and also equally vital for sensitivity, as it prevents the introduction of false-positive result (Mori & Notomi, 2009).

2.7.2 Comparison of dLAMP with other multiplex detection methods

In this study, we design 2 sets of LAMP primers using primer explorer ver. 5. The primers were designed based on MSA (multiple sequences alignment) of 123 subtypes of *KPCs* and 43 subtypes of *NDMs*. In one single reaction, two target DNA

will mixed along with reagents and water, then incubated around 30-60 minutes at 60-65°C without thermal cycler, this is the difference between dLAMP with other multiplex detection method like mPCR (Gong et al., 2018; Jang, 2021; Jang et al., 2021; Jang et al., 2022; Jang et al., 2020; Kim et al., 2021; Kim et al., 2019; Liang et al., 2012; Liu et al., 2017; Mahony et al., 2013; Moonga et al., 2020; Osterdahl et al., 2020; Sharma et al., 2021; Tanner et al., 2012; Wong et al., 2018; Zhong et al., 2019). Additionally, in this running time of incubation, there will no temperature adjustment like in the PCR that makes dLAMP assay is rapid and easy to be performed in detection of one or more than one DNA target, yet again we can obtained the results within one hour without using gel electrophoresis, instead we can add the HNB dye and see the color changing from violet to sky blue for positive result (Goto et al., 2009).

If compared with other multiplex detection methods lie microarray-based method, dLAMP typically more cost-effective and straightforward to implement which microarray may require complex sample preparation and expensive equipment (Sauer & Kliem, 2010). The next-generation sequencing (NGS) requires longer data analysis times and may be cost-prohibitive for routine diagnostics while dLAMP provides results in a shorter time frame (Mardis, 2008)o e. Lateral flow assays may require reader devices for quantification while dLAMP offers the possibility of visual detection without the need of expensive reader devices (Goto et al., 2009). Lastly, digital PCR (dPCR) requires specialized equipment and may have a longer turnaround time while dLAMP generally accessible and cost-effective (Hindson et al., 2011).

2.7.3 Strategies to overcome challenges and improve dLAMP performance

To overcome challenges and improve the performance of dLAMP assays, several strategies can be employed. These strategies aim to enhance specificity, sensitivity, speed, and reliability. The primer design and optimization by carefully optimize and design the primer sequences which are critical for dLAMP specificity and sensitivity (Mori & Notomi, 2009). The use of loop primers can enhance the efficiency and speed of dLAMP reactions, leading to improved sensitivity (Nagamine et al., 2002). Next, the optimization of reaction buffer including salt concentrations and pH can enhance dLAMP performance (Goto et al., 2009). The temperature optimization is essential for specific and efficient dLAMP amplification (Mori et al., 2001). The isothermal heating devices can ensure consistent and accurate temperature control during dLAMP reactions (Kaneko et al., 2007).

The contamination control by implementing strict control measures to prevent false-positive results in dLAMP assay is very important points (Mori et al., 2001). By multiplexing strategies is consider as optimizing primer sets and reaction conditions to expand dLAMP multiplexing capabilities for the simultaneous detection of more than two targets (Cheng et al., 2014). The implementation of visual detection methods, such as colorimetric or turbidity-based indicators, to simplify result interpretation in dLAMP (Goto et al., 2020). Optimize dLAMP for field applications by using portable, battery-operated devices is a strategy for field-friendly application (Mori & Notomi, 2009). Finally, validation and quality control should be in place to ensure the reliability of dLAMP results (Mardis, 2008).

2.8 The LAMP end point detection

The sLAMP and dLAMP product can be detected through various methods such as the addition of color dye like HNB (Goto et al., 2020), paper-based or strip (Choopara, Suea-Ngam, et al., 2021) and the standard method in detection of LAMP product, gel electrophoresis. These techniques provide different means to determine and confirm the presence of the amplified product, allowing for flexibility in experimental design and application.

The dLAMP product detection by using paper-based such as strip methods which offers a practical and visual interpretable means of confirming the presence of amplified DNA (Choopara, Teethaisong, et al., 2021). These techniques leverage the specificity of the dLAMP reaction to generate detectable signals that are then visualized on a paper strip. The strip typically contains components such as primers, enzymes, and indicators that undergo specific color changes in the presence of the target DNA. This vial readout simplified the interpretation of results, making it accessible even in resource-limited settings. On the other hand, gel electrophoresis is a traditional yet highly effective method for LAMP detection. In this technique, the amplified DNA is separated based on size and charge as it migrates through a gel matrix under the influence of an electric field. The resulting banding patterns on the gel electrophoresis is valuable for assessing the overall success of the LAMP reaction and confirming the specificity of the amplification (El-Kholy et al., 2014).

However, its important to note that gel electrophoresis requires specialized equipment, is time-consuming, and may not be as well-suited rapid, on-site diagnostics compared to strip or paper-based methods. The choice between these methods often depends on the specific requirements of the experiments, the available

resources, and the desired level of sensitivity and precision in LAMP product detection. Alternatively, we want to use the hydroxyl naphthol blue (HNB) addition in our reaction to detect the LAMP product by visualization or naked-eyes.

The selection of HNB as the color dye in LAMP detection hold significance. This choice is not arbitrary, rather, it is driven by specific characteristics that make HNB suitable for this application. Elaborating on the reasons behind choosing HNB involves discussing its sensitivity, specificity, and compatibility with the LAMP reaction. These considerations contribute to the overall efficacy and reliability of the detection method (Goto et al., 2009).

Finally, the detection of our developed dLAMP product, especially when employing HNB as a color dye, encompasses multiple dimensions, from method selection to the chemical intricacies of the chosen dye. Understanding and explaining these aspects are crucial for researchers and practitioners working in the field of molecular biology and diagnostics.

CHAPTER III
MATERIALS AND METHODS

3.1 Instruments

Autoclave: Kokusan, Shizouka, Japan

Hot air oven: Memmert, Munich, Germany

Vortex mixer: VM-10 DAIHAN Scientific, Seoul, Korea

Micro-centrifuge: Hettich, Massachusetts, USA

Laminar flow: BossTech, Hampshire, USA

UV-Cabinet: BossTech, Hampshire, USA

Nanodrop spectrophotometer: Nanodrop2000, Thermo Scientific, Northumberland, UK

Freezer 4°C MISUBISHI, Tokyo, Japan

Deep freezer -20°C: Haier, Bangkok, Thailand

Agarose Gel Electrophoresis System: GE-100, Hangzhou Bioer Technology CO., LTD. Hangzhou, China

UV transilluminator: HANGZHOU BIOER TECHNOLOGY CO., LTD.

Gel Documentation Bio-Rad, California, USA

Micropipette: Eppendorf North America, New York, USA

Dry bath incubator: Hangzhou Allsheng Instruments Co., LTD. China.

DNA Thermal Cycler: T100T^{TM} BIO-RAD, Bio-Rad laboratories LTD., Bangkok, Thailand

Balance: VALOR 7000, OHAUS Instruments (Shanghai) Co., LTD. Shanghai, China

3.2 Chemicals

Double distilled water

0.5×TBE buffer (Tris/Borate/EDTA)

1.0×TBE buffer (Tris/Borate/EDTA)

70% ethanol

Agarose powder: AMRESCO, Ohio, USA

Ethidium Bromide: AMRESCO, Ohio, USA

Novel Juice: GeneDireX, BIO-HELIX, New Taipei City, Taiwan

OneMark 100 RTU: BIO-HELIX, New Taipei City, Taiwan

Deoxynucleotide (dNTP) solution mix: New England Biolabs Ipswich, UK

Betaine solution: Sigma-aldrich, St. Louis,, USA

10×TherPol reaction buffer: New England Biolabs Ipswich, UK

Magnesium sulfate ($MgSO_4$): New England Biolabs Ipswich, UK

Bst DNA polymerase, Large fragment: New England Biolabs Ipswich, UK

Hydroxy naphthol blue (HNB): Fluka Analytical, Munich, Germany

3.3 Supplies

Microcentrifuge tubes: Bioline, massachusetts, USA

Micropipette: Labnet international, Inc., New Jersey, USA

Blade

Cuvettes

3.4 Kits

EmeraldAmp® GT PCR Master mix: TAKARA BIO INC., Shiga, Japan

PureDireX® Quick Gel extraction kit: Invintrogen, New York, USA

GF-1 Nucleic acid extraction kit: Vivantis, Malaysia

GF-1 AmbiClean Kit (Gel&PCR): Vivantis, Malaysia

3.5 Sample collections

Samples included positive controls, negative controls and laboratory references. The positive control strains were obtained from a previous study (Kerdsin et al., 2019; Takeuchi et al., 2022); The *Enterobacter asburiae* strain KU-C1235 1 (GenBank: JAJAIX010000001.1) and *E. coli* strain ECS01 (GenBank: NC_024954.1) both harboring *KPC* and *NDM* CR genes, respectively. The laboratory references, *K. pneumoniae* and *E. coli,* and negative strains were obtained from the previous study (Takeuchi et al., 2022).. All models will be extracted genomics using a DNA isolation

kit (Sangon, Shanghai, China) for reference samples or a DNeasy PowerSoil kit (Qiagen,Maryland, Germany) for clinical specimens.

3.6 Primer design for universal *KPC* and *NDM* dLAMP

123 DNA sequences of *KPC* and 43 sequences of *NDM* gene were downloaded from the NCBI GenBank database (www.ncbi.nlm.nih.gov/pathogens/refgene/#blaKPC and www.ncbi.nlm.nih.gov/pathogens/refgene/#blaNDM, respectively). Multiple sequence alignments by MUSCLE for each gene were performed using Mega11: Molecular Evolutionary Genetics Analysis software (Tamura et al., 2021). The conserved region for each type of primer (including outer primers F3 and B3, inner primers FIP and BIP, and loop primers LF and LB) was designed using Primer explorer version 5 software (http://primerexplorer.jp/lampv5e/index.html). BLASTN confirmed the specificity of every primer against the non-redundant database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and manual verification of multiple sequence alignments.

3.7 *KPC* and *NDM* PCR detection with specificity and sensitivity test

To confirm positive controls (*KPC* and NDM strains) in this study, we performed the PCR assay using F3 and B3 primers of both genes. The specificity and sensitivity of PCR was also conducted to confirm the limit of detection (LOD) and specificity of the designed universal primers of *KPC* and *NDM* in this study.

The PCR reaction in this study was using the F3 and B3 primers of each gene (10 µM each), 12.5 µL EmeraldAmp® GT PCR Master Mix (TakaRa Bio, Shiga,

Japan), and 50 ng DNA. The PCR reaction was conducted in two different tubes specifically for *KPC* and *NDM* genes. The cycling conditions were 95°C for 5 mins followed by 35 cycles of 94°C for 20 s, 61.4°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 mins. PCR product 200 bp, was determined by 2% gel electrophoresis.

In this step, we also conducted the PCR specificity using our developed universal LAMP primers (F3 and B3). The extracted DNA from twenty-one clinical bacterial obtained from the previous study (Takeuchi et al., 2022), was used as template for PCR specificity test. The ten-fold dilution of extracted DNA of both genes was used as the template in PCR sensitivity test.

3.8 The detection of *KPC* and *NDM* genes using Single LAMP (sLAMP)

The single LAMP reaction (15 µL total volume) comprises 0.2 µM each of F3 and B3 primers, 1.6 µM for FIP and BIP primers, 1.4 µM each of LF and LB primers, 1 × ThermoPol Buffer (New England BioLabs, Massachusetts, USA), 8 mM $MgSO_4$, 1.4 mM dNTP mix, 8 U Bst DNA polymerase (New England BioLabs), nuclease-free water, 0.5 M betaine and 50 ng DNA template (Choopara, Suea-Ngam, et al., 2021; Gong et al., 2018). The reaction was performed on a simple heat block. The LAMP product was analyzed by the 2% gel electrophoresis (Cambridge, UK). The appearance of band in the same temperature between both genes will be used as the estimation of the most suitable temperature and incubation time or the compromised condition of LAMP for both genes. This data then used to optimize dLAMP.

3.9 sLAMP optimization

The effectivity of sLAMP reaction was confirmed by the appearance of product band on the gel electrophoresis which then the intensity measured by ImageJ. The temperature and incubation times then ranged from 50-70°C and 30-55 minutes, respectively. The result then analyzed by two-way ANOVA to confirm the significance of different to obtained the optimum condition of sLAMP. The optimum condition of LAMP in detection of *KPC* and *NDM* is the basic understanding to find compromised condition when both genes used as templates in dLAMP reaction.

3.10 The detection of *KPC* and *NDM* using duplex LAMP (dLAMP)

The dLAMP reaction (25 µL total volume) comprises 10 µM of each F3 and B3, 40 µM of FIP, BIP, LF, and LB of both *KPC* and *NDM* primer sets, 1.4 mM dNTP mix, 12U Bst DNA polymerase, 5.5 mM of $MgSO_4$, 0.5 mM of betaine, 1x of isothermal buffer and distilled ddH_2O. The incubation was performed using simple heat block. The dLAMP product was determined by 2% agarose gel electrophoresis.

3.11 dLAMP optimization

To optimize the dLAMP reaction, we ranging the temperature based on the compromised temperature of both genes in single LAMP (63, 65, 67, 69 and 71°C), the incubation time (45, 50, 55, 60, 65, and 70 minutes), $MgSO_4$ concentration (3.5, 4.5, 5.5, 6.5, and 7.5 mM), and HNB (80, 120, and 160 µM) concentration, then the dLAMP product was determined by 2% agarose gel electrophoresis and the intensity value was analyzed by ImageJ software to compare the data then two-way ANOVA was performed to obtained the

significance difference between condition. Additionally, the specificity and sensitivity test were performed with reference positive and negative samples and using ten- fold serial dilution for sensitivity (Qin et al., 2021). The intensity was measured, and the intensity peak was plotted by using ImageJ software. Additionally, the dLAMP product was measured by using a spectrophotometer.

a. The intensity value of dLAMP using ImageJ software

To analyze the intensity value of gel electrophoresis of dLAMP product we use ImageJ software and conduct the gel plotting to compare the intensity value. The plotting results from ImageJ were analyzed using two-way ANOVA along with dLAMP product yield as the data measurement in dLAMP optimization.

b. dLAMP product yield using spectrophotometer

To obtain the dLAMP product concentration, we analyzed 3 microlite of dLAMP product using Nanodrop spectrophotometer. The data obtained was then collected and analyzed using two-way ANOVA along with intensity value as the data measurement in dLAMP optimization.

3.12 $MgSO_4$ and HNB concentration optimization

It has been demonstrated that the concentration of $MgSO_4$ has an impact on the color change of Hydroxy naphthol blue (HNB) in dLAMP. To visualize the positive outcomes in the dLAMP (Choopara, Suea-Ngam, et al., 2021; Goto et al., 2009), we conducted optimization of both $MgSO_4$ (3.5-7.5 mM) and HNB (80-160 μm) concentrations. This optimization aimed to achieve the most distinct color change from violet to sky-blue, indicative of a positive result. Additionally, the blue color

associated with positive results was quantified by measuring the highest absorbance at 650 nm using a Uv-Visible spectrophotometer. Given that $MgSO_4$ concentration is known to influence intensity and product formation in the LAMP reaction (Dadas et al., 2013), we extended our investigation to analyze the effects of varied $MgSO_4$ concentrations on both 2% gel electrophoresis intensity and dLAMP product yield. The product was assessed using Uv-Visible spectroscopy and confirmed through gel electrophoresis. subsequently, the intensity of the gel electrophoresis bands was analyzed using ImageJ software.

3.13 Sensitivity and specificity of dLAMP

The sensitivity of dLAMP was conducted by using the tenfold dilution of positive control extracted DNA as the template to determine the limit of detection (LOD) of dLAMP and compare it to PCR. The reaction was incubated at 65°C for 55 minutes with the addition of HNB and analyzed using 2% gel electrophoresis. Additionally, the specificity of dLAMP was performed by using OXA-48 genes as the negative template. The reaction was incubated at 65°C for 55 minutes. The positive result will be shown by the band appearance and the blue-sky color reaction in dLAMP.

3.14 Statistical analysis

The data obtained from the sLAMP and dLAMP optimization (intensity value, dLAMP yield, and UV-visible absorbance) in this study was then analyzed using one-way and two-way ANOVA using GraphPad Prism 9 software. The multiple comparisons using Tukey were then conducted to obtain the significant difference between factors. The p-value less or equal to (≤) 0.05 was then used as a significantly different value in this research.

CHAPTER IV
RESULTS

4.1 Targeted DNA sequences of *KPCs* and *NDMs* carbapenem-resistant (CR) genes

The multiple sequence alignment was conducted to align 123 subtypes of *KPC* and 43 subtypes of *NDM* carbapenem-resistant (CR) genes. The DNA sequences obtained from 100% conserved region covered all subtypes of *KPC* and *NDM* CR genes. As a results, 854 bp of *KPC* and 813 bp (Table 1) of *NDM* DNA sequences were obtained from the software. Then to confirm the specificity of the DNA sequences, checking using BLASTN was conducted.

Table 1 The universal DNA sequences of *KPC* and *NDM*

Genes	Targeted DNA sequences	Length
KPC	ATGTCACTGTATCGCCGTCTAGTTCTGCTGTCTTGTCTTCATGGCCGCTGG CTGGCTTTTCTGCCACCGCGCTGACCAACCTCGTCGCGGAACCATTCGCT AAACTCGAACAGGACTTTGGCGGCTCCATCGGTGTGTACGCGATGGATA CCGGCTCAGGCGCAACTGTAAGTTACCGCGCTGAGGAGCGCTTCCCACT GTGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCC AGCAGCAGGCCGGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGC GCTGGTTCGGTGGTCACCCATCTCGGAAAAATATCTGACAACAGGCATG ACGGTGGCGGAGCTGTCCGCGGCCGCCGTGCAATACAGTGATAACGCCG CCGCCAATTTGTTGCTGAAGGAGTTGGGCGGCCCGGCCGGGCTGACGGC CTTCATGCGCTCTATCGGCGATACCACGTTCCGTCTGGACCGCTGGGAGC CCGCCCCAGGCGATGCGCGCGATACCTCATCGCCGCGCGCCGTGACGGA AAGCTTACAAAAACTGACACTGGGCTCTGCACTGGCTGCGCCGCAGCGG CAGCAGTTTGTTGATTGGCTAAAGGGAAACACGACCGGCAACCACCGCA TCCGCGCGGCGGTGCCGGCAGACTGGGCAGTCGGAGACAAAACCGGAAC CTGCTATGCAAATGACTATGCCGTCGTCTGGCCCACTGGGCGCGCACCTA TTGTGTTGGCCGTCTACACCCGGGCGCCTAACAAGGATGACAAGTACAG CGAGGCCGTCATCGCCGCTGCGGCTAGACTCGCGCTCGAGGGATTGGGC GTCAACGGGCAGTAA	854
NDM	ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATT AGCCGCTGCATTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCG ACGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTGG TTTTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGAC ATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTCAGGGATGG CGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCC CAGATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGG CGGTGGTGACTCACGCGCATCAGGACAAGATGGGCGGTATGGACGCGCT GCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTG CCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGC CAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAG GTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGAT CGACGGCACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAG GCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCACTACGCCG CGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTG ATGAGCCATTCCGCCCCCAATAGCCGCGCCGCAATCACTCATACGGCCCG CATGGCCAACAAGCTGCGCTGA	813

4.2 Specificity check result of *KPCs* and *NDMs* DNA sequences

4.2.1 *KPCs* universal DNA sequences

The specificity checks of *KPC* DNA sequences using 5000 hit BLAST N from NCBI software showed that *KPC* DNA sequences showed 87.32% found in plasmid, 5.17% in chromosomes, and 7.51% clearly stated that it is *KPC* sequences that mostly found in 78.82% of *K. pneumoniae* among bacterial strains (Figure 4). It has also already been confirmed that every chromosome that listed harbored this DNA sequence are same sequence as the *KPC* DNA sequence.

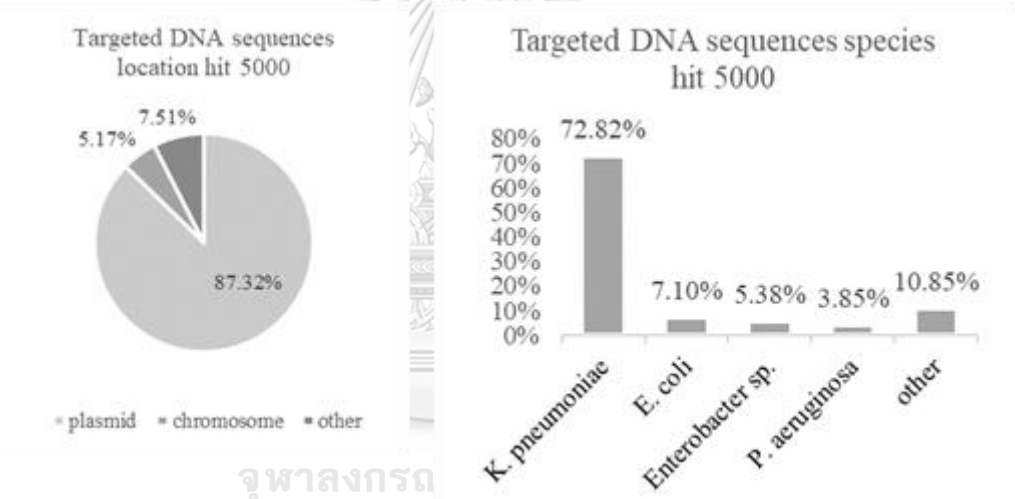

Figure 4 The specificity of the universal *KPC* sequence

4.2.2 *NDMs* universal DNA sequences

The specificity check result showed that 74.20% of *NDM* DNA sequence is harbored in the plasmid, 9.84% in the chromosome, and 15.96% clearly stated that it is listed as the DNA sequence of NDM in the NCBI database. *K. pneumoniae* is the bacterial strain that mostly might harbored and already listed in NCBI as shown as 31.92% among many species (Figure 5).

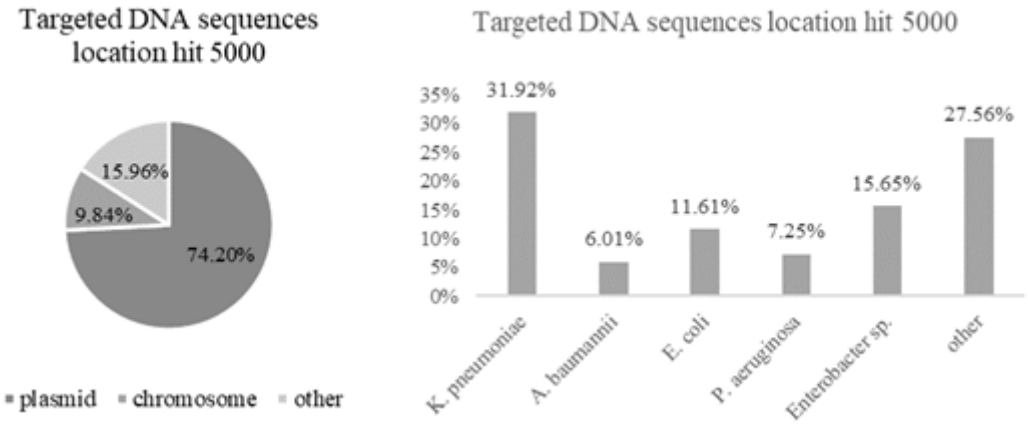

Figure 5 The specificity of the universal NDM sequence

4.3 Universal LAMP primers for *KPC* and *NDM*

The specific DNA sequences of *KPC* and *NDM* are then used as sequence templates to design LAMP primers consisting of outer primers (F3 and B3), inner primers (FIP and BIP), and two more optional loop primers (LF and LB). In total, we designed 2 sets of primers for *KPC* and *NDM* CR genes (Table 2).

After we designed the universal primers for *KPC* and *NDM*, we conducted a specificity test using hit 5000 BLAST N from NCBI software and the result showed similar results to the DNA sequences specificity check (Figure 6). Both sets of the primers (*KPC* and *NDM*) will bind to the DNA sequences or templates that are harbored or found in plasmids with *K. pneumoniae* (*KPC*) and *E. coli* (*NDM*) will be the most common species that might harbor this DNA sequence that represents as CR gene DNA sequences (Figure 4.3).

Table 2 The list of LAMP primer sets of KPC and NDM

Genes	Primers	Sequences
KPC	F3	TGGCTTTTCTGCCACCG
	B3	TGCGAGCCAGCACAGC
	FIP	TACACACCGATGGAGCCGCC-TTTT-CCTCGTCGCGGAACCAT

	BIP	GGCTCAGGCGCAACTGTAAGT-TTTT-GCAGCAAGAAAGCCCTTGAA
	LF	AAAGTCCTGTTCGAGTTTAGC
	LB	GAGCGCTTCCCACTGT
NDM	F3	GGCGACCAACGGTTTGG
	B3	CCTGCTTGATCCAGTTGAGG
	FIP	CGAAACCCGGCATGTCGAGATA-TTTT-TTTTCCGCCAGCTCGCAC
	BIP	GGCAGTCGCTTCCAACGGT-TTTT-TGGTCATCGGTCCAGGC
	LF	GAAGTGTGCTGCCAGACATTC
	LB	GCGTGCTGGTGGTCGATA

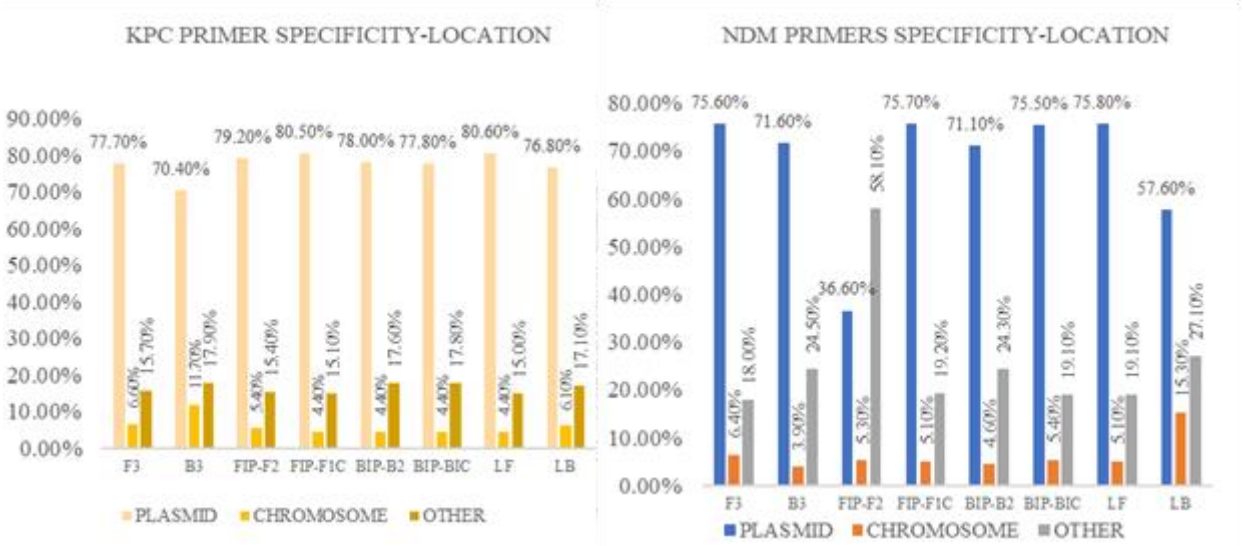

Figure 6 The specificity of the universal primers KPC and NDM

4.4 Detection of *KPC* and *NDM* genes using Polymerase-chained reaction (PCR)

To confirm positive controls (*KPC* and *NDM* strains) in this study, we performed the PCR assay using F3 and B3 primers of both genes. The result showed the PCR product around 200 bp as in Figure 7.

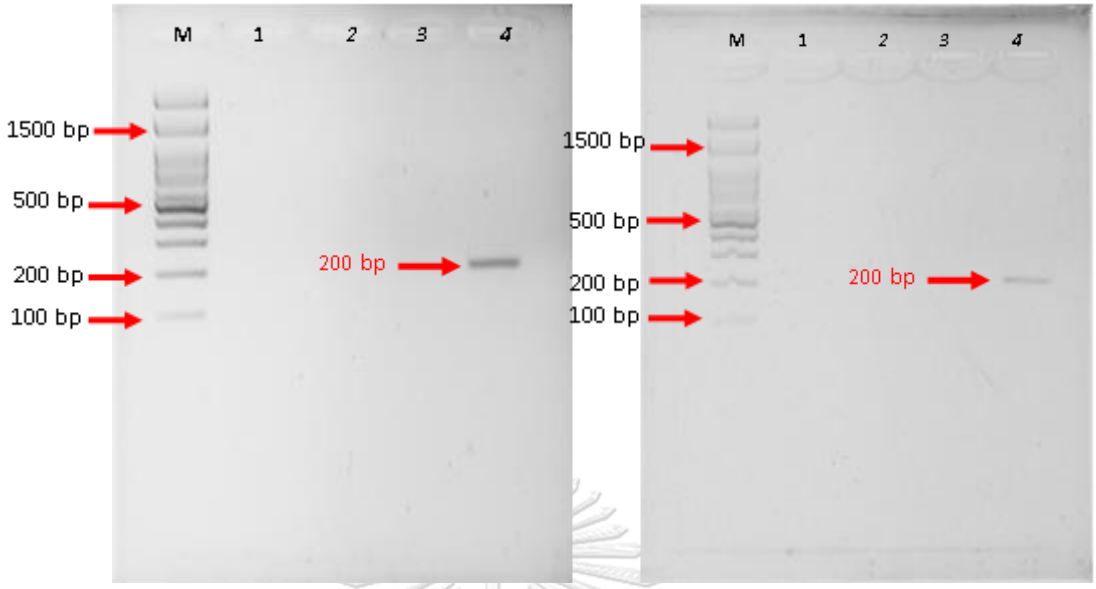

Figure 7 KPC and NDM PCR detection

a. Lane M: 100 bp DNA ladder bio-helix; lane 2-4: PCR (KPC primers with) ddH_2O, NDM, OXA-48, and KPC template, respectively.
b. Lane M: 100 bp DNA ladder bio-helix: lane 2-4: PCR (NDM primers with) ddH_2O, KPC, OXA-48, and NDM template respectively

As for the specificity of the PCR using clinical bacterial DNA samples, we confirm that the PCR method using F3, and B3 of LAMP primers can identify the different subtypes of *NDM* gene with specificity 100% and no false negative result (Figure 8).

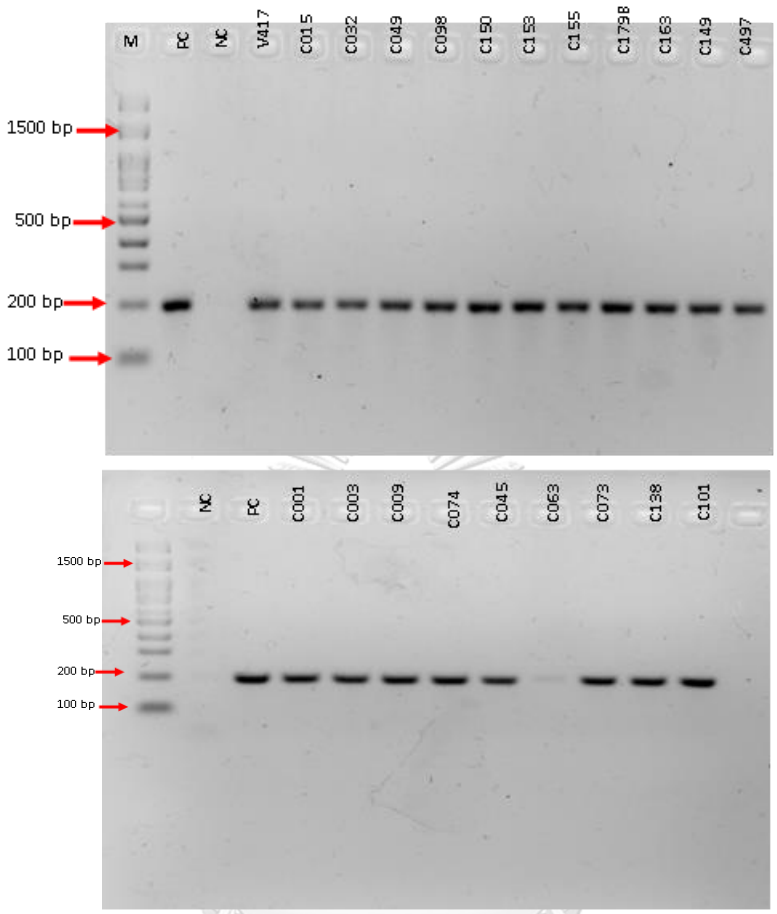

Figure 8 The PCR specificity using clinical bacterial samples

a. Lane M: 100 bp DNA ladder bio-helix; Lane 4-15: clinical bacterial sample (*E. coli*) *NDM-1, NDM-4, NDM-5, NDM-1, NDM-1, NDM-7, NDM-7, NDM-4, NDM-4, NDM-1, NDM-1,* and *NDM-1* harboring gene, respectively.
b. Lane M: 100 bp DNA ladder bio-helix; Lane 4-12: clinical bacterial sample (*K. pneumoniae*) *NDM-1, NDM-1, NDM-1, NDM-1, NDM-1, NDM-5, NDM-1, NDM-1,* and *NDM-1* harboring gene, respectively.

Furthermore, the sensitivity of *KPC-NDM-PCR* was also analyzed (Figure 9). The result showed that the PCR method in detection in detection of *KPC* and *NDM* genes has able to detect the genes until limit of detection 520 fg or equal to 10 copies of DNA in one reaction.

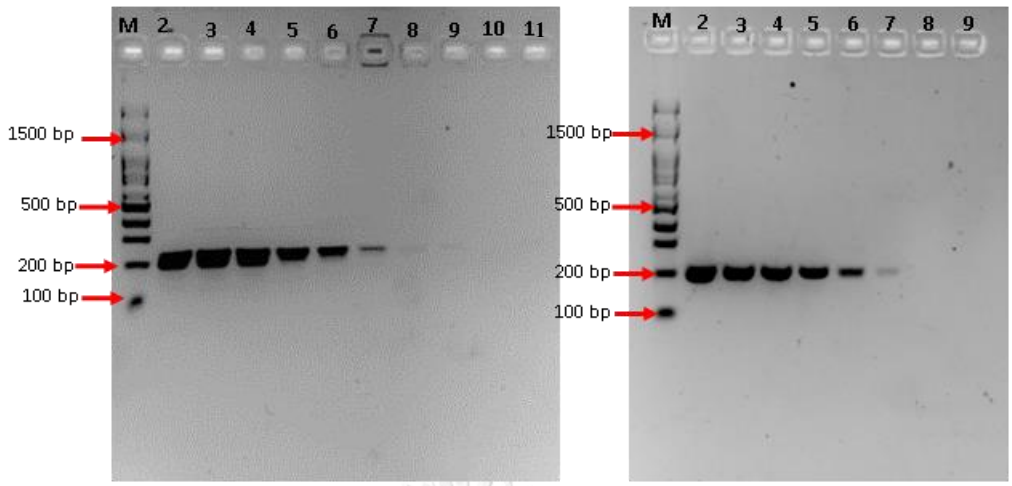

Figure 9 The PCR sensitivity test

a. Lane M: 100 bp DNA ladder bio-helix; Lane 2-10: 10-fold serial dilution of KPC positive control 1×10^{5}-1×10^{-3}, respectively. Lane 11: negative control,
b. Lane M: 100 bp DNA ladder bio-helix; Lane 2-8: 10-fold serial dilution of NDM positive control 1×10^{5}-1×10^{-1}, respectively. Lane 9: Negative control.

4.5 The detection and optimization of *KPC* and *NDM* genes using Single LAMP (sLAMP)

To find the optimal condition of sLAMP of *KPC* and *NDM* genes, 2% of gel electrophoresis was analyzed. The intensity measurement using ImageJ and analyzed by two-way ANOVA confirmed that the temperature 65, 65, and 67 showed a significant higher intensity among other temperature ($p < 0.0001$) (Figure10). The results suggested that the optimum temperature of sLAMP in detection of both genes are between 65-67°C (Figure 11 (a). The optimization of incubation time of sLAMP in both genes showed that the sLAMP were able to amplify started from 45 minutes (Figure 11 (b). These compromised temperatures and incubation time are then used as compromised temperatures that will be used early condition of dLAMP in the detection of both genes.

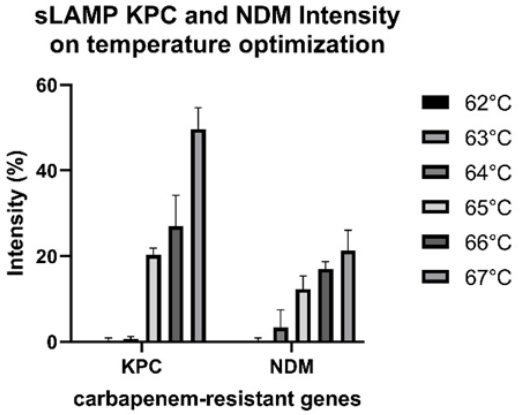

Figure 10 The two-way ANOVA graph on analysis of sLAMP intensity for temperature optimization

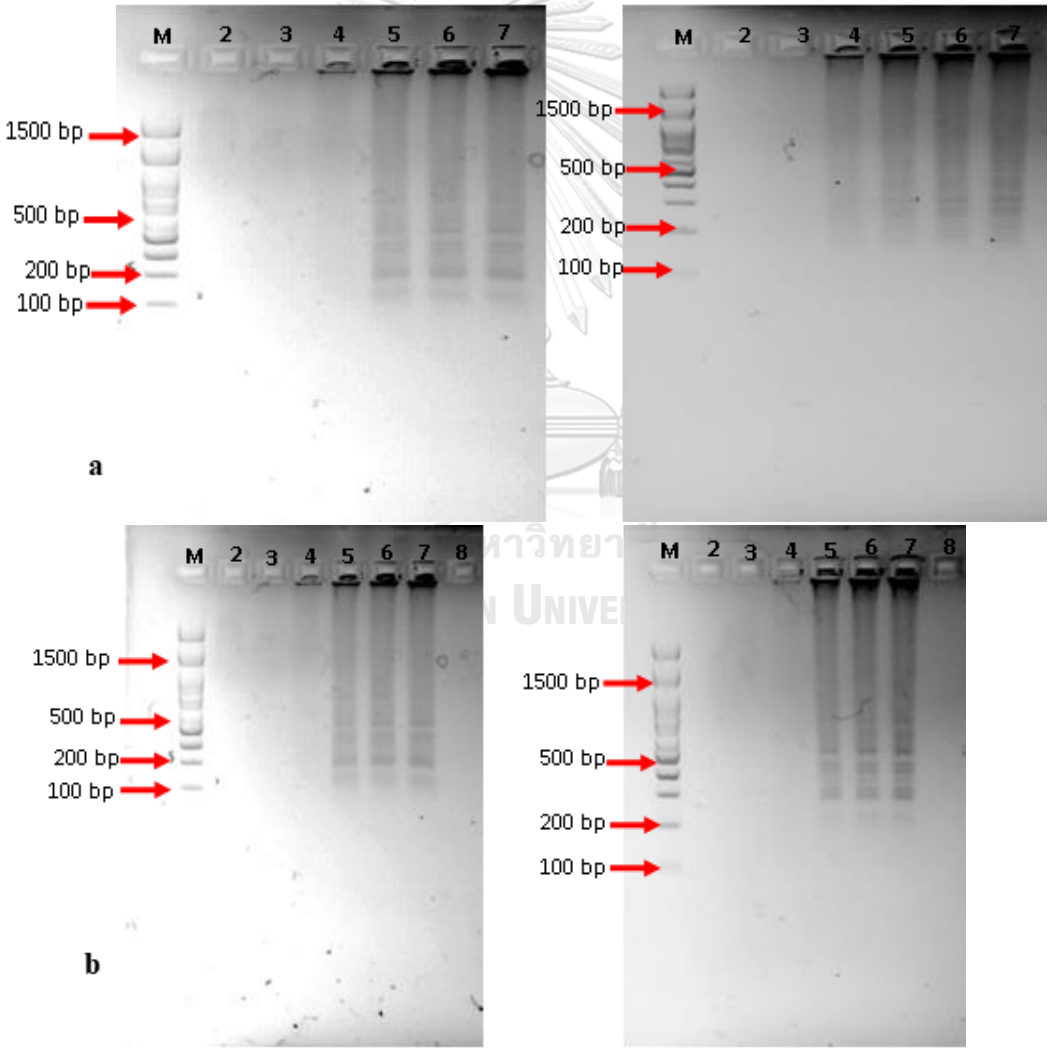

Figure 11 The temperature and incubation time optimization in dLAMP

a. Ladder M: 100 bp DNA ladder bio-helix; Lane 2-7: KPC sLAMP temperature 62, 63, 64, 65, 66, and 67℃, respectively.
b. Ladder M: 100 bp DNA ladder bio-helix; Lane 2-7: NDM sLAMP temperature 62, 63, 64, 65, 66, and 67, respectively.
c. Ladder M: 100 bp DNA ladder bio-helix; Lane 2-7: KPC sLAMP incubation time 30,35,40,45,50, and 55 minutes, respectively. Lane 8: Negative control
d. Ladder M: 100 bp DNA ladder bio-helix; Lane 2-7: NDM sLAMP temperature 65℃ with incubation time 30,35,40,45,50, and 55 minutes, respectively. Lane 8: Negative control

4.6 The detection and optimization of *KPC* and *NDM* using dLAMP

After we obtained the optimum condition of sLAMP of both *KPC* and *NDM*, we performed a dLAMP assay that contained both gene DNA as the template. 2% gel electrophoresis of dLAMP were analyzed. As a result, we found two temperatures that give a clear band and three incubation times. The temperature of 63°C with incubation times 55 and 60 minutes, and the temperature of 65 with incubation times 50 and 55 minutes give the clear product band as well (Figure 12).

To obtain this conclusion, we analyze the yield concentration (Table 3) and intensity (Table 4) that we present in the table of summary as the average value plus-minus (±) of the standard error with the comparison graph to see a significant difference (Figure 13).

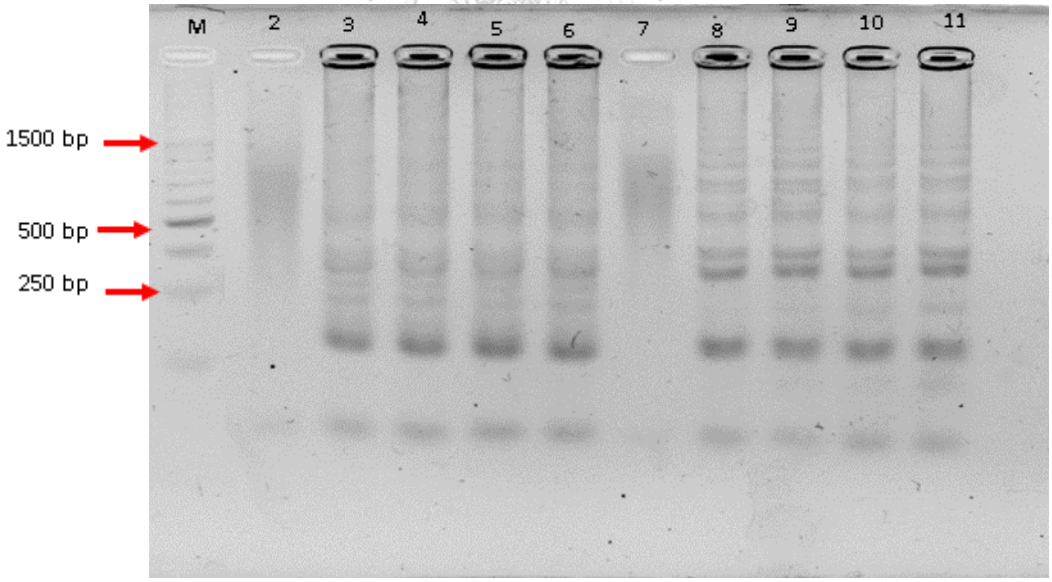

Figure 12 The possible optimum temperatures and incubation time of dLAMP

Lane M: 100 bp DNA ladder bio-helix; Lane 2 and 7 : Negative controls; Lane 3 and 4: dLAMP 63°C 55 minutes Lane 5 and 6: dLAMP 63°C 60 minutes; Lane 8 and 9: dLAMP 65°C 50 minutes; Lane 10 and 11: : dLAMP 65°C 55 minutes

Table 3 The product yield of dLAMP optimization

Incubation time (°C)	DNA concentration (ng/µl)					
	Incubation times (minutes)					
	45	50	55	60	65	70
63	34±0.58	34±0.00	20±1.15	30±4.04	0	0
65	16±2.65	27±2.08	26±3.06	21±2.89	26±0.58	19±2.65
67	21±4.36	21±2.65	21±2.31	17±3.79	21±5.13	20±1.53
69	0	0	0	13±2.52	16±0.58	17±3.06
71	16±4.58	13±1.73	13±1.15	13±1.73	14±4.04	14±2.00

Table 4 The intensity in dLAMP optimization

Incubation time (°C)	Intensity comparison to ladder (%) ± SD					
	Incubation times (minutes)					
	45	50	55	60	65	70
63	15±1.85	12±1.31	14±0.72	15±1.89	8±3.79	3±2.67
65	**21±1.49**	**22±3.12**	**23±3.68**	**17±6.70**	**22±4.09**	**9±5.01**
67	10±4.00	9±0.56	8±1.18	10±1.15	9±2.82	9±6.69
69	1±0.24	1±0.70	2±0.27	7±2.93	9±4.32	15±2.11
71	2±0.99	2±1.83	2±1.31	2±1.93	4±0.20	6±3.51

4.6.1 The intensity value of dLAMP

The intensity of dLAMP product on 2% gel electrophoresis was measured and analyzed. In Figure 13 showing the overall comparison of the intensity compared to the ladder of each temperature and incubation time.

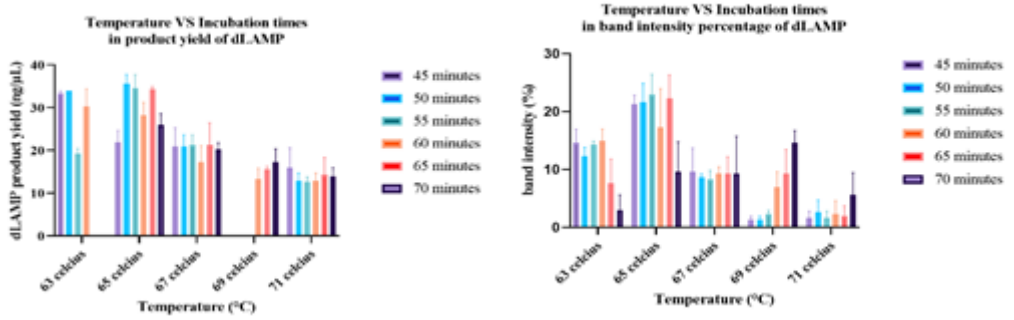

Figure 13 The graph summary of temperature and incubation time optimization in dLAMP

We performed ANOVA two-way to get the significant difference between temperature and incubation time. The ANOVA test showed that the intensity based on each temperature shows a significant difference (P<0.0001), but for the incubation time, there is no significant difference (P>0.6339) (table 5).

Table 5 Two-way ANOVA for the intensity value based on varied temperatures and incubation time

Two-way ANOVA	**Ordinary**				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary		Significant?
Interaction	25.75	<0.0001	****		Yes
Row Factor	61.45	<0.0001	****		Yes
Column Factor	0.6952	0.6339	ns		No
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1179	20	58.94	F (20, 60) = 6.376	P<0.0001
Row Factor	2814	4	703.4	F (4, 60) = 76.09	P<0.0001
Column Factor	31.83	5	6.367	F (5, 60) = 0.6887	P=0.6339
Residual	554.7	60	9.244		

Furthermore, based on the result we concluded that the temperature of 65°C with 55 minutes of incubation time showed the highest value of band intensity compared to 67,69, and 71°C in the same incubation time (p<0.0001) (Figure 14).

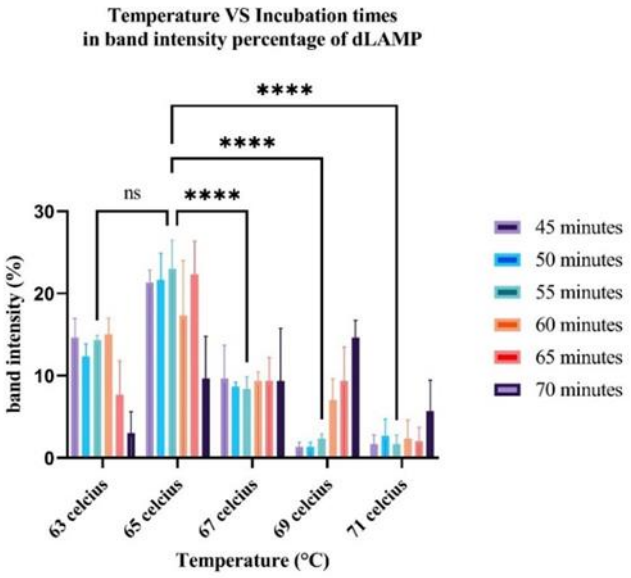

Figure 14 The two-way ANOVA graph for dLAMP intensity

4.6.2 dLAMP product yield

We performed ANOVA two-way to get the significant difference between temperature and incubation time. The ANOVA test showed that the dLAMP yield on both each temperature and incubation time (P<0.0001) (table 6).

Table 6 Two-way ANOVA for dLAMP product yield based on varied temperatures and incubation

Two-way ANOVA	**Ordinary**				
Alpha	0.05				
Source of Variation	% of total variation		P value	P value summary	Significant ?
Interaction	47.50		<0.0001	****	Yes
Row Factor	46.15		<0.0001	****	Yes
Column Factor	2.783		<0.0001	****	Yes
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	5159	20	257.9	F (20, 60) = 40.02	P<0.0001
Row Factor	5012	4	1253	F (4, 60) = 194.4	P<0.0001
Column Factor	302.2	5	60.44	F (5, 60) = 9.378	P<0.0001
Residual	386.7	60	6.444		

Furthermore, based on the result we concluded that the temperature 65°C with 55 minutes incubation time showed as the highest value of band intensity compared to 63, 67,69, and 71°C in the same incubation time ($p<0.0001$) (Figure 15).

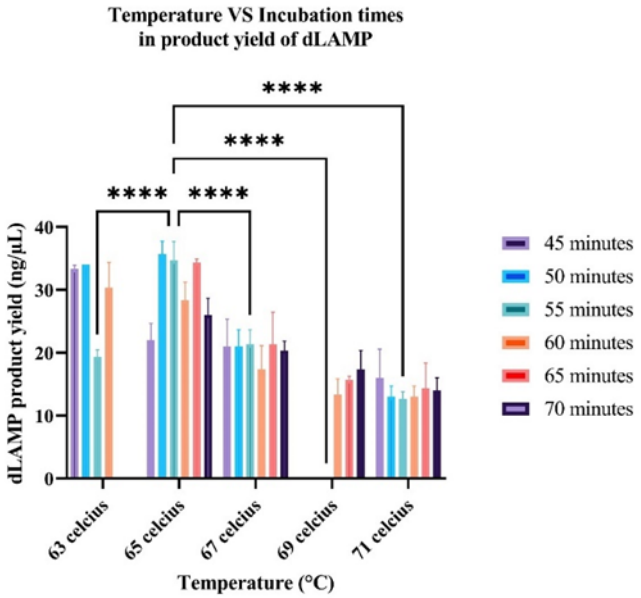

Figure 15 The two-way ANOVA graph for dLAMP product yield

Based on the results of intensity and dLAMP product of each temperature in varied incubation time, we concluded that temperature 65°C with incubation time 55 minutes give a clear intensity and comparable dLAMP product concentration. Next, we used this optimum temperature and incubation time dLAMP condition in optimization of $MgSO_4$ along with HNB volume to obtain the best condition of our developed dLAMP as well to be able to distinguish the positive and negative results by naked eyes.

4.6.3 $MgSO_4$ and HNB optimization

The optimum of $MgSO_4$ correlation with the addition of HNB gives a significant result in this study. Based on the two-way ANOVA analysis (Table 7) on wavelength 650 nm, we concluded that as high the $MgSO_4$ and HNB concentration we added to the reaction, as high the absorbance on wavelength 650 nm ($p \leq 0.0001$) (Figure 16).

Table 7 The two-way ANOVA table result of $MgSO_4$ and HNB concentration optimization

Two-way ANOVA	**Ordinary**			
Alpha	0.05			
Source of Variation	% of total variation	P value	P value summary	Significant ?
Interaction	10.19	<0.0001	****	Yes
Row Factor	81.74	<0.0001	****	Yes
Column Factor	6.576	<0.0001	****	Yes

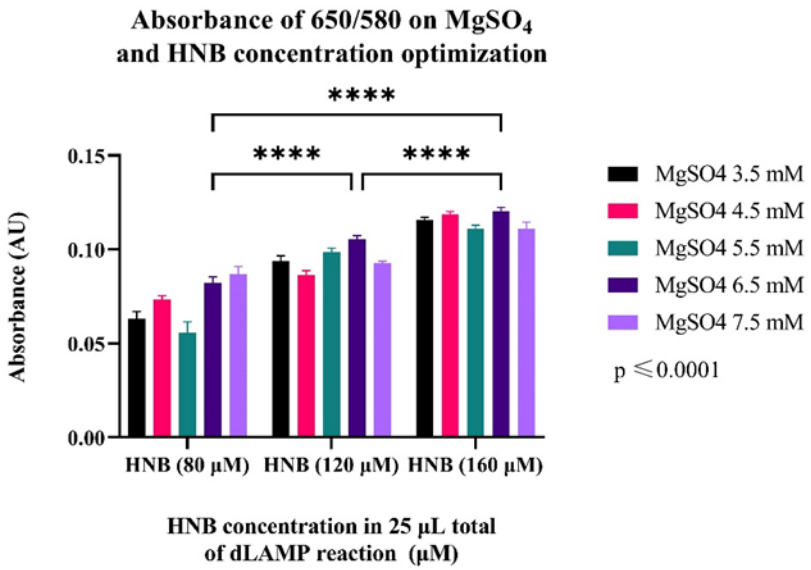

Figure 16 The absorbance value for MgSO4 and HNB optimization

As for the conclusion, the optimum $MgSO_4$ in its correlation on HNB concentration optimization on dLAMP assay based on the analysis of the absorbance on wavelength 650 nm using UV-Vis spectrophotometry instrument is at 6.5 mM

$MgSO_4$ with the addition of 160 µM HNB in 25 µL total volume of dLAMP reaction which show a significant higher than other condition of $MgSO_4$ and HNB volume ($p \leq 0.0001$) that also shown in Figure 17 of the optimum $MgSO_4$ and HNB concentration for visualization.

Figure 17 The reaction tube for MgSO4 and HNB optimization

Additionally, the optimization of $MgSO_4$ and HNB concentration shows no significant differences on intensity (Table 8) and dLAMP product yield (Table 9), that also able to be analyzed by graphic summary (Figure 18). The dLAMP product also analyzed using 2% gel electrophoresis (Figure 19).

Table 8 The one-way ANOVA of $MgSO_4$ on intensity

ANOVA summary	
F	0.6956
P value	0.6021
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R squared	0.1001

Table 9 The one-way ANOVA of $MgSO_4$ on product yield

ANOVA summary	
F	1.375
P value	0.2780
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R squared	0.2157

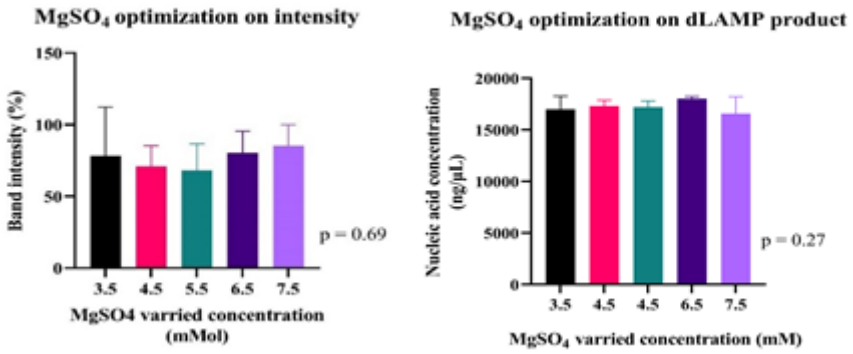

Figure 18 The graph summary of $MgSO_4$ and HNB concentration optimization

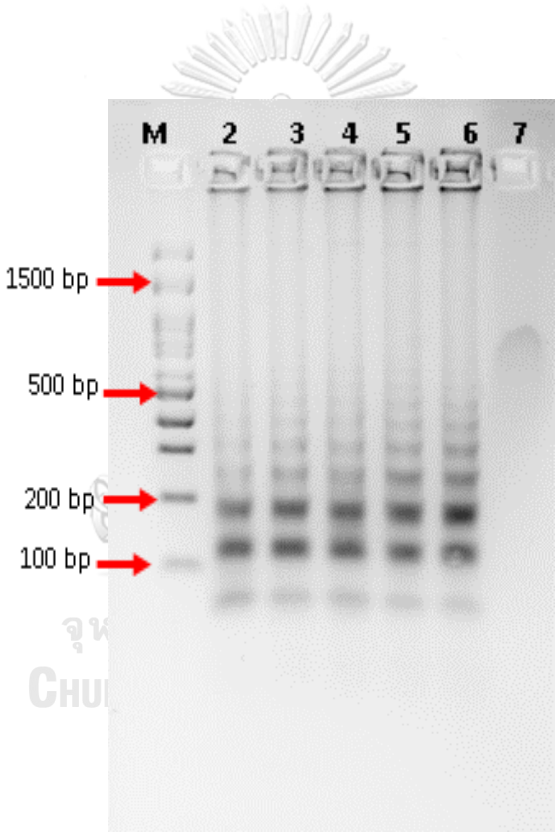

Figure 19 The varied $MgSO_4$ and HNB concentration on dLAMP gel electrophoresis result

Lane M: 100 bp DNA ladder bio-helix; Lane 2-6: $MgSO_4$ varied on dLAMP 3.5, 4.5, 5.5, 6.5, and 7.5 mM, respectively; Lane 7: Negative control.

Furthermore, the sensitivity of dLAMP was analyzed using ten-fold serial dilution of positive controls of *KPC* (Figure 20 (a)) and *NDM* (Figure 21 (a)). The result showed that the dLAMP can detect the *KPC* and *NDM* genes until the limit of detection 52 fg or equal to a copy of DNA each gene, respectively in one reaction.

The positive result also can be seen on the blue-sky color on the reaction of dLAMP with single template *KPC* (Figure 20 (b)) and *NDM* (Figure 21 (b).

The specificity of dLAMP reaction was confirmed by cross react the template used in dLAMP with different genes, *OXA-48*. As the result, the specificity of dLAMP shown by Figure 22 (a) was specific and did not show any band product on gel electrophoresis and color changing in the reaction with addition of HNB (22 (b)).

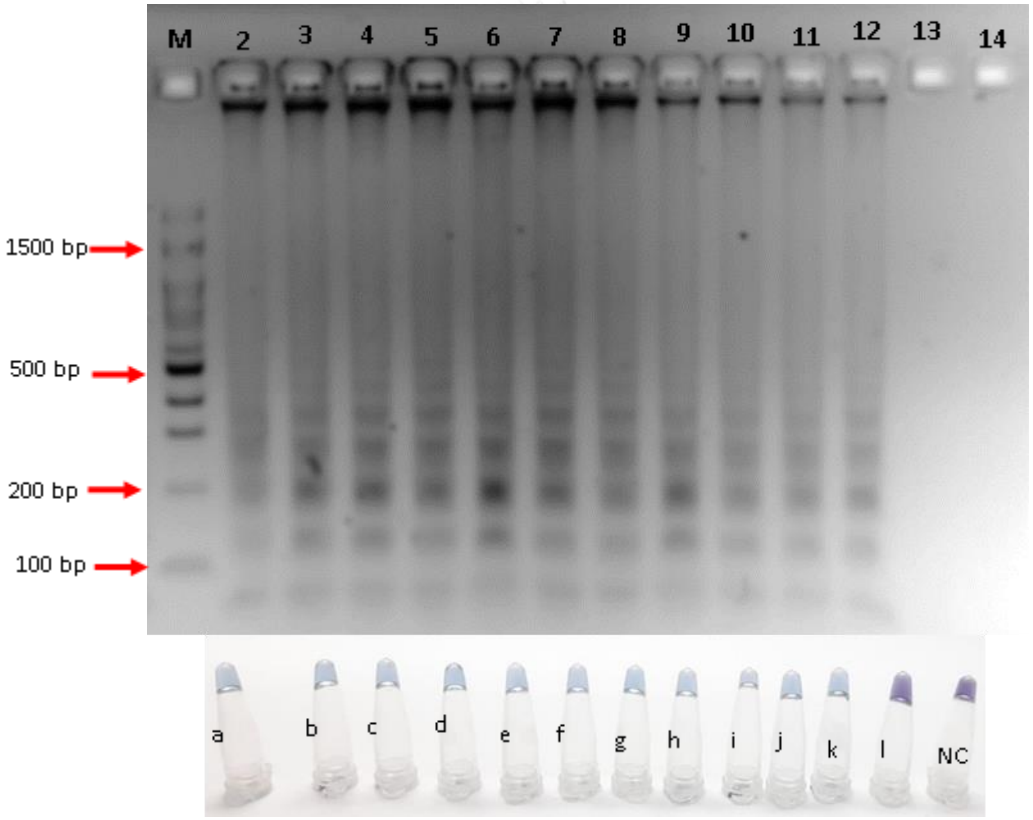

Figure 20 The sensitivity of dLAMP *KPC*

a. Lane M: 100 bp DNA ladder bio-helix; Lane 2-13: ten-fold serial dilution for dLAMP sensitivity test, 1.0×10^{7}, 10^{6},10^{5}, 10^{4}, 10^{3}, 10^{2}, 10^{1}, 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, respectively.
b. dLAMP tube reaction, a-l: , 1.0×10^{7}, 10^{6},10^{5}, 10^{4}, 10^{3}, 10^{2}, 10^{1}, 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, respectively; NC: negative control.

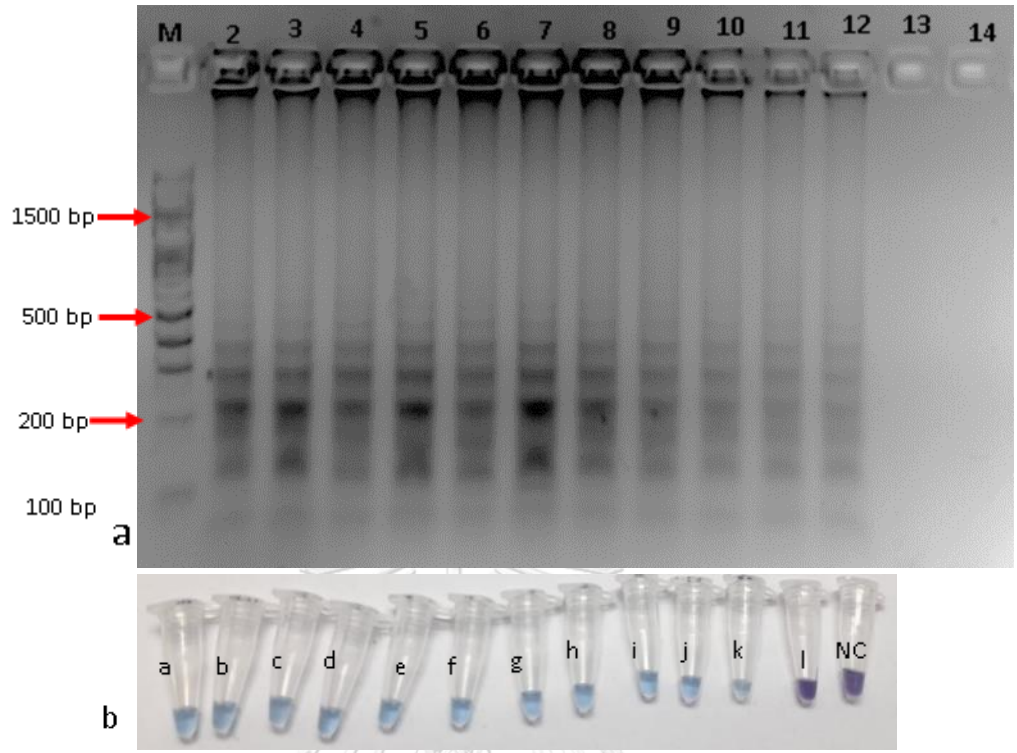

Figure 21 The sensitivity of dLAMP NDM

1 Lane M: 100 bp DNA ladder bio-helix; Lane 2-13: ten-fold serial dilution for dLAMP sensitivity test, 1.0×10^7, 10^6,10^5, 10^4, 10^3, 10^2, 10^1, 10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, respectively.

2 dLAMP tube reaction, a-l: 1.0×10^7, 10^6,10^5, 10^4, 10^3, 10^2, 10^1, 10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, respectively; NC: negative control

The specificity of dLAMP reaction was confirmed by cross react the template used in dLAMP with different genes, *OXA-48*. As the result, the specificity of dLAMP shown by Figure 4.18 (a) was specific and did not show any band product on gel electrophoresis and color changing in the reaction with addition of HNB (4.18 (b)).

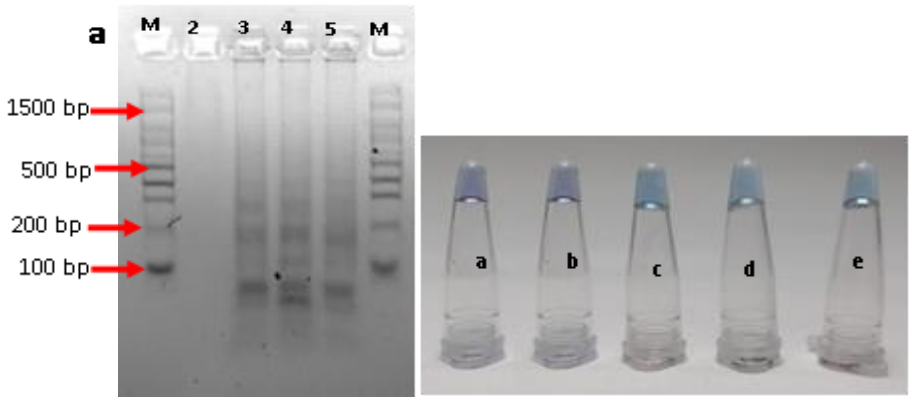

Figure 22 The specificity of dLAMP

a. Lane M: 100 bp DNA ladder bio-helix; Lane 2-5: dLAMP reaction with *OXA-48* DNA template, dLAMP reaction with *KPC* template, dLAMP reaction with *KPC* and *NDM* template, dLAMP reaction with *NDM* template.
b. dLAMP tube reaction, a-e: Negative control, dLAMP reaction with *OXA-48* DNA template, dLAMP reaction with *KPC* template, dLAMP reaction with *KPC* and *NDM* template, dLAMP reaction with *NDM* template.

CHAPTER V
DISCUSSION

5.1 Targeted DNA sequences of *KPCs* and *NDMs* carbapenem-resistant (CR) genes

One hundred and twenty-three subtypes of *KPC* (*K. pneumoniae* carbapenemase) and forty-three subtypes of *NDM* (new-delhi-β-Lactamase) were aligned with one hundred percent conserved region were used to ensure that the LAMP primers designed are specific to the desired DNA sequences of *KPCs* and *NDMs* CR genes.

5.2 Specificity of *KPCs* and *NDMs* DNA sequences

After the confirmation of the CR DNA sequences, the specificity of the sequences was checked using NCBI BLASTN software. The results showed that the *KPC* sequence is mostly found in plasmid (87.32%), and chromosome (5.17%), and clearly stated that it is the *KPC* sequence (7.51%). The *NDM* showed that the gene is mostly found in a plasmid (74.20%), chromosome (9.84%), and clearly states that it is the *NDM* sequence (15.96%). We checked their sequences in the chromosome and aligned them to make sure that they are both the same DNA sequences and confirmed that all the DNA sequences harbored in the chromosome are correctly the *KPC* and *NDM* CR DNA sequences. Then we ensure that these DNA sequences are specific enough to be used as the template for designing the primers of LAMP.

5.3 The universal LAMP primers of *KPC* and *NDM* genes

The LAMP primers were designed using the Primer Explorer version 5 software. The template for designing the primers used the DNA sequences of both

genes that previously aligned with a hundred percent of the conserved region from 123 subtypes of *KPC* and 43 subtypes of *NDM*. This makes the DNA sequences universal, meaning that the sequences are present in almost all these subtypes of *KPC* and *NDM* CR genes. By using these universal DNA sequences of both genes, we designed LAMP primers for both which are expected to be able to bind in all types of *KPC* and *NDM* CR genes making them the universal primers in the detection of both CR genes. The specificity of both sets of primers of *KPC* and *NDM* was then checked using BLASTN of the NCBI database to confirm their specificity. As a result, both *KPC* and *NDM* primer sets confirm that most will bind with the targeted sequence that is harbored in a plasmid with *K. pneumoniae* and *E. coli* are the species that mostly will be detected or bind with these primer sets. In the previous study, the LAMP primers were designed only based on 5 and 4 subtypes of *KPC* and *NDM* genes, respectively (Feng et al., 2021). This study represents a larger range of subtypes of both types of genes, which are later expected to be more reliable in the detection of *KPC* and *NDM* CR genes.

5.4 Detection of *KPC* and *NDM* genes using Polymerase-chained reaction (PCR)

The positive controls obtained from the previous study (Kerdsin et al., 2019; Takeuchi et al., 2022) which were previously confirmed as *KPC* and *NDM* CR genes by whole genome sequencing (WGS). The extracted DNA of *E. asburiae* as the positive control of the *KPC* gene and *E. coli* as the *NDM* positive controls were confirmed using polymerase-chained reaction (PCR) using F3 and B3 that were designed for *KPC* and *NDM*. The result showed that both genes can be detected by the PCR band on 2% gel electrophoresis around 200 base pair products. In this research,

the PCR products obtained were shown in 200 bp. products (Figure 4.4), through the template of *KPC* and *NDM* we used to be around 800 bp. This is due to the designed LAMP F3 and B3 primers of both genes are designed to amplify the template and will stop amplifying if the product already produces 200 bp. product, to avoid hairpin formation in LAMP reaction (Notomi et al., 2000).

Our designed universal primers of *KPC* and *NDM* were used in PCR to check their specificity. We first check their specificity in our positive and negative controls. The band showed only in the specific DNA template that we mixed in the reaction tube. But didn't show any band when we mixed with crossed template to each other genes and our negative control, *OXA-48* CR genes. Furthermore, we check their specificity in different types of *NDM* by using extracted DNA of twenty-one clinical bacterial strains that were previously confirmed with *NDM* in a previous study (Takeuchi et al., 2022). The PCR result showed in 2% gel electrophoresis with F3 and B3 primers that the different subtypes of *NDM* can be detected using our developed universal primers.

Additionally, we perform the sensitivity test using our design primers in PCR using our positive controls *KPC* and *NDM*. The 10-fold serial dilution was performed to dilute the extracted bacterial DNA as the template to decrease the concentration as low as we could. The result showed that the PCR can detect the *KPC* and *NDM* gene in the limit around 520 fg or equal to approximately 10 copies/reaction, for both *KPC* and *NDM*.

5.5 The detection and optimization of *KPC* and *NDM* genes using Single LAMP (sLAMP)

The loop-mediated isothermal amplification (LAMP) performed in this study is aimed to determine the compromised temperatures of both CR genes, *KPC* and *NDM*. Both LAMP reactions contained with each of the *KPC* and *NDM* templates showed the same temperature in working well, which are around 65, 66, and 67 Celsius degree (Figure 4.8). This reaction was incubated around 30-70 minutes. We confirmed that the reaction was working well and started to show a band on 2% gel electrophoresis when we incubated them for 45 minutes. We use this result as a compromised temperature for both genes in duplex loop-mediated isothermal amplification (dLAMP) assay, as we expect that both genes can be detected using this method using the compromised temperature of both in the LAMP reaction. This finding also compromises with the previous study in the detection of CR genes as the incubation temperature was 65°C (Feng et al., 2021) which may also be an optimum temperature in the detection of *KPC* and *NDM* in all subtypes using LAMP.

5.6 The detection and optimization of *KPC* and *NDM* using dLAMP

The dLAMP method in this study was performed first using the compromised temperature of both genes around 65, 67, and 68°C with 45 minutes of incubation time. The result showed that the reaction can detect both genes, which is confirmed by a single template in the dLAMP mixture. Additionally, the band also showed when we added both templates in one single dLAMP reaction tube. After we confirmed the dLAMP reaction was working, the optimization of dLAMP in temperature, incubation time, and $MgSO_4$ along with HNB concentration was conducted. As the result of the

optimization, the optimum condition of the dLAMP in the detection of CR genes, *KPC,* and *NDM* are as follows; temperature 65°C with 55 minutes incubation time and 6.5 mM of $MgSO_4$ along with the addition of 160 µM of HNB in total volume of 25 µL dLAMP reaction.

The sensitivity of dLAMP primers in the detection of *KPC* and *NDM* CR genes was conducted to confirm the limit of detection of our developed dLAMP method. In this part, the template used was the bacterial DNA extraction from positive controls of both genes. The concentration of the extracted DNA is then measured using nanodrop and then diluted with a 10-fold serial dilution method. As a result, the limit of detection showed the dLAMP can detect the CR genes at the limit of detection of around 52 fg, which showed ten times more sensitivity than the PCR method.

Previous research in the detection of *KPC* and *NDM* CR genes using LAMP showed that the LAMP assay was found to be more sensitive than conventional PCR which confirmed that the PCR was not able to detect some isolates of that LAMP assay can detect it. Furthermore, the turnaround time of the LAMP assay is only 2-3 hours, which might be an alternative method for rapid detection of both genes (Solanki et al., 2013). The detection of CR genes using the dLAMP technique is a new technique yet gives a very limited comparison result in this study. Therefore, in this study, the comparison study is mostly compared to the LAMP method.

Finally, the specificity of the dLAMP method in the detection of other CR genes; *OXA-48* showed a very specific result, as no band presence on the gel electrophoresis and no color changing in the reaction by the addition of the HNB into sky-blue color. This finding is also consistent with a previous study about the detection of the *mcr-1* to *mcr-5* gene using multi-LAMP in comparison to the

conventional PCR method. The PCR method is known to possess high specificity, therefore the multi-LAMP also showed good consistency with PCR in the detection of the *mcr-1* to *mcr-5* gene (Zhong et al., 2019).

CHAPTER VI

CONCLUSION

This study has developed a duplex method of loop-mediated isothermal amplification (dLAMP) for enhancing the universality, rapid, sensitivity, and specificity of detecting CR genes; *KPC,* and *NDM* compared to the conventional PCR method. The dLAMP, utilizing a specially designed primers set, which can identify various subtypes of *KPC* and *NDM*, allows for visible results within an hour, even without the need for specialized equipment. In contrast, the standard PCR method requires 3.5 hours, including 30 minutes of gel electrophoresis, to achieve the same level of gene detection. Furthermore, when applied to clinical bacterial samples, the universal primers developed in this study demonstrated their ability to detect multiple types of *NDM* genes, affirming their suitability for identifying not only one subtype but multiple subtypes of CR genes, making them valuable for future application.

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APPENDIX

A. Subtypes of KPC and NDM

123 Subtypes of KPC

Allele	Product name	RefSeq nucleotide	GenBank nucleotide
blaKPC-100	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-100	NG_081070.1	ON521726.1
blaKPC-101	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-101	NG_088394.1	OK086805.1
blaKPC-102	carbapenem-hydrolyzing class A beta-lactamase KPC-102	NG_078063.1	OK652013.1
blaKPC-103	class A beta-lactamase KPC-103	NG_078051.1	OL445423.1
blaKPC-104	class A beta-lactamase KPC-104	NG_078052.1	OL445424.1
blaKPC-105	class A beta-lactamase KPC-105	NG_078054.1	OL445426.1
blaKPC-106	class A beta-lactamase KPC-106	NG_078056.1	OL445428.1
blaKPC-107	class A beta-lactamase KPC-107	NG_078053.1	OL445425.1
blaKPC-108	class A beta-lactamase KPC-108	NG_078055.1	OL445427.1
blaKPC-109	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-109	NG_149659.1	OL744263.1
blaKPC-10	carbapenem-hydrolyzing class A beta-lactamase KPC-10	NG_049243.1	GQ140348.1
blaKPC-110	inhibitor-resistant class A beta-lactamase KPC-110	NG_088395.1	CP100313.1
blaKPC-111	inhibitor-resistant class A beta-lactamase KPC-111	NG_081791.1	OL744330.1
blaKPC-112	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-112	NG_079230.1	OM177660.1
blaKPC-113	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-113	NG_079888.1	OM728506.1
blaKPC-114	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-114	NG_079889.1	OM728507.1
blaKPC-115	inhibitor-resistant class A beta-lactamase KPC-115	NG_079890.1	OM714909.1
blaKPC-116	carbapenem-hydrolyzing class A beta-lactamase KPC-116	NG_079891.1	OM729575.1

blaKPC-117	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-117	NG_079892.1	OM933711.1
blaKPC-118	carbapenem-hydrolyzing class A beta-lactamase KPC-118	NG_079893.1	OM933712.1
blaKPC-119	carbapenem-hydrolyzing class A beta-lactamase KPC-119	NG_079894.1	OM933713.1
blaKPC-11	carbapenem-hydrolyzing class A beta-lactamase KPC-11	NG_049244.1	HM066995.1
blaKPC-120	carbapenem-hydrolyzing class A beta-lactamase KPC-120	NG_079895.1	OM933715.1
blaKPC-121	carbapenem-hydrolyzing class A beta-lactamase KPC-121	NG_079896.1	OM933717.1
blaKPC-122	carbapenem-hydrolyzing class A beta-lactamase KPC-122	NG_079897.1	OM933720.1
blaKPC-123	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-123	NG_079898.1	ON012820.1
blaKPC-124	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-124	NG_203393.1	ON221403.1
blaKPC-125	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-125	NG_080778.1	CP095778.1
blaKPC-126	carbapenem-hydrolyzing class A beta-lactamase KPC-126	NG_080779.1	OM830488.1
blaKPC-127	carbapenem-hydrolyzing class A beta-lactamase KPC-127	NG_081071.1	ON521725.1
blaKPC-128	carbapenem-hydrolyzing class A beta-lactamase KPC-128	NG_081072.1	ON521727.1
blaKPC-129	class A beta-lactamase KPC-129	NG_203394.1	ON751738.1
blaKPC-12	extended-spectrum class A beta-lactamase KPC-12	NG_049245.1	HQ641421.1
blaKPC-130	class A beta-lactamase KPC-130	NG_081699.1	ON794466.1
blaKPC-131	carbapenem-hydrolyzing class A beta-lactamase KPC-131	NG_081700.1	ON823194.1
blaKPC-132	inhibitor-resistant class A beta-lactamase KPC-132	NG_081783.1	OP081092.1
blaKPC-133	carbapenem-hydrolyzing class A beta-lactamase KPC-133	NG_081784.1	OP081531.1
blaKPC-134	inhibitor-resistant class A beta-lactamase KPC-134	NG_088396.1	OP293349.1
blaKPC-135	inhibitor-resistant class A beta-lactamase KPC-135	NG_088397.1	OP205646.1
blaKPC-136	carbapenem-hydrolyzing class A beta-lactamase KPC-136	NG_157007.1	OQ579152.1
blaKPC-138	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-138	NG_088398.1	OP432320.1
blaKP	inhibitor-resistant carbapenem-hydrolyzing class	NG_0883	OP503887.

C-139	A beta-lactamase KPC-139	99.1	1
blaKPC-13	carbapenem-hydrolyzing class A beta-lactamase KPC-13	NG_049246.1	HQ342889.1
blaKPC-140	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-140	NG_088400.1	OP503888.1
blaKPC-141	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-141	NG_088401.1	OP503889.1
blaKPC-142	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-142	NG_088402.1	OP503890.1
blaKPC-143	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-143	NG_088403.1	OP503891.1
blaKPC-144	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-144	NG_088404.1	OP559533.1
blaKPC-145	class A beta-lactamase KPC-145	NG_148622.1	OP626310.1
blaKPC-146	carbapenem-hydrolyzing class A beta-lactamase KPC-146	NG_148623.1	OP696903.1
blaKPC-147	carbapenem-hydrolyzing class A beta-lactamase KPC-147	NG_148624.1	OP696904.1
blaKPC-148	class A beta-lactamase KPC-148	NG_148625.1	JAOZYA010000028.1
blaKPC-14	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-14	NG_049247.1	JX524191.1
blaKPC-151	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-151	NG_148626.1	OP823148.1
blaKPC-153	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-153	NG_148627.1	OP884096.1
blaKPC-154	class A beta-lactamase KPC-154	NG_231545.1	OQ096263.1
blaKPC-155	inhibitor-resistant class A beta-lactamase KPC-155	NG_149660.1	OQ139542.1
blaKPC-156	carbapenem-hydrolyzing class A beta-lactamase KPC-156	NG_149661.1	OQ390084.1
blaKPC-157	carbapenem-hydrolyzing class A beta-lactamase KPC-157	NG_149662.1	JAPQEX020000004.1
blaKPC-158	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-158	NG_228670.1	OQ305823.1
blaKPC-159	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-159	NG_157008.1	OQ450354.1
blaKPC-15	carbapenem-hydrolyzing class A beta-lactamase KPC-15	NG_049248.1	KC433553.1
blaKPC-160	carbapenem-hydrolyzing class A beta-lactamase KPC-160	NG_157009.1	OQ579136.1
blaKPC-161	carbapenem-hydrolyzing class A beta-lactamase KPC-161	NG_157010.1	OQ579137.1
blaKPC-162	carbapenem-hydrolyzing class A beta-lactamase KPC-162	NG_157011.1	OQ579138.1

blaKPC-163	carbapenem-hydrolyzing class A beta-lactamase KPC-163	NG_157012.1	OQ579139.1
blaKPC-164	carbapenem-hydrolyzing class A beta-lactamase KPC-164	NG_157013.1	OQ579140.1
blaKPC-165	carbapenem-hydrolyzing class A beta-lactamase KPC-165	NG_157014.1	OQ579141.1
blaKPC-166	carbapenem-hydrolyzing class A beta-lactamase KPC-166	NG_157015.1	OQ592369.1
blaKPC-167	class A beta-lactamase KPC-167	NG_157016.1	OQ592370.1
blaKPC-16	carbapenem-hydrolyzing class A beta-lactamase KPC-16	NG_049249.1	KC465199.1
blaKPC-170	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-170	NG_231546.1	OR449906.1
blaKPC-178	inhibitor-resistant class A beta-lactamase KPC-178	NG_203395.1	OQ926587.1
blaKPC-179	class A beta-lactamase KPC-179	NG_203396.1	OR115556.1
blaKPC-17	carbapenem-hydrolyzing class A beta-lactamase KPC-17	NG_049250.1	KC465200.1
blaKPC-180	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-180	NG_203397.1	OR206047.1
blaKPC-181	carbapenem-hydrolyzing class A beta-lactamase KPC-181	NG_228671.1	OR282795.1
blaKPC-182	carbapenem-hydrolyzing class A beta-lactamase KPC-182	NG_228672.1	OR282796.1
blaKPC-183	carbapenem-hydrolyzing class A beta-lactamase KPC-183	NG_228673.1	OR282800.1
blaKPC-184	carbapenem-hydrolyzing class A beta-lactamase KPC-184	NG_228674.1	OR282801.1
blaKPC-185	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-185	NG_231547.1	OR359279.1
blaKPC-186	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-186	NG_231548.1	OR466746.1
blaKPC-187	inhibitor-resistant class A beta-lactamase KPC-187	NG_231549.1	OR466751.1
blaKPC-189	extended-spectrum class A beta-lactamase KPC-189	NG_231550.1	OR501577.1
blaKPC-18	carbapenem-hydrolyzing class A beta-lactamase KPC-18	NG_049251.1	KP681699.1
blaKPC-190	inhibitor-resistant class A beta-lactamase KPC-190	NG_231551.1	OR499110.1
blaKPC-191	inhibitor-resistant class A beta-lactamase KPC-191	NG_231552.1	OR499111.1
blaKPC-192	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-192	NG_231553.1	OR529436.1
blaKP	carbapenem-hydrolyzing class A beta-lactamase	NG_0492	KJ775801.

C-19	KPC-19	52.1	1
blaKPC-21	carbapenem-hydrolyzing class A beta-lactamase KPC-21	NG_049254.1	LN609376.1
blaKPC-22	carbapenem-hydrolyzing class A beta-lactamase KPC-22	NG_049255.1	KM379100.1
blaKPC-23	carbapenem-hydrolyzing class A beta-lactamase KPC-23	NG_060569.1	MH450213.1
blaKPC-24	carbapenem-hydrolyzing class A beta-lactamase KPC-24	NG_049256.1	KR052099.1
blaKPC-25	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-25	NG_051167.1	KU216748.1
blaKPC-26	carbapenem-hydrolyzing class A beta-lactamase KPC-26	NG_051469.1	KX619622.1
blaKPC-27	carbapenem-hydrolyzing class A beta-lactamase KPC-27	NG_052862.1	KX828722.1
blaKPC-28	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-28	NG_052581.1	KY282958.1
blaKPC-29	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-29	NG_055580.1	KY563764.1
blaKPC-2	carbapenem-hydrolyzing class A beta-lactamase KPC-2	NG_049253.1	AY034847.1
blaKPC-30	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-30	NG_054685.1	KY646302.1
blaKPC-31	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-31	NG_055494.1	MAPH01000113.1
blaKPC-32	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-32	NG_055495.1	MAPO01000050.1
blaKPC-33	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-33	NG_056170.1	CP025144.1
blaKPC-34	carbapenem-hydrolyzing class A beta-lactamase KPC-34	NG_057447.1	KU985429.1
blaKPC-35	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-35	NG_060524.1	MH404098.1
blaKPC-36	carbapenem-hydrolyzing class A beta-lactamase KPC-36	NG_061389.1	MH593787.1
blaKPC-37	carbapenem-hydrolyzing class A beta-lactamase KPC-37	NG_061612.1	MH718730.1
blaKPC-38	carbapenem-hydrolyzing class A beta-lactamase KPC-38	NG_062357.1	MK098861.1
blaKPC-39	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-39	NG_063841.1	MK118771.1
blaKPC-3	carbapenem-hydrolyzing class A beta-lactamase KPC-3	NG_049257.1	AF395881.1
blaKPC-40	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-40	NG_064726.1	QRBR01000058.1
blaKPC-41	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-41	NG_065876.1	MK497255.1

blaKPC-42	carbapenem-hydrolyzing class A beta-lactamase KPC-42	NG_064727.1	MK467612.1
blaKPC-43	carbapenem-hydrolyzing class A beta-lactamase KPC-43	NG_064728.1	MK628511.1
blaKPC-44	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-44	NG_065427.1	MK823188.1
blaKPC-45	carbapenem-hydrolyzing class A beta-lactamase KPC-45	NG_065877.1	MN104596.1
blaKPC-46	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-46	NG_065878.1	MN267701.1
blaKPC-47	carbapenem-hydrolyzing class A beta-lactamase KPC-47	NG_074714.1	MN422012.1
blaKPC-48	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-48	NG_074715.1	MN422013.1
blaKPC-49	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-49	NG_071203.1	MN619655.1
blaKPC-4	carbapenem-hydrolyzing class A beta-lactamase KPC-4	NG_049258.1	EU447304.1
blaKPC-50	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-50	NG_068507.1	MN654342.1
blaKPC-51	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-51	NG_067224.1	MN725731.1
blaKPC-52	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-52	NG_067225.1	MN725732.1
blaKPC-53	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-53	NG_068176.1	CP058327.1
blaKPC-54	carbapenem-hydrolyzing class A beta-lactamase KPC-54	NG_067226.1	MN854706.1
blaKPC-55	carbapenem-hydrolyzing class A beta-lactamase KPC-55	NG_068177.1	MT028409.1
blaKPC-56	carbapenem-hydrolyzing class A beta-lactamase KPC-56	NG_068016.1	MT040751.1
blaKPC-57	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-57	NG_068508.1	MT358626.1
blaKPC-58	inhibitor-resistant class A beta-lactamase KPC-58	NG_070177.1	MT463289.1
blaKPC-59	carbapenem-hydrolyzing class A beta-lactamase KPC-59	NG_070178.1	MT463290.1
blaKPC-5	carbapenem-hydrolyzing class A beta-lactamase KPC-5	NG_049259.1	EU400222.2
blaKPC-60	carbapenem-hydrolyzing class A beta-lactamase KPC-60	NG_070179.1	MT482411.1
blaKPC-61	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-61	NG_070180.1	MK559426.1
blaKPC-62	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-62	NG_073465.1	MT604163.1
blaKP	inhibitor-resistant extended-spectrum class A	NG_0734	MT604164.

C-63	beta-lactamase KPC-63	66.1	1
blaKPC-64	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-64	NG_073467.1	MT604165.1
blaKPC-65	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-65	NG_073468.1	MT604166.1
blaKPC-66	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-66	NG_070739.1	MT833884.1
blaKPC-67	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-67	NG_074716.1	MT809697.1
blaKPC-68	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-68	NG_074717.1	MT809698.1
blaKPC-69	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-69	NG_074718.1	MT809700.1
blaKPC-6	carbapenem-hydrolyzing class A beta-lactamase KPC-6	NG_049260.1	EU555534.1
blaKPC-70	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-70	NG_074719.1	MT809701.1
blaKPC-71	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-71	NG_070895.1	MW015092.1
blaKPC-72	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-72	NG_070740.1	MT833885.1
blaKPC-73	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-73	NG_070741.1	MT833886.1
blaKPC-74	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-74	NG_070742.1	MT856045.1
blaKPC-75	carbapenem-hydrolyzing class A beta-lactamase KPC-75	NG_070743.1	MT920645.1
blaKPC-76	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-76	NG_070896.1	MT550690.1
blaKPC-77	carbapenem-hydrolyzing class A beta-lactamase KPC-77	NG_070897.1	MW030519.1
blaKPC-78	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-78	NG_071204.1	MW319056.1
blaKPC-79	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-79	NG_071205.1	MT875328.1
blaKPC-7	carbapenem-hydrolyzing class A beta-lactamase KPC-7	NG_049261.1	EU729727.1
blaKPC-80	carbapenem-hydrolyzing class A beta-lactamase KPC-80	NG_073469.1	MW444845.1
blaKPC-81	carbapenem-hydrolyzing class A beta-lactamase KPC-81	NG_073470.1	MW444846.1
blaKPC-82	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-82	NG_073471.1	MW485086.1
blaKPC-83	carbapenem-hydrolyzing class A beta-lactamase KPC-83	NG_079231.1	MW581775.1
blaKPC-84	inhibitor-resistant extended-spectrum carbapenem-hydrolyzing class A beta-lactamase	NG_074720.1	MW657985.1

	KPC-84		
blaKPC-85	carbapenem-hydrolyzing class A beta-lactamase KPC-85	NG_074721.1	MW896839.1
blaKPC-86	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-86	NG_074722.1	MZ067229.1
blaKPC-87	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-87	NG_074723.1	MZ067230.1
blaKPC-88	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-88	NG_074724.1	MZ067231.1
blaKPC-89	carbapenem-hydrolyzing class A beta-lactamase KPC-89	NG_079232.1	MZ401141.1
blaKPC-8	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-8	NG_049262.1	FJ234412.1
blaKPC-90	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-90	NG_076666.1	MZ404504.1
blaKPC-91	carbapenem-hydrolyzing class A beta-lactamase KPC-91	NG_076667.1	MZ404505.1
blaKPC-92	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-92	NG_079233.1	MZ461464.1
blaKPC-93	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-93	NG_080780.1	MZ569034.1
blaKPC-94	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-94	NG_076680.1	MZ646140.1
blaKPC-95	inhibitor-resistant extended-spectrum class A beta-lactamase KPC-95	NG_076681.1	MZ646141.1
blaKPC-96	carbapenem-hydrolyzing class A beta-lactamase KPC-96	NG_078037.1	OK086970.1
blaKPC-97	class A beta-lactamase KPC-97	NG_078038.1	OK086971.1
blaKPC-98	carbapenem-hydrolyzing class A beta-lactamase KPC-98	NG_078032.1	MZ893466.1
blaKPC-99	inhibitor-resistant carbapenem-hydrolyzing class A beta-lactamase KPC-99	NG_088405.1	OK086803.1

43 subtypes of NDM

Allele	**Product name**	**RefSeq nucleotide**	**GenBank nucleotide**
blaNDM-10	subclass B1 metallo-beta-lactamase NDM-10	NG_049327.1	KF361506.1
blaNDM-11	subclass B1 metallo-beta-lactamase NDM-11	NG_049328.1	KP265939.1
blaNDM-12	subclass B1 metallo-beta-lactamase NDM-12	NG_049329.1	AB926431.1
blaNDM-13	subclass B1 metallo-beta-lactamase NDM-13	NG_049330.1	LC012596.1

blaNDM-14	subclass B1 metallo-beta-lactamase NDM-14	NG_049331.1	KM210086.1
blaNDM-15	subclass B1 metallo-beta-lactamase NDM-15	NG_049332.1	KP735848.1
blaNDM-16a	subclass B1 metallo-beta-lactamase NDM-16a	NG_049333.1	KP862821.1
blaNDM-16b	subclass B1 metallo-beta-lactamase NDM-16b	NG_074726.1	AP024206.1
blaNDM-17	subclass B1 metallo-beta-lactamase NDM-17	NG_052662.1	KX812714.1
blaNDM-18	subclass B1 metallo-beta-lactamase NDM-18	NG_052866.1	KY503030.1
blaNDM-19	subclass B1 metallo-beta-lactamase NDM-19	NG_055498.1	MF370080.1
blaNDM-1	subclass B1 metallo-beta-lactamase NDM-1	NG_049326.1	FN396876.1
blaNDM-20	subclass B1 metallo-beta-lactamase NDM-20	NG_057455.1	KY654092.1
blaNDM-21	subclass B1 metallo-beta-lactamase NDM-21	NG_055664.1	MG183694.1
blaNDM-22	subclass B1 metallo-beta-lactamase NDM-22	NG_057612.1	MH243357.1
blaNDM-23	subclass B1 metallo-beta-lactamase NDM-23	NG_060570.1	MH450214.1
blaNDM-24	subclass B1 metallo-beta-lactamase NDM-24	NG_060571.1	MH450215.1
blaNDM-25	subclass B1 metallo-beta-lactamase NDM-25	NG_066711.1	MH986670.1
blaNDM-26	subclass B1 metallo-beta-lactamase NDM-26	NG_067144.1	MK079575.1
blaNDM-27	subclass B1 metallo-beta-lactamase NDM-27	NG_062358.1	MK105832.1
blaNDM-28	subclass B1 metallo-beta-lactamase NDM-28	NG_064729.1	MK425035.1
blaNDM-29	subclass B1 metallo-beta-lactamase NDM-29	NG_067145.1	MN624980.1
blaNDM-2	subclass B1 metallo-beta-lactamase NDM-2	NG_049334.1	JF703135.1
blaNDM-30	subclass B1 metallo-beta-lactamase NDM-30	NG_071206.1	MW306748.1
blaNDM-31	subclass B1 metallo-beta-lactamase NDM-31	NG_071207.1	MW306749.1
blaNDM-33	subclass B1 metallo-beta-lactamase NDM-33	NG_080782.1	MZ004933.1
blaNDM-34	subclass B1 metallo-beta-lactamase NDM-34	NG_076661.1	MZ254705.1
blaND	subclass B1 metallo-beta-lactamase NDM-	NG_07666	MZ265788.1

M-35	35	2.1	
blaNDM-36	subclass B1 metallo-beta-lactamase NDM-36	NG_076641.1	JAHAWL010000074.1
blaNDM-37	subclass B1 metallo-beta-lactamase NDM-37	NG_076642.1	CP091926.1
blaNDM-38	subclass B1 metallo-beta-lactamase NDM-38	NG_076664.1	MZ359766.1
blaNDM-39	subclass B1 metallo-beta-lactamase NDM-39	NG_076842.1	MZ748325.1
blaNDM-3	subclass B1 metallo-beta-lactamase NDM-3	NG_049335.1	JQ734687.1
blaNDM-40	subclass B1 metallo-beta-lactamase NDM-40	NG_076843.1	MZ748326.1
blaNDM-41	subclass B1 metallo-beta-lactamase NDM-41	NG_078034.1	MZ913436.1
blaNDM-42	subclass B1 metallo-beta-lactamase NDM-42	NG_080783.1	ON205946.1
blaNDM-43	subclass B1 metallo-beta-lactamase NDM-43	NG_081701.1	ON954084.1
blaNDM-44	subclass B1 metallo-beta-lactamase NDM-44	NG_088409.1	OP288001.1
blaNDM-45	subclass B1 metallo-beta-lactamase NDM-45	NG_148636.1	OP696898.1
blaNDM-46	subclass B1 metallo-beta-lactamase NDM-46	NG_148637.1	OP696899.1
blaNDM-47	subclass B1 metallo-beta-lactamase NDM-47	NG_148638.1	OP696900.1
blaNDM-48	subclass B1 metallo-beta-lactamase NDM-48	NG_148639.1	OP696902.1
blaNDM-49	subclass B1 metallo-beta-lactamase NDM-49	NG_149663.1	OP966824.1
blaNDM-4	subclass B1 metallo-beta-lactamase NDM-4	NG_049336.1	JQ348841.1
blaNDM-50	subclass B1 metallo-beta-lactamase NDM-50	NG_149664.1	ABJWWM020000052.1
blaNDM-51	subclass B1 metallo-beta-lactamase NDM-51	NG_157017.1	OQ442836.1
blaNDM-52	subclass B1 metallo-beta-lactamase NDM-52	NG_157018.1	OQ564973.1
blaNDM-53	subclass B1 metallo-beta-lactamase NDM-53	NG_157019.1	OQ595422.1
blaNDM-54	subclass B1 metallo-beta-lactamase NDM-54	NG_157020.1	OQ595423.1
blaNDM-55	subclass B1 metallo-beta-lactamase NDM-55	NG_157021.1	OQ708894.1
blaNDM-56	subclass B1 metallo-beta-lactamase NDM-56	NG_203399.1	OQ870699.1

blaNDM-57	subclass B1 metallo-beta-lactamase NDM-57	NG_203400.1	OQ870700.1
blaNDM-58	subclass B1 metallo-beta-lactamase NDM-58	NG_203401.1	OR081828.1
blaNDM-5	subclass B1 metallo-beta-lactamase NDM-5	NG_049337.1	JN104597.1
blaNDM-60	subclass B1 metallo-beta-lactamase NDM-60	NG_203402.1	OR139852.1
blaNDM-61	subclass B1 metallo-beta-lactamase NDM-61	NG_231554.1	DAPGEA010000082.1
blaNDM-6	subclass B1 metallo-beta-lactamase NDM-6	NG_049338.1	JN967644.1
blaNDM-7	subclass B1 metallo-beta-lactamase NDM-7	NG_049339.1	JX262694.1
blaNDM-8	subclass B1 metallo-beta-lactamase NDM-8	NG_049340.1	AB744718.1
blaNDM-9	taniborbactam-resistant subclass B1 metallo-beta-lactamase NDM-9	NG_049341.1	KC999080.2

B. Two-way ANOVA and Multiple comparisons of dLAMP optimization

a. Temperature 63°C

Two-way ANOVA of temperature 63 with different incubation times in band intensity and dLAMP product

Table Analyzed	Temperature 63°C band intensity and dLAMP product yield				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	23.80	<0.0001	****	Yes	
Row Factor	55.23	<0.0001	****	Yes	
Column Factor	13.80	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1729	5	345.8	F (5, 24) = 15.94	P<0.0001
Row Factor	4012	5	802.4	F (5, 24) = 36.99	P<0.0001
Column Factor	1003	1	1003	F (1, 24) = 46.22	P<0.0001
Residual	520.7	24	21.69		
Difference between columns means					
Mean of Band intensity (%)	11.17				
Mean of dLAMP product yield (ng/µL)	21.72				
Difference between means	-10.56				
SE of difference	1.553				
95% CI of difference	-13.76 to -7.351				
Data summary					
Number of columns (Column Factor)	2				
Number of rows (Row Factor)	6				
Number of values	36				

Tukey multiple comparisons of temperature 63 in different incubation times in band intensity and dLAMP product

WITHIN EACH COLUMN, COMPARE ROWS (SIMPLE EFFECTS WITHIN COLUMNS)					
NUMBER OF FAMILIES	2				
NUMBER OF COMPARISONS PER FAMILY	15				
ALPHA	0.05				
TUKEY'S MULTIPLE COMPARISONS TEST	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
BAND INTESITY (%)					
45 MINUTES VS. 50 MINUTES	2.333	-9.425 to 14.09	No	ns	0.9890
45 MINUTES VS. 55 MINUTES	0.3333	-11.43 to 12.09	No	ns	>0.9999
45 MINUTES VS. 60 MINUTES	-0.3333	-12.09 to 11.43	No	ns	>0.9999
45 MINUTES VS. 65 MINUTES	7.000	-4.759 to 18.76	No	ns	0.4603
45 MINUTES VS. 70 MINUTES	11.67	-0.09201 to 23.43	No	ns	0.0527
50 MINUTES VS. 55 MINUTES	-2.000	-13.76 to 9.759	No	ns	0.9946
50 MINUTES VS. 60 MINUTES	-2.667	-14.43 to 9.092	No	ns	0.9800
50 MINUTES VS. 65 MINUTES	4.667	-7.092 to 16.43	No	ns	0.8195
50 MINUTES VS. 70 MINUTES	9.333	-2.425 to 21.09	No	ns	0.1779
55 MINUTES VS. 60 MINUTES	-0.6667	-12.43 to 11.09	No	ns	>0.9999
55 MINUTES VS. 65 MINUTES	6.667	-5.092 to 18.43	No	ns	0.5125
55 MINUTES VS. 70 MINUTES	11.33	-0.4253 to 23.09	No	ns	0.0634
60 MINUTES VS. 65 MINUTES	7.333	-4.425 to 19.09	No	ns	0.4102
60 MINUTES VS. 70 MINUTES	12.00	0.2413 to 23.76	Yes	*	0.0436
65 MINUTES VS. 70 MINUTES	4.667	-7.092 to 16.43	No	ns	0.8195
DLAMP PRODUCT YIELD (NG/ML)					
45 MINUTES VS. 50 MINUTES	12.67	0.9080 to 24.43	Yes	*	0.0296
45 MINUTES VS. 55 MINUTES	27.33	15.57 to 39.09	Yes	****	<0.0001
45 MINUTES VS. 60 MINUTES	16.33	4.575 to 28.09	Yes	**	0.0030
45 MINUTES VS. 65 MINUTES	46.67	34.91 to 58.43	Yes	****	<0.0001
45 MINUTES VS. 70 MINUTES	46.67	34.91 to 58.43	Yes	****	<0.0001
50 MINUTES VS. 55 MINUTES	14.67	2.908 to 26.43	Yes	**	0.0087
50 MINUTES VS. 60 MINUTES	3.667	-8.092 to 15.43	No	ns	0.9248

50 MINUTES VS. 65 MINUTES	34.00	22.24 to 45.76	Yes	****	<0.0001			
50 MINUTES VS. 70 MINUTES	34.00	22.24 to 45.76	Yes	****	<0.0001			
55 MINUTES VS. 60 MINUTES	-11.00	-22.76 to 0.7587	No	ns	0.0761			
55 MINUTES VS. 65 MINUTES	19.33	7.575 to 31.09	Yes	***	0.0004			
55 MINUTES VS. 70 MINUTES	19.33	7.575 to 31.09	Yes	***	0.0004			
60 MINUTES VS. 65 MINUTES	30.33	18.57 to 42.09	Yes	****	<0.0001			
60 MINUTES VS. 70 MINUTES	30.33	18.57 to 42.09	Yes	****	<0.0001			
65 MINUTES VS. 70 MINUTES	0.000	-11.76 to 11.76	No	ns	>0.9999			
TEST DETAILS	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
BAND INTENSITY (%)								
45 MINUTES VS. 50 MINUTES	14.67	12.33	2.333	3.803	3	3	0.8677	24.00
45 MINUTES VS. 55 MINUTES	14.67	14.33	0.3333	3.803	3	3	0.1240	24.00
45 MINUTES VS. 60 MINUTES	14.67	15.00	-0.3333	3.803	3	3	0.1240	24.00
45 MINUTES VS. 65 MINUTES	14.67	7.667	7.000	3.803	3	3	2.603	24.00
45 MINUTES VS. 70 MINUTES	14.67	3.000	11.67	3.803	3	3	4.338	24.00
50 MINUTES VS. 55 MINUTES	12.33	14.33	-2.000	3.803	3	3	0.7437	24.00
50 MINUTES VS. 60 MINUTES	12.33	15.00	-2.667	3.803	3	3	0.9916	24.00
50 MINUTES VS. 65 MINUTES	12.33	7.667	4.667	3.803	3	3	1.735	24.00
50 MINUTES VS. 70 MINUTES	12.33	3.000	9.333	3.803	3	3	3.471	24.00
55 MINUTES VS. 60 MINUTES	14.33	15.00	-0.6667	3.803	3	3	0.2479	24.00
55 MINUTES VS. 65 MINUTES	14.33	7.667	6.667	3.803	3	3	2.479	24.00
55 MINUTES VS. 70 MINUTES	14.33	3.000	11.33	3.803	3	3	4.214	24.00
60 MINUTES VS. 65 MINUTES	15.00	7.667	7.333	3.803	3	3	2.727	24.00
60 MINUTES VS. 70 MINUTES	15.00	3.000	12.00	3.803	3	3	4.462	24.00

65 MINUTES VS. 70 MINUTES	7.667	3.000	4.667	3.803	3	3	1.735	24.00
DLAMP PRODUCT YIELD (NG/ML)								
45 MINUTES VS. 50 MINUTES	46.67	34.00	12.67	3.803	3	3	4.710	24.00
45 MINUTES VS. 55 MINUTES	46.67	19.33	27.33	3.803	3	3	10.16	24.00
45 MINUTES VS. 60 MINUTES	46.67	30.33	16.33	3.803	3	3	6.074	24.00
45 MINUTES VS. 65 MINUTES	46.67	0.000	46.67	3.803	3	3	17.35	24.00
45 MINUTES VS. 70 MINUTES	46.67	0.000	46.67	3.803	3	3	17.35	24.00
50 MINUTES VS. 55 MINUTES	34.00	19.33	14.67	3.803	3	3	5.454	24.00
50 MINUTES VS. 60 MINUTES	34.00	30.33	3.667	3.803	3	3	1.364	24.00
50 MINUTES VS. 65 MINUTES	34.00	0.000	34.00	3.803	3	3	12.64	24.00
50 MINUTES VS. 70 MINUTES	34.00	0.000	34.00	3.803	3	3	12.64	24.00
55 MINUTES VS. 60 MINUTES	19.33	30.33	-11.00	3.803	3	3	4.091	24.00
55 MINUTES VS. 65 MINUTES	19.33	0.000	19.33	3.803	3	3	7.189	24.00
55 MINUTES VS. 70 MINUTES	19.33	0.000	19.33	3.803	3	3	7.189	24.00
60 MINUTES VS. 65 MINUTES	30.33	0.000	30.33	3.803	3	3	11.28	24.00
60 MINUTES VS. 70 MINUTES	30.33	0.000	30.33	3.803	3	3	11.28	24.00

b. Temperature 65°C

Two-way ANOVA of temperature 65 with different incubation times in band intensity and dLAMP product

Table Analyzed	Temperature 65°C band intensity and dLAMP product yield				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P valu	P value summary	Significant?	

		e			
Interaction	9.807	0.0154	*	Yes	
Row Factor	28.51	<0.0001	****	Yes	
Column Factor	48.39	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	218.5	5	43.69	F (5, 24) = 3.543	P=0.0154
Row Factor	635.1	5	127.0	F (5, 24) = 10.30	P<0.0001
Column Factor	1078	1	1078	F (1, 24) = 87.41	P<0.0001
Residual	296.0	24	12.33		
Difference between column means					
Mean of Band intesity (%)	19.22				
Mean of dLAMP product yield (ng/µL)	30.17				
Difference between means	-10.94				
SE of difference	1.171				
95% CI of difference	-13.36 to -8.528				
Data summary					
Number of columns (Column Factor)	2				
Number of rows (Row Factor)	6				
Number of values	36				

Tukey multiple comparisons of temperature 65 in different incubation times in band intensity and dLAMP product

Within each column, compare rows (simple effects within columns)					
Number of families	2				
Number of comparisons per family	15				
Alpha	0.05				
Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold ?	Summary	Adjusted P Value
Band intesity (%)					
45 minutes vs. 50 minutes	-0.3333	-9.648 to 8.982	No	ns	>0.9999
45 minutes vs. 55 minutes	-1.667	-10.98 to 7.648	No	ns	>0.9999
45 minutes vs. 60 minutes	4.000	-5.315 to	No	ns	0.9450

		13.32						
45 minutes vs. 65 minutes	-1.000	-10.32 to 8.315	No	ns	>0.9999			
45 minutes vs. 70 minutes	11.67	2.352 to 20.98	Yes	**	0.0066			
50 minutes vs. 55 minutes	-1.333	-10.65 to 7.982	No	ns	>0.9999			
50 minutes vs. 60 minutes	4.333	-4.982 to 13.65	No	ns	0.9026			
50 minutes vs. 65 minutes	-0.6667	-9.982 to 8.648	No	ns	>0.9999			
50 minutes vs. 70 minutes	12.00	2.685 to 21.32	Yes	**	0.0049			
55 minutes vs. 60 minutes	5.667	-3.648 to 14.98	No	ns	0.6030			
55 minutes vs. 65 minutes	0.6667	-8.648 to 9.982	No	ns	>0.9999			
55 minutes vs. 70 minutes	13.33	4.018 to 22.65	Yes	**	0.0015			
60 minutes vs. 65 minutes	-5.000	-14.32 to 4.315	No	ns	0.7726			
60 minutes vs. 70 minutes	7.667	-1.648 to 16.98	No	ns	0.1817			
65 minutes vs. 70 minutes	12.67	3.352 to 21.98	Yes	**	0.0027			
dLAMP product yield (ng/µL)								
45 minutes vs. 50 minutes	-13.67	-22.98 to -4.352	Yes	**	0.0011			
45 minutes vs. 55 minutes	-12.67	-21.98 to -3.352	Yes	**	0.0027			
45 minutes vs. 60 minutes	-6.333	-15.65 to 2.982	No	ns	0.4319			
45 minutes vs. 65 minutes	-12.33	-21.65 to -3.018	Yes	**	0.0037			
45 minutes vs. 70 minutes	-4.000	-13.32 to 5.315	No	ns	0.9450			
50 minutes vs. 55 minutes	1.000	-8.315 to 10.32	No	ns	>0.9999			
50 minutes vs. 60 minutes	7.333	-1.982 to 16.65	No	ns	0.2301			
50 minutes vs. 65 minutes	1.333	-7.982 to 10.65	No	ns	>0.9999			
50 minutes vs. 70 minutes	9.667	0.3515 to 18.98	Yes	*	0.0373			
55 minutes vs. 60 minutes	6.333	-2.982 to 15.65	No	ns	0.4319			
55 minutes vs. 65 minutes	0.3333	-8.982 to 9.648	No	ns	>0.9999			
55 minutes vs. 70 minutes	8.667	-0.6485 to 17.98	No	ns	0.0847			
60 minutes vs. 65 minutes	-6.000	-15.32 to 3.315	No	ns	0.5154			
60 minutes vs. 70 minutes	2.333	-6.982 to 11.65	No	ns	0.9997			
65 minutes vs. 70 minutes	8.333	-0.9818 to 17.65	No	ns	0.1101			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N 2	t	DF
Band intesity (%)								
45 minutes vs. 50 minutes	21.33	21.67	-0.3333	2.867	3	3	0.1162	24.00
45 minutes vs. 55 minutes	21.33	23.00	-1.667	2.867	3	3	0.5	24.

45 minutes vs. 60 minutes	21.33	17.33	4.000	2.867	3	3	1.812	0.0024
45 minutes vs. 65 minutes	21.33	22.33	-1.000	2.867	3	3	0.395	0.0024
45 minutes vs. 70 minutes	21.33	9.667	11.67	2.867	3	3	4.087	0.0024
50 minutes vs. 55 minutes	21.67	23.00	-1.333	2.867	3	3	0.469	0.0024
50 minutes vs. 60 minutes	21.67	17.33	4.333	2.867	3	3	1.516	0.0024
50 minutes vs. 65 minutes	21.67	22.33	-0.6667	2.867	3	3	0.211	0.0024
50 minutes vs. 70 minutes	21.67	9.667	12.00	2.867	3	3	4.185	0.0024
55 minutes vs. 60 minutes	23.00	17.33	5.667	2.867	3	3	1.976	0.0024
55 minutes vs. 65 minutes	23.00	22.33	0.6667	2.867	3	3	0.211	0.0024
55 minutes vs. 70 minutes	23.00	9.667	13.33	2.867	3	3	4.650	0.0024
60 minutes vs. 65 minutes	17.33	22.33	-5.000	2.867	3	3	1.744	0.0024
60 minutes vs. 70 minutes	17.33	9.667	7.667	2.867	3	3	2.674	0.0024
65 minutes vs. 70 minutes	22.33	9.667	12.67	2.867	3	3	4.417	0.0024
dLAMP product yield (ng/µL)								
45 minutes vs. 50 minutes	22.00	35.67	-13.67	2.867	3	3	4.766	0.0024
45 minutes vs. 55 minutes	22.00	34.67	-12.67	2.867	3	3	4.417	0.0024
45 minutes vs. 60 minutes	22.00	28.33	-6.333	2.867	3	3	2.209	0.0024
45 minutes vs. 65 minutes	22.00	34.33	-12.33	2.867	3	3	4.301	0.0024
45 minutes vs. 70 minutes	22.00	26.00	-4.000	2.867	3	3	1.395	0.0024
50 minutes vs. 55 minutes	35.67	34.67	1.000	2.867	3	3	0.348	0.0024
50 minutes vs. 60 minutes	35.67	28.33	7.333	2.867	3	3	2.557	0.0024
50 minutes vs. 65 minutes	35.67	34.33	1.333	2.867	3	3	0.465	0.0024
50 minutes vs. 70 minutes	35.67	26.00	9.667	2.867	3	3	3.371	0.0024
55 minutes vs. 60 minutes	34.67	28.33	6.333	2.867	3	3	2.209	0.0024
55 minutes vs. 65 minutes	34.67	34.33	0.3333	2.867	3	3	0.116	0.0024
55 minutes vs. 70 minutes	34.67	26.00	8.667	2.867	3	3	3.022	0.0024
60 minutes vs. 65 minutes	28.33	34.33	-6.000	2.867	3	3	2.092	0.0024
60 minutes vs. 70 minutes	28.33	26.00	2.333	2.867	3	3	0.813	0.0024
65 minutes vs. 70 minutes	34.33	26.00	8.333	2.867	3	3	2.906	0.0024

c. Temperature 67°C

Two-way ANOVA of temperature 67 with different incubation times in band intensity and dLAMP product

Table Analyzed	Temperature 67°C band intensity and dLAMP product yield				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	3.030	0.5133	ns	No	
Row Factor	2.426	0.6288	ns	No	
Column Factor	75.30	<0.0001	****	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	39.55	5	7.910	F (5, 23) = 0.8747	P=0.5133
Row Factor	31.67	5	6.333	F (5, 23) = 0.7003	P=0.6288
Column Factor	983.0	1	983.0	F (1, 23) = 108.7	P<0.0001
Residual	208.0	23	9.043		
Difference between column means					
Predicted (LS) mean of Band intesity (%)	9.722				
Predicted (LS) mean of dLAMP product yield (ng/μL)	20.39				
Difference between predicted means	-10.67				
SE of difference	1.023				
95% CI of difference	-12.78 to -8.550				
Data summary					
Number of columns (Column Factor)	2				
Number of rows (Row	6				

Factor)					
Number of values	35				

Tukey multiple comparisons of temperature 67 in different incubation times in band intensity and dLAMP product

WITHIN EACH COLUMN, COMPARE ROWS (SIMPLE EFFECTS WITHIN COLUMNS)

NUMBER OF FAMILIES	2				
NUMBER OF COMPARISONS PER FAMILY	15				
ALPHA	0.05				
ŠÍDÁK'S MULTIPLE COMPARISONS TEST	Predicted (LS) mean diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
BAND INTESITY (%)					
45 MINUTES VS. 50 MINUTES	1.000	-7.014 to 9.014	No	ns	>0.9999
45 MINUTES VS. 55 MINUTES	1.333	-6.681 to 9.348	No	ns	>0.9999
45 MINUTES VS. 60 MINUTES	0.3333	-7.681 to 8.348	No	ns	>0.9999
45 MINUTES VS. 65 MINUTES	0.3333	-7.681 to 8.348	No	ns	>0.9999
45 MINUTES VS. 70 MINUTES	-3.333	-12.29 to 5.627	No	ns	0.9827
50 MINUTES VS. 55 MINUTES	0.3333	-7.681 to 8.348	No	ns	>0.9999
50 MINUTES VS. 60 MINUTES	-0.6667	-8.681 to 7.348	No	ns	>0.9999
50 MINUTES VS. 65 MINUTES	-0.6667	-8.681 to 7.348	No	ns	>0.9999
50 MINUTES VS. 70 MINUTES	-4.333	-13.29 to 4.627	No	ns	0.8721
55 MINUTES VS. 60 MINUTES	-1.000	-9.014 to 7.014	No	ns	>0.9999
55 MINUTES VS. 65 MINUTES	-1.000	-9.014 to 7.014	No	ns	>0.9999
55 MINUTES VS. 70 MINUTES	-4.667	-13.63 to 4.294	No	ns	0.8030
60 MINUTES VS. 65 MINUTES	0.000	-8.014 to 8.014	No	ns	>0.9999
60 MINUTES VS. 70 MINUTES	-3.667	-12.63 to 5.294	No	ns	0.9612
65 MINUTES VS. 70 MINUTES	-3.667	-12.63 to 5.294	No	ns	0.9612
DLAMP PRODUCT YIELD (NG/ML)					
45 MINUTES VS. 50 MINUTES	0.000	-8.014 to 8.014	No	ns	>0.9999
45 MINUTES VS. 55 MINUTES	-0.3333	-8.348 to 7.681	No	ns	>0.9999
45 MINUTES VS. 60 MINUTES	3.667	-4.348 to 11.68	No	ns	0.9110
45 MINUTES VS. 65 MINUTES	-0.3333	-8.348 to 7.681	No	ns	>0.9999
45 MINUTES VS. 70 MINUTES	0.6667	-7.348 to 8.681	No	ns	>0.9999
50 MINUTES VS. 55 MINUTES	-0.3333	-8.348 to 7.681	No	ns	>0.9999
50 MINUTES VS. 60 MINUTES	3.667	-4.348 to 11.68	No	ns	0.9110

50 MINUTES VS. 65 MINUTES	-0.3333	-8.348 to 7.681	No	ns	>0.9999			
50 MINUTES VS. 70 MINUTES	0.6667	-7.348 to 8.681	No	ns	>0.9999			
55 MINUTES VS. 60 MINUTES	4.000	-4.014 to 12.01	No	ns	0.8451			
55 MINUTES VS. 65 MINUTES	0.000	-8.014 to 8.014	No	ns	>0.9999			
55 MINUTES VS. 70 MINUTES	1.000	-7.014 to 9.014	No	ns	>0.9999			
60 MINUTES VS. 65 MINUTES	-4.000	-12.01 to 4.014	No	ns	0.8451			
60 MINUTES VS. 70 MINUTES	-3.000	-11.01 to 5.014	No	ns	0.9817			
65 MINUTES VS. 70 MINUTES	1.000	-7.014 to 9.014	No	ns	>0.9999			
TEST DETAILS	Predicted (LS) mean 1	Predicted (LS) mean 2	Predicted (LS) mean diff.	SE of diff.	N1	N2	t	DF
BAND INTESITY (%)								
45 MINUTES VS. 50 MINUTES	9.667	8.667	1.000	2.455	3	3	0.4073	23.00
45 MINUTES VS. 55 MINUTES	9.667	8.333	1.333	2.455	3	3	0.5430	23.00
45 MINUTES VS. 60 MINUTES	9.667	9.333	0.3333	2.455	3	3	0.1358	23.00
45 MINUTES VS. 65 MINUTES	9.667	9.333	0.3333	2.455	3	3	0.1358	23.00
45 MINUTES VS. 70 MINUTES	9.667	13.00	-3.333	2.745	3	2	1.214	23.00
50 MINUTES VS. 55 MINUTES	8.667	8.333	0.3333	2.455	3	3	0.1358	23.00
50 MINUTES VS. 60 MINUTES	8.667	9.333	-0.6667	2.455	3	3	0.2715	23.00
50 MINUTES VS. 65 MINUTES	8.667	9.333	-0.6667	2.455	3	3	0.2715	23.00
50 MINUTES VS. 70 MINUTES	8.667	13.00	-4.333	2.745	3	2	1.579	23.00
55 MINUTES VS. 60 MINUTES	8.333	9.333	-1.000	2.455	3	3	0.4073	23.00
55 MINUTES VS. 65 MINUTES	8.333	9.333	-1.000	2.455	3	3	0.4073	23.0

55 MINUTES VS. 70 MINUTES	8.333	13.00	-4.667	2.745	3	2	1.700	0.0023
60 MINUTES VS. 65 MINUTES	9.333	9.333	0.000	2.455	3	3	0.0000	0.0023
60 MINUTES VS. 70 MINUTES	9.333	13.00	-3.667	2.745	3	2	1.336	0.0023
65 MINUTES VS. 70 MINUTES	9.333	13.00	-3.667	2.745	3	2	1.336	0.0023
DLAMP PRODUCT YIELD (NG/ML)								
45 MINUTES VS. 50 MINUTES	21.00	21.00	0.000	2.455	3	3	0.0000	0.0023
45 MINUTES VS. 55 MINUTES	21.00	21.33	-0.3333	2.455	3	3	0.1358	0.0023
45 MINUTES VS. 60 MINUTES	21.00	17.33	3.667	2.455	3	3	1.493	0.0023
45 MINUTES VS. 65 MINUTES	21.00	21.33	-0.3333	2.455	3	3	0.1358	0.0023
45 MINUTES VS. 70 MINUTES	21.00	20.33	0.6667	2.455	3	3	0.2715	0.0023
50 MINUTES VS. 55 MINUTES	21.00	21.33	-0.3333	2.455	3	3	0.1358	0.0023
50 MINUTES VS. 60 MINUTES	21.00	17.33	3.667	2.455	3	3	1.493	0.0023
50 MINUTES VS. 65 MINUTES	21.00	21.33	-0.3333	2.455	3	3	0.1358	0.0023
50 MINUTES VS. 70 MINUTES	21.00	20.33	0.6667	2.455	3	3	0.2715	0.0023
55 MINUTES VS. 60 MINUTES	21.33	17.33	4.000	2.455	3	3	1.629	0.0023
55 MINUTES VS. 65 MINUTES	21.33	21.33	0.000	2.455	3	3	0.0000	0.0023
55 MINUTES VS. 70 MINUTES	21.33	20.33	1.000	2.45	3	3	0.	2

				5			40 73	3.000
60 MINUTES VS. 65 MINUTES	17.33	21.33	-4.000	2.455	3	3	1.629	23.00
60 MINUTES VS. 70 MINUTES	17.33	20.33	-3.000	2.455	3	3	1.222	23.00
65 MINUTES VS. 70 MINUTES	21.33	20.33	1.000	2.455	3	3	0.4073	23.00

d. Temperature 69°C

Two-way ANOVA of temperature 69 with different incubation times in band intensity and dLAMP product

Table Analyzed	Temperature 69°C band intensity and dLAMP product yield				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	7.147	0.0008	***	Yes	
Row Factor	85.69	<0.0001	****	Yes	
Column Factor	1.620	0.0141	*	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	117.8	5	23.56	F (5, 24) = 6.191	P=0.0008
Row Factor	1412	5	282.5	F (5, 24) = 74.23	P<0.0001
Column Factor	26.69	1	26.69	F (1, 24) = 7.015	P=0.0141
Residual	91.33	24	3.806		
Difference between column means					
Mean of Band intesity (%)	6.000				
Mean of dLAMP	7.722				

product yield (ng/µL)					
Difference between means	-1.722				
SE of difference	0.6503				
95% CI of difference	-3.064 to -0.3801				
Data summary					
Number of columns (Column Factor)	2				
Number of rows (Row Factor)	6				
Number of values	36				

Tukey multiple comparisons of temperature 69 in different incubation times in band intensity and dLAMP product

WITHIN EACH COLUMN, COMPARE ROWS (SIMPLE EFFECTS WITHIN COLUMNS)					
NUMBER OF FAMILIES	2				
NUMBER OF COMPARISONS PER FAMILY	15				
ALPHA	0.05				
TUKEY'S MULTIPLE COMPARISONS TEST	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
BAND INTESITY (%)					
45 MINUTES VS. 50 MINUTES	0.000	-4.925 to 4.925	No	ns	>0.9999
45 MINUTES VS. 55 MINUTES	-1.000	-5.925 to 3.925	No	ns	0.9878
45 MINUTES VS. 60 MINUTES	-5.667	-10.59 to -0.7418	Yes	*	0.0176
45 MINUTES VS. 65 MINUTES	-8.000	-12.92 to -3.075	Yes	***	0.0005
45 MINUTES VS. 70 MINUTES	-13.33	-18.26 to -8.408	Yes	****	<0.0001
50 MINUTES VS. 55 MINUTES	-1.000	-5.925 to 3.925	No	ns	0.9878
50 MINUTES VS. 60 MINUTES	-5.667	-10.59 to -0.7418	Yes	*	0.0176
50 MINUTES VS. 65 MINUTES	-8.000	-12.92 to -3.075	Yes	***	0.0005
50 MINUTES VS. 70 MINUTES	-13.33	-18.26 to -8.408	Yes	****	<0.0001
55 MINUTES VS. 60 MINUTES	-4.667	-9.592 to 0.2582	No	ns	0.0705
55 MINUTES VS. 65 MINUTES	-7.000	-11.92 to -	Yes	**	0.0024

	0	2.075						
55 MINUTES VS. 70 MINUTES	-12.33	-17.26 to -7.408	Yes	****	<0.0001			
60 MINUTES VS. 65 MINUTES	-2.333	-7.258 to 2.592	No	ns	0.6886			
60 MINUTES VS. 70 MINUTES	-7.667	-12.59 to -2.742	Yes	***	0.0008			
65 MINUTES VS. 70 MINUTES	-5.333	-10.26 to -0.4085	Yes	*	0.0284			
DLAMP PRODUCT YIELD (NG/ML)								
45 MINUTES VS. 50 MINUTES	0.000	-4.925 to 4.925	No	ns	>0.9999			
45 MINUTES VS. 55 MINUTES	0.000	-4.925 to 4.925	No	ns	>0.9999			
45 MINUTES VS. 60 MINUTES	-13.33	-18.26 to -8.408	Yes	****	<0.0001			
45 MINUTES VS. 65 MINUTES	-15.67	-20.59 to -10.74	Yes	****	<0.0001			
45 MINUTES VS. 70 MINUTES	-17.33	-22.26 to -12.41	Yes	****	<0.0001			
50 MINUTES VS. 55 MINUTES	0.000	-4.925 to 4.925	No	ns	>0.9999			
50 MINUTES VS. 60 MINUTES	-13.33	-18.26 to -8.408	Yes	****	<0.0001			
50 MINUTES VS. 65 MINUTES	-15.67	-20.59 to -10.74	Yes	****	<0.0001			
50 MINUTES VS. 70 MINUTES	-17.33	-22.26 to -12.41	Yes	****	<0.0001			
55 MINUTES VS. 60 MINUTES	-13.33	-18.26 to -8.408	Yes	****	<0.0001			
55 MINUTES VS. 65 MINUTES	-15.67	-20.59 to -10.74	Yes	****	<0.0001			
55 MINUTES VS. 70 MINUTES	-17.33	-22.26 to -12.41	Yes	****	<0.0001			
60 MINUTES VS. 65 MINUTES	-2.333	-7.258 to 2.592	No	ns	0.6886			
60 MINUTES VS. 70 MINUTES	-4.000	-8.925 to 0.9249	No	ns	0.1603			
65 MINUTES VS. 70 MINUTES	-1.667	-6.592 to 3.258	No	ns	0.8972			
TEST DETAILS	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
BAND INTESITY (%)								
45 MINUTES VS. 50 MINUTES	1.333	1.333	0.000	1.593	3	3	0.000	24.00

45 MINUTES VS. 55 MINUTES	1.333	2.333	-1.000	1.593	3	3	0.8879	24.00
45 MINUTES VS. 60 MINUTES	1.333	7.000	-5.667	1.593	3	3	5.031	24.00
45 MINUTES VS. 65 MINUTES	1.333	9.333	-8.000	1.593	3	3	7.103	24.00
45 MINUTES VS. 70 MINUTES	1.333	14.67	-13.33	1.593	3	3	11.84	24.00
50 MINUTES VS. 55 MINUTES	1.333	2.333	-1.000	1.593	3	3	0.8879	24.00
50 MINUTES VS. 60 MINUTES	1.333	7.000	-5.667	1.593	3	3	5.031	24.00
50 MINUTES VS. 65 MINUTES	1.333	9.333	-8.000	1.593	3	3	7.103	24.00
50 MINUTES VS. 70 MINUTES	1.333	14.67	-13.33	1.593	3	3	11.84	24.00
55 MINUTES VS. 60 MINUTES	2.333	7.000	-4.667	1.593	3	3	4.143	24.00
55 MINUTES VS. 65 MINUTES	2.333	9.333	-7.000	1.593	3	3	6.215	24.00
55 MINUTES VS. 70 MINUTES	2.333	14.67	-12.33	1.593	3	3	10.95	24.00
60 MINUTES VS. 65 MINUTES	7.000	9.333	-2.333	1.593	3	3	2.072	24.00
60 MINUTES VS. 70 MINUTES	7.000	14.67	-7.667	1.593	3	3	6.807	24.00
65 MINUTES VS. 70 MINUTES	9.333	14.67	-5.333	1.593	3	3	4.735	24.00
DLAMP PRODUCT YIELD (NG/ML)								
45 MINUTES VS. 50 MINUTES	0.000	0.000	0.000	1.593	3	3	0.000	24.00
45 MINUTES VS. 55 MINUTES	0.000	0.000	0.000	1.593	3	3	0.000	24.00
45 MINUTES VS. 60 MINUTES	0.000	13.33	-13.33	1.593	3	3	11.84	24.00
45 MINUTES VS. 65 MINUTES	0.000	15.67	-15.67	1.593	3	3	13.91	24.00
45 MINUTES VS. 70 MINUTES	0.000	17.33	-17.33	1.593	3	3	15.39	24.00
50 MINUTES VS. 55 MINUTES	0.000	0.000	0.000	1.593	3	3	0.000	24.00
50 MINUTES VS. 60 MINUTES	0.000	13.33	-13.33	1.593	3	3	11.84	24.0

50 MINUTES VS. 65 MINUTES	0.000	15.67	-15.67	1.593	3	3	13.91	0.0024
50 MINUTES VS. 70 MINUTES	0.000	17.33	-17.33	1.593	3	3	15.39	0.0024
55 MINUTES VS. 60 MINUTES	0.000	13.33	-13.33	1.593	3	3	11.84	0.0024
55 MINUTES VS. 65 MINUTES	0.000	15.67	-15.67	1.593	3	3	13.91	0.0024
55 MINUTES VS. 70 MINUTES	0.000	17.33	-17.33	1.593	3	3	15.39	0.0024
60 MINUTES VS. 65 MINUTES	13.33	15.67	-2.333	1.593	3	3	2.072	0.0024
60 MINUTES VS. 70 MINUTES	13.33	17.33	-4.000	1.593	3	3	3.551	0.0024
65 MINUTES VS. 70 MINUTES	15.67	17.33	-1.667	1.593	3	3	1.480	0.0024

e. Temperature 71°C

Two-way ANOVA of temperature 71 with different incubation times in band intensity and dLAMP product

Table Analyzed	Temperature 71°C band intensity and dLAMP product yield				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	0.2837	0.9678	ns	No	
Row Factor	1.531	0.4766	ns	No	
Column Factor	92.76	<0.0001	****	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	3.014	5	0.6027	F (5, 16) = 0.1759	P=0.9678
Row Factor	16.27	5	3.253	F (5, 16)	P=0.

				= 0.9493	4766
Column Factor	985.5	1	985.5	F (1, 16) = 287.6	P<0.0001
Residual	54.83	16	3.427		
Difference between column means					
Predicted (LS) mean of Band intesity (%)	1.500				
Predicted (LS) mean of dLAMP product yield (ng/μL)	13.58				
Difference between predicted means	-12.08				
SE of difference	0.7125				
95% CI of difference	-13.59 to -10.57				
Data summary					
Number of columns (Column Factor)	2				
Number of rows (Row Factor)	6				
Number of values	28				

Tukey multiple comparisons of temperature 71 in different incubation times in band intensity and dLAMP product

WITHIN EACH COLUMN, COMPARE ROWS (SIMPLE EFFECTS WITHIN COLUMNS)					
NUMBER OF FAMILIES	2				
NUMBER OF COMPARISONS PER FAMILY	15				
ALPHA	0.05				
TUKEY'S MULTIPLE COMPARISONS TEST	Predicted (LS) mean diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
BAND INTESITY (%)					
45 MINUTES VS. 50 MINUTES	-0.5000	-6.465 to 5.465	No	ns	0.9998
45 MINUTES VS. 55 MINUTES	0.000	-5.965 to 5.965	No	ns	>0.9999
45 MINUTES VS. 60 MINUTES	0.000	-5.965 to 5.965	No	ns	>0.9999
45 MINUTES VS. 65 MINUTES	0.000	-5.965 to 5.965	No	ns	>0.9999
45 MINUTES VS. 70 MINUTES	-2.500	-8.465 to 3.465	No	ns	0.7540
50 MINUTES VS. 55 MINUTES	0.5000	-5.465 to 6.465	No	ns	0.9998
50 MINUTES VS. 60 MINUTES	0.5000	-5.465 to 6.465	No	ns	0.9998
50 MINUTES VS. 65 MINUTES	0.5000	-5.465 to 6.465	No	ns	0.9998

50 MINUTES VS. 70 MINUTES	-2.000	-7.965 to 3.965	No	ns	0.8821			
55 MINUTES VS. 60 MINUTES	0.000	-5.965 to 5.965	No	ns	>0.9999			
55 MINUTES VS. 65 MINUTES	0.000	-5.965 to 5.965	No	ns	>0.9999			
55 MINUTES VS. 70 MINUTES	-2.500	-8.465 to 3.465	No	ns	0.7540			
60 MINUTES VS. 65 MINUTES	0.000	-5.965 to 5.965	No	ns	>0.9999			
60 MINUTES VS. 70 MINUTES	-2.500	-8.465 to 3.465	No	ns	0.7540			
65 MINUTES VS. 70 MINUTES	-2.500	-8.465 to 3.465	No	ns	0.7540			
DLAMP PRODUCT YIELD (NG/ML)								
45 MINUTES VS. 50 MINUTES	0.5000	-4.945 to 5.945	No	ns	0.9996			
45 MINUTES VS. 55 MINUTES	0.8333	-4.612 to 6.279	No	ns	0.9957			
45 MINUTES VS. 60 MINUTES	0.5000	-4.945 to 5.945	No	ns	0.9996			
45 MINUTES VS. 65 MINUTES	-0.8333	-6.279 to 4.612	No	ns	0.9957			
45 MINUTES VS. 70 MINUTES	-1.500	-7.465 to 4.465	No	ns	0.9614			
50 MINUTES VS. 55 MINUTES	0.3333	-4.537 to 5.204	No	ns	>0.9999			
50 MINUTES VS. 60 MINUTES	0.000	-4.870 to 4.870	No	ns	>0.9999			
50 MINUTES VS. 65 MINUTES	-1.333	-6.204 to 3.537	No	ns	0.9455			
50 MINUTES VS. 70 MINUTES	-2.000	-7.445 to 3.445	No	ns	0.8383			
55 MINUTES VS. 60 MINUTES	-0.3333	-5.204 to 4.537	No	ns	>0.9999			
55 MINUTES VS. 65 MINUTES	-1.667	-6.537 to 3.204	No	ns	0.8733			
55 MINUTES VS. 70 MINUTES	-2.333	-7.779 to 3.112	No	ns	0.7374			
60 MINUTES VS. 65 MINUTES	-1.333	-6.204 to 3.537	No	ns	0.9455			
60 MINUTES VS. 70 MINUTES	-2.000	-7.445 to 3.445	No	ns	0.8383			
65 MINUTES VS. 70 MINUTES	-0.6667	-6.112 to 4.779	No	ns	0.9985			
TEST DETAILS	Predicted (LS) mean 1	Predicted (LS) mean 2	Predicted (LS) mean diff.	SE of diff.	N1	N2	q	DF
BAND INTESITY (%)								
45 MINUTES VS. 50 MINUTES	1.000	1.500	-0.5000	1.851	2	2	0.3820	16.00
45 MINUTES VS. 55 MINUTES	1.000	1.000	0.000	1.851	2	2	0.000	16.00
45 MINUTES VS. 60 MINUTES	1.000	1.000	0.000	1.851	2	2	0.000	16.00
45 MINUTES VS. 65 MINUTES	1.000	1.000	0.000	1.851	2	2	0.00	16.

							0	0 0
45 MINUTES VS. 70 MINUTES	1.000	3.500	-2.500	1.851	2	2	1.910	16.00
50 MINUTES VS. 55 MINUTES	1.500	1.000	0.5000	1.851	2	2	0.3820	16.00
50 MINUTES VS. 60 MINUTES	1.500	1.000	0.5000	1.851	2	2	0.3820	16.00
50 MINUTES VS. 65 MINUTES	1.500	1.000	0.5000	1.851	2	2	0.3820	16.00
50 MINUTES VS. 70 MINUTES	1.500	3.500	-2.000	1.851	2	2	1.528	16.00
55 MINUTES VS. 60 MINUTES	1.000	1.000	0.000	1.851	2	2	0.000	16.00
55 MINUTES VS. 65 MINUTES	1.000	1.000	0.000	1.851	2	2	0.000	16.00
55 MINUTES VS. 70 MINUTES	1.000	3.500	-2.500	1.851	2	2	1.910	16.00
60 MINUTES VS. 65 MINUTES	1.000	1.000	0.000	1.851	2	2	0.000	16.00
60 MINUTES VS. 70 MINUTES	1.000	3.500	-2.500	1.851	2	2	1.910	16.00
65 MINUTES VS. 70 MINUTES	1.000	3.500	-2.500	1.851	2	2	1.910	16.00
DLAMP PRODUCT YIELD (NG/ML)								
45 MINUTES VS. 50 MINUTES	13.50	13.00	0.5000	1.690	2	3	0.4184	16.00
45 MINUTES VS. 55 MINUTES	13.50	12.67	0.8333	1.690	2	3	0.6974	16.00
45 MINUTES VS. 60 MINUTES	13.50	13.00	0.5000	1.690	2	3	0.4184	16.00
45 MINUTES VS. 65 MINUTES	13.50	14.33	-0.8333	1.690	2	3	0.6974	16.00

45 MINUTES VS. 70 MINUTES	13.50	15.00	-1.500	1.851	2	2	1.146	16.00
50 MINUTES VS. 55 MINUTES	13.00	12.67	0.3333	1.512	3	3	0.3119	16.00
50 MINUTES VS. 60 MINUTES	13.00	13.00	0.000	1.512	3	3	0.0000	16.00
50 MINUTES VS. 65 MINUTES	13.00	14.33	-1.333	1.512	3	3	1.247	16.00
50 MINUTES VS. 70 MINUTES	13.00	15.00	-2.000	1.690	3	2	1.674	16.00
55 MINUTES VS. 60 MINUTES	12.67	13.00	-0.3333	1.512	3	3	0.3119	16.00
55 MINUTES VS. 65 MINUTES	12.67	14.33	-1.667	1.512	3	3	1.559	16.00
55 MINUTES VS. 70 MINUTES	12.67	15.00	-2.333	1.690	3	2	1.953	16.00
60 MINUTES VS. 65 MINUTES	13.00	14.33	-1.333	1.512	3	3	1.247	16.00
60 MINUTES VS. 70 MINUTES	13.00	15.00	-2.000	1.690	3	2	1.674	16.00
65 MINUTES VS. 70 MINUTES	14.33	15.00	-0.6667	1.690	3	2	0.5579	16.00

2. Two-way ANOVA on MgSO4 and HNB concentration di dLAMP

Two-way ANAVA on MgSO4 and HNB concentration in dLAMP

Table Analyzed	UVVIS				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant ?	
Interaction	10.19	<0.0001	****	Yes	
Row Factor	81.74	<0.0001	****	Yes	
Column Factor	6.576	<0.0	****	Yes	

		001			
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.001731	8	0.0002164	F (8, 30) = 25.43	P<0.0001
Row Factor	0.01389	2	0.006946	F (2, 30) = 816.1	P<0.0001
Column Factor	0.001118	4	0.0002794	F (4, 30) = 32.83	P<0.0001
Residual	0.0002553	30	8.511e-006		
Data summary					
Number of columns (Column Factor)	5				
Number of rows (Row Factor)	3				
Number of values	45				

Tukey test for multiple comparison in MgSO4 and HNB concentration in dLAMP

COMPARE CELL MEANS REGARDLESS OF ROWS AND COLUMNS					
NUMBER OF FAMILIES	1				
NUMBER OF COMPARISONS PER FAMILY	105				
ALPHA	0.05				
TUKEY'S MULTIPLE COMPARISONS TEST	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (80 MM):MGSO4 4.5 MMOL	-0.01000	-0.01878 to -0.001222	Yes	*	0.0144
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (80 MM):MGSO4 5.5 MMOL	0.007667	-0.001111 to 0.01644	No	ns	0.1378
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (80 MM):MGSO4 6.5 MMOL	-0.01900	-0.02778 to -0.01022	Yes	****	<0.0001
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (80 MM):MGSO4 7.5 MMOL	-0.02367	-0.03244 to -0.01489	Yes	****	<0.0001
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 3.5 MMOL	-0.03067	-0.03944 to -0.02189	Yes	****	<0.0001
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 4.5 MMOL	-0.02300	-0.03178 to -0.01422	Yes	****	<0.0001
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 5.5 MMOL	-0.03533	-0.04411 to -0.02656	Yes	****	<0.0001
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	-0.04200	-0.05078 to -0.03322	Yes	****	<0.0001
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	-0.02933	-0.03811 to -0.02056	Yes	****	<0.0001
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	-0.052	-0.06111 to -0.04356	Yes	****	<0.0001

HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	-0.05533	-0.06411 to -0.04656	Yes	****	<0.0001
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	-0.04767	-0.05644 to -0.03889	Yes	****	<0.0001
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.05700	-0.06578 to -0.04822	Yes	****	<0.0001
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	-0.04767	-0.05644 to -0.03889	Yes	****	<0.0001
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (80 MM):MGSO4 5.5 MMOL	0.01767	0.008889 to 0.02644	Yes	****	<0.0001
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (80 MM):MGSO4 6.5 MMOL	-0.009000	-0.01778 to -0.0002222	Yes	*	0.0402
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (80 MM):MGSO4 7.5 MMOL	-0.01367	-0.02244 to -0.004889	Yes	***	0.0002
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 3.5 MMOL	-0.02067	-0.02944 to -0.01189	Yes	****	<0.0001
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 4.5 MMOL	-0.01300	-0.02178 to -0.004222	Yes	***	0.0005
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 5.5 MMOL	-0.02533	-0.03411 to -0.01656	Yes	****	<0.0001
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	-0.03200	-0.04078 to -0.02322	Yes	****	<0.0001
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	-0.01933	-0.02811 to -0.01056	Yes	****	<0.0001
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	-0.04233	-0.05111 to -0.03356	Yes	****	<0.0001
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	-0.04533	-0.05411 to -0.03656	Yes	****	<0.0001
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	-0.03767	-0.04644 to -0.02889	Yes	****	<0.0001
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.04700	-0.05578 to -0.03822	Yes	****	<0.0001
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	-0.03767	-0.04644 to -0.02889	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (80 MM):MGSO4 6.5 MMOL	-0.02667	-0.03544 to -0.01789	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (80 MM):MGSO4 7.5 MMOL	-0.03133	-0.04011 to -0.02256	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 3.5 MMOL	-0.03833	-0.04711 to -0.02956	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 4.5 MMOL	-0.03067	-0.03944 to -0.02189	Yes	****	<0.0001

HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 5.5 MMOL	-0.04300	-0.05178 to -0.03422	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	-0.04967	-0.05844 to -0.04089	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	-0.03700	-0.04578 to -0.02822	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	-0.06000	-0.06878 to -0.05122	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	-0.06300	-0.07178 to -0.05422	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	-0.05533	-0.06411 to -0.04656	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.06467	-0.07344 to -0.05589	Yes	****	<0.0001
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	-0.05533	-0.06411 to -0.04656	Yes	****	<0.0001
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (80 MM):MGSO4 7.5 MMOL	-0.004667	-0.01344 to 0.004111	No	ns	0.8053
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 3.5 MMOL	-0.01167	-0.02044 to -0.002889	Yes	**	0.0023
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 4.5 MMOL	-0.004000	-0.01278 to 0.004778	No	ns	0.9236
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 5.5 MMOL	-0.01633	-0.02511 to -0.007556	Yes	****	<0.0001
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	-0.02300	-0.03178 to -0.01422	Yes	****	<0.0001
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	-0.01033	-0.01911 to -0.001556	Yes	*	0.0101
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	-0.03333	-0.04211 to -0.02456	Yes	****	<0.0001
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	-0.03633	-0.04511 to -0.02756	Yes	****	<0.0001
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	-0.02867	-0.03744 to -0.01989	Yes	****	<0.0001
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.03800	-0.04678 to -0.02922	Yes	****	<0.0001
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	-0.02867	-0.03744 to -0.01989	Yes	****	<0.0001
HNB (80 MM):MGSO4 7.5 MMOL VS. HNB (120 MM):MGSO4 3.5 MMOL	-0.007000	-0.01578 to 0.001778	No	ns	0.2352
HNB (80 MM):MGSO4 7.5 MMOL VS. HNB (120 MM):MGSO4 4.5 MMOL	0.0006667	-0.008111 to 0.009444	No	ns	>0.9999
HNB (80 MM):MGSO4 7.5 MMOL VS.	-	-0.02044 to	Yes	**	0.0023

HNB (120 MM):MGSO4 5.5 MMOL	0.01167	-0.002889			
HNB (80 MM):MGSO4 7.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	-0.01833	-0.02711 to -0.009556	Yes	****	<0.0001
HNB (80 MM):MGSO4 7.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	-0.005667	-0.01444 to 0.003111	No	ns	0.5465
HNB (80 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	-0.02867	-0.03744 to -0.01989	Yes	****	<0.0001
HNB (80 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	-0.03167	-0.04044 to -0.02289	Yes	****	<0.0001
HNB (80 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	-0.02400	-0.03278 to -0.01522	Yes	****	<0.0001
HNB (80 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.03333	-0.04211 to -0.02456	Yes	****	<0.0001
HNB (80 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	-0.02400	-0.03278 to -0.01522	Yes	****	<0.0001
HNB (120 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 4.5 MMOL	0.007667	-0.001111 to 0.01644	No	ns	0.1378
HNB (120 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 5.5 MMOL	-0.004667	-0.01344 to 0.004111	No	ns	0.8053
HNB (120 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	-0.01133	-0.02011 to -0.002556	Yes	**	0.0034
HNB (120 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	0.001333	-0.007444 to 0.01011	No	ns	>0.9999
HNB (120 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	-0.02167	-0.03044 to -0.01289	Yes	****	<0.0001
HNB (120 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	-0.02467	-0.03344 to -0.01589	Yes	****	<0.0001
HNB (120 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	-0.01700	-0.02578 to -0.008222	Yes	****	<0.0001
HNB (120 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.02633	-0.03511 to -0.01756	Yes	****	<0.0001
HNB (120 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	-0.01700	-0.02578 to -0.008222	Yes	****	<0.0001
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 5.5 MMOL	-0.01233	-0.02111 to -0.003556	Yes	**	0.0011
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	-0.01900	-0.02778 to -0.01022	Yes	****	<0.0001
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	-0.006333	-0.01511 to 0.002444	No	ns	0.3740
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	-0.02933	-0.03811 to -0.02056	Yes	****	<0.0001
HNB (120 MM):MGSO4 4.5 MMOL	-	-0.04111 to	Yes	****	<0.0001

VS. HNB (160 MM):MGSO4 4.5 MMOL	0.03233	-0.02356			
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	-0.02467	-0.03344 to -0.01589	Yes	****	<0.0001
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.03400	-0.04278 to -0.02522	Yes	****	<0.0001
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	-0.02467	-0.03344 to -0.01589	Yes	****	<0.0001
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	-0.006667	-0.01544 to 0.002111	No	ns	0.2995
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	0.006000	-0.002778 to 0.01478	No	ns	0.4572
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	-0.01700	-0.02578 to -0.008222	Yes	****	<0.0001
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	-0.02000	-0.02878 to -0.01122	Yes	****	<0.0001
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	-0.01233	-0.02111 to -0.003556	Yes	**	0.0011
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.02167	-0.03044 to -0.01289	Yes	****	<0.0001
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	-0.01233	-0.02111 to -0.003556	Yes	**	0.0011
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	0.01267	0.003889 to 0.02144	Yes	***	0.0008
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	-0.01033	-0.01911 to -0.001556	Yes	*	0.0101
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	-0.01333	-0.02211 to -0.004556	Yes	***	0.0004
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	-0.005667	-0.01444 to 0.003111	No	ns	0.5465
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.01500	-0.02378 to -0.006222	Yes	****	<0.0001
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	-0.005667	-0.01444 to 0.003111	No	ns	0.5465
HNB (120 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	-0.02300	-0.03178 to -0.01422	Yes	****	<0.0001
HNB (120 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	-0.02600	-0.03478 to -0.01722	Yes	****	<0.0001
HNB (120 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	-0.01833	-0.02711 to -0.009556	Yes	****	<0.0001
HNB (120 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.02767	-0.03644 to -0.01889	Yes	****	<0.0001
HNB (120 MM):MGSO4 7.5 MMOL	-	-0.02711 to	Yes	****	<0.0001

VS. HNB (160 MM):MGSO4 7.5 MMOL	0.01833	-0.009556						
HNB (160 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	-0.003000	-0.01178 to 0.005778	No	ns	0.9925			
HNB (160 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	0.004667	-0.004111 to 0.01344	No	ns	0.8053			
HNB (160 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.004667	-0.01344 to 0.004111	No	ns	0.8053			
HNB (160 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.004667	-0.004111 to 0.01344	No	ns	0.8053			
HNB (160 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	0.007667	-0.001111 to 0.01644	No	ns	0.1378			
HNB (160 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.001667	-0.01044 to 0.007111	No	ns	>0.9999			
HNB (160 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.007667	-0.001111 to 0.01644	No	ns	0.1378			
HNB (160 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	-0.009333	-0.01811 to -0.0005555	Yes	*	0.0288			
HNB (160 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.000	-0.008778 to 0.008778	No	ns	>0.9999			
HNB (160 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.009333	0.0005555 to 0.01811	Yes	*	0.0288			
TEST DETAILS	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (80 MM):MGSO4 4.5 MMOL	0.06333	0.07333	-0.01000	0.002382	3	3	5.937	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (80 MM):MGSO4 5.5 MMOL	0.06333	0.05567	0.007667	0.002382	3	3	4.552	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (80 MM):MGSO4 6.5 MMOL	0.06333	0.08233	-0.01900	0.002382	3	3	11.28	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (80 MM):MGSO4 7.5 MMOL	0.06333	0.08700	-0.02367	0.002382	3	3	14.05	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 3.5 MMOL	0.06333	0.09400	-0.03067	0.002382	3	3	18.21	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 4.5 MMOL	0.06333	0.08633	-0.02300	0.002382	3	3	13.66	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 5.5 MMOL	0.06333	0.09867	-0.03533	0.002382	3	3	20.98	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	0.06333	0.1053	-0.04200	0.002382	3	3	24.94	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	0.06333	0.09267	-0.02933	0.002382	3	3	17.42	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS.	0.063	0.1157	-	0.00	3	3	31.	30

HNB (160 MM):MGSO4 3.5 MMOL	33		0.05233	2382			07	.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	0.063 33	0.1187	-0.05533	0.00 2382	3	3	32.85	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	0.063 33	0.1110	-0.04767	0.00 2382	3	3	28.30	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	0.063 33	0.1203	-0.05700	0.00 2382	3	3	33.84	30.00
HNB (80 MM):MGSO4 3.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.063 33	0.1110	-0.04767	0.00 2382	3	3	28.30	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (80 MM):MGSO4 5.5 MMOL	0.073 33	0.05567	0.01767	0.00 2382	3	3	10.49	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (80 MM):MGSO4 6.5 MMOL	0.073 33	0.08233	-0.009000	0.00 2382	3	3	5.343	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (80 MM):MGSO4 7.5 MMOL	0.073 33	0.08700	-0.01367	0.00 2382	3	3	8.114	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 3.5 MMOL	0.073 33	0.09400	-0.02067	0.00 2382	3	3	12.27	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 4.5 MMOL	0.073 33	0.08633	-0.01300	0.00 2382	3	3	7.718	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 5.5 MMOL	0.073 33	0.09867	-0.02533	0.00 2382	3	3	15.04	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	0.073 33	0.1053	-0.03200	0.00 2382	3	3	19.00	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	0.073 33	0.09267	-0.01933	0.00 2382	3	3	11.48	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	0.073 33	0.1157	-0.04233	0.00 2382	3	3	25.13	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	0.073 33	0.1187	-0.04533	0.00 2382	3	3	26.91	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	0.073 33	0.1110	-0.03767	0.00 2382	3	3	22.36	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	0.073 33	0.1203	-0.04700	0.00 2382	3	3	27.90	30.00
HNB (80 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.073 33	0.1110	-0.03767	0.00 2382	3	3	22.36	30.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (80 MM):MGSO4 6.5 MMOL	0.055 67	0.08233	-0.02667	0.00 2382	3	3	15.83	30.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (80 MM):MGSO4 7.5 MMOL	0.055 67	0.08700	-0.03133	0.00 2382	3	3	18.60	30.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 3.5 MMOL	0.055 67	0.09400	-0.03833	0.00 2382	3	3	22.76	30.00
HNB (80 MM):MGSO4 5.5 MMOL VS.	0.055	0.08633	-	0.00	3	3	18.	30

HNB (120 MM):MGSO4 4.5 MMOL	67		0.03067	2382			21	.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 5.5 MMOL	0.05567	0.09867	-0.04300	0.002382	3	3	25.53	.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	0.05567	0.1053	-0.04967	0.002382	3	3	29.49	.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	0.05567	0.09267	-0.03700	0.002382	3	3	21.97	.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	0.05567	0.1157	-0.06000	0.002382	3	3	35.62	.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	0.05567	0.1187	-0.06300	0.002382	3	3	37.40	.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	0.05567	0.1110	-0.05533	0.002382	3	3	32.85	.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	0.05567	0.1203	-0.06467	0.002382	3	3	38.39	.00
HNB (80 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.05567	0.1110	-0.05533	0.002382	3	3	32.85	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (80 MM):MGSO4 7.5 MMOL	0.08233	0.08700	-0.004667	0.002382	3	3	2.771	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 3.5 MMOL	0.08233	0.09400	-0.01167	0.002382	3	3	6.927	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 4.5 MMOL	0.08233	0.08633	-0.004000	0.002382	3	3	2.375	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 5.5 MMOL	0.08233	0.09867	-0.01633	0.002382	3	3	9.697	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	0.08233	0.1053	-0.02300	0.002382	3	3	13.66	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	0.08233	0.09267	-0.01033	0.002382	3	3	6.135	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	0.08233	0.1157	-0.03333	0.002382	3	3	19.79	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	0.08233	0.1187	-0.03633	0.002382	3	3	21.57	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	0.08233	0.1110	-0.02867	0.002382	3	3	17.02	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	0.08233	0.1203	-0.03800	0.002382	3	3	22.56	.00
HNB (80 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.08233	0.1110	-0.02867	0.002382	3	3	17.02	.00
HNB (80 MM):MGSO4 7.5 MMOL VS. HNB (120 MM):MGSO4 3.5 MMOL	0.08700	0.09400	-0.007000	0.002382	3	3	4.156	.00
HNB (80 MM):MGSO4 7.5 MMOL VS.	0.087	0.08633	0.00066	0.00	3	3	0.3	30

HNB (120 MM):MGSO4 4.5 MMOL	00		67	2382			95	.0
							8	0
HNB (80 MM):MGSO4 7.5 MMOL VS.	0.087	0.09867	-	0.00	3	3	6.9	30
HNB (120 MM):MGSO4 5.5 MMOL	00		0.01167	2382			27	.0
								0
HNB (80 MM):MGSO4 7.5 MMOL VS.	0.087	0.1053	-	0.00	3	3	10.	30
HNB (120 MM):MGSO4 6.5 MMOL	00		0.01833	2382			88	.0
								0
HNB (80 MM):MGSO4 7.5 MMOL VS.	0.087	0.09267	-	0.00	3	3	3.3	30
HNB (120 MM):MGSO4 7.5 MMOL	00		0.00566	2382			64	.0
			7					0
HNB (80 MM):MGSO4 7.5 MMOL VS.	0.087	0.1157	-	0.00	3	3	17.	30
HNB (160 MM):MGSO4 3.5 MMOL	00		0.02867	2382			02	.0
								0
HNB (80 MM):MGSO4 7.5 MMOL VS.	0.087	0.1187	-	0.00	3	3	18.	30
HNB (160 MM):MGSO4 4.5 MMOL	00		0.03167	2382			80	.0
								0
HNB (80 MM):MGSO4 7.5 MMOL VS.	0.087	0.1110	-	0.00	3	3	14.	30
HNB (160 MM):MGSO4 5.5 MMOL	00		0.02400	2382			25	.0
								0
HNB (80 MM):MGSO4 7.5 MMOL VS.	0.087	0.1203	-	0.00	3	3	19.	30
HNB (160 MM):MGSO4 6.5 MMOL	00		0.03333	2382			79	.0
								0
HNB (80 MM):MGSO4 7.5 MMOL VS.	0.087	0.1110	-	0.00	3	3	14.	30
HNB (160 MM):MGSO4 7.5 MMOL	00		0.02400	2382			25	.0
								0
HNB (120 MM):MGSO4 3.5 MMOL	0.094	0.08633	0.00766	0.00	3	3	4.5	30
VS. HNB (120 MM):MGSO4 4.5	00		7	2382			52	.0
MMOL								0
HNB (120 MM):MGSO4 3.5 MMOL	0.094	0.09867	-	0.00	3	3	2.7	30
VS. HNB (120 MM):MGSO4 5.5	00		0.00466	2382			71	.0
MMOL			7					0
HNB (120 MM):MGSO4 3.5 MMOL	0.094	0.1053	-	0.00	3	3	6.7	30
VS. HNB (120 MM):MGSO4 6.5	00		0.01133	2382			29	.0
MMOL								0
HNB (120 MM):MGSO4 3.5 MMOL	0.094	0.09267	0.00133	0.00	3	3	0.7	30
VS. HNB (120 MM):MGSO4 7.5	00		3	2382			91	.0
MMOL							6	0
HNB (120 MM):MGSO4 3.5 MMOL	0.094	0.1157	-	0.00	3	3	12.	30
VS. HNB (160 MM):MGSO4 3.5	00		0.02167	2382			86	.0
MMOL								0
HNB (120 MM):MGSO4 3.5 MMOL	0.094	0.1187	-	0.00	3	3	14.	30
VS. HNB (160 MM):MGSO4 4.5	00		0.02467	2382			64	.0
MMOL								0
HNB (120 MM):MGSO4 3.5 MMOL	0.094	0.1110	-	0.00	3	3	10.	30
VS. HNB (160 MM):MGSO4 5.5	00		0.01700	2382			09	.0
MMOL								0
HNB (120 MM):MGSO4 3.5 MMOL	0.094	0.1203	-	0.00	3	3	15.	30
VS. HNB (160 MM):MGSO4 6.5	00		0.02633	2382			63	.0
MMOL								0
HNB (120 MM):MGSO4 3.5 MMOL	0.094	0.1110	-	0.00	3	3	10.	30
VS. HNB (160 MM):MGSO4 7.5	00		0.01700	2382			09	.0
MMOL								0
HNB (120 MM):MGSO4 4.5 MMOL	0.086	0.09867	-	0.00	3	3	7.3	30
VS. HNB (120 MM):MGSO4 5.5	33		0.01233	2382			22	.0
MMOL								0
HNB (120 MM):MGSO4 4.5 MMOL	0.086	0.1053	-	0.00	3	3	11.	30
VS. HNB (120 MM):MGSO4 6.5	33		0.01900	2382			28	.0
MMOL								0
HNB (120 MM):MGSO4 4.5 MMOL	0.086	0.09267	-	0.00	3	3	3.7	30
VS. HNB (120 MM):MGSO4 7.5	33		0.00633	2382			60	.0
MMOL			3					0
HNB (120 MM):MGSO4 4.5 MMOL	0.086	0.1157	-	0.00	3	3	17.	30

VS. HNB (160 MM):MGSO4 3.5 MMOL	33		0.02933	2382			42	.0 0
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	0.086 33	0.1187	- 0.03233	0.00 2382	3	3	19. 20	30 .0 0
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	0.086 33	0.1110	- 0.02467	0.00 2382	3	3	14. 64	30 .0 0
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	0.086 33	0.1203	- 0.03400	0.00 2382	3	3	20. 19	30 .0 0
HNB (120 MM):MGSO4 4.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.086 33	0.1110	- 0.02467	0.00 2382	3	3	14. 64	30 .0 0
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 6.5 MMOL	0.098 67	0.1053	- 0.00666 7	0.00 2382	3	3	3.9 58	30 .0 0
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	0.098 67	0.09267	0.00600 0	0.00 2382	3	3	3.5 62	30 .0 0
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	0.098 67	0.1157	- 0.01700	0.00 2382	3	3	10. 09	30 .0 0
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	0.098 67	0.1187	- 0.02000	0.00 2382	3	3	11. 87	30 .0 0
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	0.098 67	0.1110	- 0.01233	0.00 2382	3	3	7.3 22	30 .0 0
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	0.098 67	0.1203	- 0.02167	0.00 2382	3	3	12. 86	30 .0 0
HNB (120 MM):MGSO4 5.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.098 67	0.1110	- 0.01233	0.00 2382	3	3	7.3 22	30 .0 0
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (120 MM):MGSO4 7.5 MMOL	0.105 3	0.09267	0.01267	0.00 2382	3	3	7.5 20	30 .0 0
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	0.105 3	0.1157	- 0.01033	0.00 2382	3	3	6.1 35	30 .0 0
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	0.105 3	0.1187	- 0.01333	0.00 2382	3	3	7.9 16	30 .0 0
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	0.105 3	0.1110	- 0.00566 7	0.00 2382	3	3	3.3 64	30 .0 0
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 6.5 MMOL	0.105 3	0.1203	- 0.01500	0.00 2382	3	3	8.9 06	30 .0 0
HNB (120 MM):MGSO4 6.5 MMOL VS. HNB (160 MM):MGSO4 7.5 MMOL	0.105 3	0.1110	- 0.00566 7	0.00 2382	3	3	3.3 64	30 .0 0
HNB (120 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 3.5 MMOL	0.092 67	0.1157	- 0.02300	0.00 2382	3	3	13. 66	30 .0 0
HNB (120 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 4.5 MMOL	0.092 67	0.1187	- 0.02600	0.00 2382	3	3	15. 44	30 .0 0
HNB (120 MM):MGSO4 7.5 MMOL VS. HNB (160 MM):MGSO4 5.5 MMOL	0.092 67	0.1110	- 0.01833	0.00 2382	3	3	10. 88	30 .0 0
HNB (120 MM):MGSO4 7.5 MMOL	0.092	0.1203	-	0.00	3	3	16.	30

VS. HNB (160 MM):MGSO4 6.5 MMOL	67		0.02767	2382			43	.0 0
HNB (120 MM):MGSO4 7.5 MMOL	0.092	0.1110	-	0.00	3	3	10.	30
VS. HNB (160 MM):MGSO4 7.5 MMOL	67		0.01833	2382			88	.0 0
HNB (160 MM):MGSO4 3.5 MMOL	0.115	0.1187	-	0.00	3	3	1.7	30
VS. HNB (160 MM):MGSO4 4.5 MMOL	7		0.00300 0	2382			81	.0 0
HNB (160 MM):MGSO4 3.5 MMOL	0.115	0.1110	0.00466	0.00	3	3	2.7	30
VS. HNB (160 MM):MGSO4 5.5 MMOL	7		7	2382			71	.0 0
HNB (160 MM):MGSO4 3.5 MMOL	0.115	0.1203	-	0.00	3	3	2.7	30
VS. HNB (160 MM):MGSO4 6.5 MMOL	7		0.00466 7	2382			71	.0 0
HNB (160 MM):MGSO4 3.5 MMOL	0.115	0.1110	0.00466	0.00	3	3	2.7	30
VS. HNB (160 MM):MGSO4 7.5 MMOL	7		7	2382			71	.0 0
HNB (160 MM):MGSO4 4.5 MMOL	0.118	0.1110	0.00766	0.00	3	3	4.5	30
VS. HNB (160 MM):MGSO4 5.5 MMOL	7		7	2382			52	.0 0
HNB (160 MM):MGSO4 4.5 MMOL	0.118	0.1203	-	0.00	3	3	0.9	30
VS. HNB (160 MM):MGSO4 6.5 MMOL	7		0.00166 7	2382			89 5	.0 0
HNB (160 MM):MGSO4 4.5 MMOL	0.118	0.1110	0.00766	0.00	3	3	4.5	30
VS. HNB (160 MM):MGSO4 7.5 MMOL	7		7	2382			52	.0 0
HNB (160 MM):MGSO4 5.5 MMOL	0.111	0.1203	-	0.00	3	3	5.5	30
VS. HNB (160 MM):MGSO4 6.5 MMOL	0		0.00933 3	2382			41	.0 0
HNB (160 MM):MGSO4 5.5 MMOL	0.111	0.1110	0.000	0.00	3	3	0.0	30
VS. HNB (160 MM):MGSO4 7.5 MMOL	0			2382			00	.0 0
HNB (160 MM):MGSO4 6.5 MMOL	0.120	0.1110						
VS. HNB (160 MM):MGSO4 7.5 MMOL	3							

C. List of clinical bacterial

List of *Escherichia coli* clinical bacteria harboring NDM gene

Iso No.	Hospital	Organism	MLST	NGS Carbapenemase	NGS blaOXA
V417	Phetchabul	Escherichia coli	410	NDM-1	blaOXA-1
C015	Sakon Nakhon	Escherichia coli	410	NDM-5	blaOXA-1
C032	Sakon Nakhon	Escherichia coli	361	NDM-5	none
C049	Udonthani	Escherichia coli	354	NDM-1	none
C098	Surin	Escherichia coli	48	NDM-1, OXA-232	blaOXA-1
C150	Mae Sot	Escherichia coli	448	NDM-7	none
C153	Mae Sot	Escherichia coli	448	NDM-7	none
C155	Suratthani	Escherichia coli	448	NDM-4	blaOXA-1
C176	Suratthani	Escherichia coli	1340	OXA-181,hugA	none
C787B	Surin	Escherichia coli	410	OXA-232	none
C161	Suratthani	Escherichia coli	410	OXA-181	blaOXA-1
C179B	Suratthani	Escherichia coli	448	NDM-4	blaOXA-1
C214	Suratthani	Escherichia coli	410	OXA-484	none
C163	Suratthani	Escherichia coli	410	NDM-1,OXA-181	blaOXA-1
C149	Surin	Escherichia coli	2144	NDM-1	none
C168B	Suratthani	Escherichia coli	410	OXA-181	blaOXA-1
C497	Surin	Escherichia coli	88	NDM-1	none
C163	Suratthani	Escherichia coli	410	NDM-1,OXA-181	blaOXA-1

List of Klebsiella pneumoniae clinical bacterial

Iso No.	Hospital	Specmen	KmerIden	MLST	NGS Carbapenemase	NGS blaOXA
C001	Sakon Nakhon	Abdomen	Klebsiella pneumonia	**147**	NDM-1	blaOXA-1, blaOXA-9
C002	Sakon Nakhon	Sputum	Klebsiella pneumonia	**340**	OXA-181	blaOXA-1
C003	Sakon Nakhon	Sputum	Klebsiella pneumonia	**147**	NDM-1	blaOXA-1, blaOXA-9
49	C049	Udonthani	Escherichia coli	**354**	blaCTX-M-24, blaCTX-M-55	none
C009	Sakon Nakhon	Stump	Klebsiella pneumonia	**336**	NDM-1	blaOXA-1
C038	Sakon Nakhon	pleural fluid	Klebsiella pneumonia	**340**	OXA-181	blaOXA-1
C074	Sakon Nakhon	Urine	Klebsiella pneumonia	**147**	NDM-1, OXA-181	blaOXA-1, blaOXA-9
C045	Udonthani	Blood	Klebsiella pneumonia	**231**	NDM-1	none
C054	Udonthani	Sputum	Klebsiella pneumonia	**15**	none	none
C063	Udonthani	Urine	Klebsiella pneumonia	**1089**	NDM-5	none
C073	Sakon Nakhon	Sputum	Klebsiella pneumonia	**16**	NDM-1 OXA-232	blaOXA-9
C120	Sakon Nakhon	Urine	Klebsiella pneumonia	**340**	OXA-181	blaOXA-1
C138	Surin	Sputum	Klebsiella pneumonia	**16**	NDM-1 OXA-232	blaOXA-9
C143	Surin	Sputum	Klebsiella pneumonia	**231**	OXA-232	none
C101	Surin	pus	Klebsiella pneumoniae	**147**	NDM-1	none

VITA

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