Identification of SNP barcode for classify carbapenem-resistant enterobacteriaceae (CRE) and non-CRE using Nanopore sequencing

Miss Nicha Sangpiromapichai

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การระบุหาสนิปบาร์โค้ดเพื่อตรวจหาเชื้อเอนเทอโรแบคทีเรียซีอีที่ดื้อต่อยาคาบาพีเนมและไม่ดื้อต่อยาคาบาพีเนมโดยการหาลำดับเบสด้วยนาโนพอร์

น.ส.ณิชา แสงภิรมย์อภิชัย

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By	Miss Nicha Sangpiromapichai
Field of Study	Medical Sciences
Thesis Advisor	VORAPHOJ NILARATANAKUL, M.D., Ph.D.
Thesis Co Advisor	NATTHAYA CHUAYPEN, Ph.D.

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

..... Dean of the FACULTY OF MEDICINE
(Associate Professor CHANCHAI SITTIPUNT, M.D.)

THESIS COMMITTEE

..... Chairman
(Professor PADET SIRIYASATIEN, M.D., Ph.D.)

..... Thesis Advisor
(VORAPHOJ NILARATANAKUL, M.D., Ph.D.)

..... Thesis Co-Advisor
(NATTHAYA CHUAYPEN, Ph.D.)

..... Examiner
(Associate Professor SUNCHAI PAYUNGPORN, Ph.D.)

..... Examiner
(TANITTHA CHATSUWAN, Ph.D.)

..... Examiner
(Piroon Jenjaroenpun, Ph.D.)

ณิชา แสงภิรมย์อภิชัย : การระบุหาสนิปบาร์โค้ดเพื่อตรวจหาเชื้อเอนเทอโรแบคทีเรียซีอีที่ดื้อต่อยาคาบาพีเนมและไม่ดื้อต่อยาคาบาพีเนมโดยการหาลำดับเบสด้วยนาโนพอร์. (Identification of SNP barcode for classify carbapenem-resistant enterobacteriaceae (CRE) and non-CRE using Nanopore sequencing) อ.ที่ปรึกษาหลัก : ดร. นพ.วรพจน์ นิลรัตนกุล, อ.ที่ปรึกษาร่วม : ดร.ณัฐธยาน์ ช่วยเพ็ญ

หลักการ: ความชุกของเชื้อ carbapenem-resistant Enterobacteriaceae หรือ CRE ที่เพิ่มขึ้นในทุกปีเป็นปัญหาสำคัญของสาธารณสุขทั่วโลก การศึกษาก่อนหน้านี้พบว่าจำนวนตัวอย่างเชื้อ *Klebsiella pneumoniae* (KP) ทั้งหมด 3864 ตัวอย่าง พบเชื้อ KP ที่มีการดื้อต่อยา carbapenem ถึง 25% ถือว่าเป็นตัวเลขที่สูงมาก การดื้อยามีปัจจัยหลายอย่างซึ่งหนึ่งในนั้นก็คือการใช้ยาต้านจุลชีพเกินความจำเป็น อันเนื่องมาจากโดยปกติแล้วนั้นการวินิจฉัยหาเชื้อแบคทีเรียในห้องปฏิบัติการจะต้องใช้เวลาในการเพาะเชื้อและทดสอบความไวของยาเป็นเวลา 2 ถึง 3 วัน ซึ่งทำให้เกิดความล่าช้าในการให้ยาต้านจุลชีพอย่างเหมาะสม วัตถุประสงค์: เพื่อหาแนวทางแยกเชื้อดื้อยา CRE และ non-CRE ด้วยการใช้ SNP barcode วิธีการศึกษา: เก็บ *Klebsiella pneumoniae* ทั้งหมด 40 ตัวอย่างในปี 2563 ถึง 2564 ภายในโรงพยาบาลจุฬาลงกรณ์สภากาชาดไทย โดย 20 ตัวอย่างแรกคือเชื้อ *Klebsiella pneumoniae* ที่ดื้อต่อยาcarbapenem (CRKP) และ 20 ตัวอย่างหลังคือเชื้อ *Klebsiella pneumoniae* ที่ไม่ดื้อต่อยา carbapenem (non-CRKP) จากนั้นนำตัวอย่างมาเพาะเชื้อที่ 37 องศาเป็นเวลา 1 คืน เพื่อมาสกัดดีเอนเอและทำการหาลำดับเบสด้วยเทคนิค nanopore จากนั้นนำมาทำ whole genome assembly แล้วสร้าง phylogenetic tree ของ KP chromosome ด้วยโปรแกรมSnippy และ OrthoFinder รวมทั้งหา SNP barcode ที่ช่วยแยก CRKP ออกจาก non-CRKP ผลการศึกษา: ผลจากโปรแกรม Snippy พบว่า phylogenetic tree แบ่งออกได้เป็น 3 clade โดยแต่ละส่วนใหญ่เชื้อ non-CRKP จะอยู่ใน clade 1 และ 2 ส่วนเชื้อ CRKP ส่วนใหญ่จะอยู่ใน clade 3 อย่างไรก็ตามไม่พบว่ามีชุด SNP ใดที่สามารถใช้ในการแยก CRKPออกจาก non-CRKP ได้ ในขณะที่ phylogenetic tree ที่ได้จากโปรแกรม OrthoFinder สามารถแบ่งได้เป็น 4 clade โดยมี CRKP ใน clade 1–2 แยกกันอย่างชัดเจนกับ non-CRKP ใน clade 3–4 ทั้งนี้มีเพียง 2 ตัวอย่างของเชื้อ CRKP ที่อยู่ใน clade 3–4 และมีเพียง1 ตัวอย่างของเชื้อ non-CRKP ที่อยู่ใน clade 1–2สรุปผลการศึกษา: การสร้าง phylogenetic tree ในแต่ละโปรแกรมมีการใช้หลักการในการสร้าง phylogenetic tree แตกต่างกันออกไป ทำให้มีผลที่ออกมาที่แตกต่างกัน การที่ phylogenetic tree ไม่สามารถแบ่ง CRKP และ non-CRKP ออกเป็น clade ที่แยกจากกันชัดเจน รวมถึงไม่สามารถสร้าง SNP barcode ได้ อาจเป็นเพราะ ยีนดื้อยาส่วนมากอยู่บนพลาสมิดในกรณีของเชื้อ KP ทำให้ความสัมพันธ์ระหว่างการดื้อยากับ SNP บนโครโมโซมของ KP ไม่ชัดเจน

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Nicha Sangpiromapichai : Identification of SNP barcode for classify carbapenem-resistant enterobacteriaceae (CRE) and non-CRE using Nanopore sequencing. Advisor: VORAPHOJ NILARATANAKUL, M.D., Ph.D. Co-advisor: NATTHAYA CHUAYPEN, Ph.D.

Background: The continuous increase of carbapenem-resistant Enterobacteriaceae (CRE) prevalence has been a serious problem for public health worldwide. In the previous study, 25% of 3864 *Klebsiella pneumoniae* isolates were carbapenem-resistant. One of the most important risks of bacterial drug resistance is the improper use of antibiotics. The routine antimicrobial-susceptibility test requires overnight and has a turnaround time of approximately 2–3 days which delays the proper antibiotics. This study aims to develop a technique to classify CRE and non-CRE by SNP barcode. Method: Forty *Klebsiella pneumoniae* (KP) clinical isolates were collected from 2020–2021 at King Chulalongkorn Memorial Hospital. Twenty samples were carbapenem-resistant *Klebsiella pneumoniae* (CRKP), and 20 samples were not carbapenem-resistant (non-CRKP). Each isolate was sub-cultured at 37°C overnight for DNA extraction, library preparation, and Nanopore sequencing. Then, we assembled the bacterial whole genomes and analyzed their chromosomes by bioinformatic tools, including Snippy and OrthoFinder. Result: The phylogenetic tree generated by the Snippy tool can classify 40 KP clinical isolates into 3 clades. Most of non-CRKPs are in clades 1 and 2, while the CRKPs are mainly in clade 3. However, none of the SNPs is unique and useful in differentiation of CRKP from non-CRKP. Thus, SNP barcode cannot be defined from this study. In contrast, OrthoFinder, which compares amino acid sequences between orthologous genes, can clearly distinguish KPs into 4 clades, 1–2 for CRKPs and 3–4 for non-CRKPs. Two of twenty CRKPs are members of clades 3–4, while only 1 non-CRKP is in clade 1–2. Conclusion: Different tools generate quite different phylogenetic trees. SNPs from KP chromosomal DNA are not very different between CRKP and non-CRKP. In fact, plasmids and other mobile genetic elements, harboring drug-resistant genes, are frequently found in KPs. These can be horizontal transferred and may weaken the association between SNPs in bacterial chromosome and the carbapenem-resistance in KPs.

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LIST OF ABBREVIATIONS

CRE = Carbapenem-resistant Enterobacteriaceae

CRKP = Carbapenem-resistant *Klebsiella pneumoniae*

AST = Antimicrobial susceptibility test

AMR = Antimicrobial-resistant

SP = *Streptococcus pneumoniae*

SNP = Single nucleotide polymorphism

SNV = Single nucleotide variation

DNA = Deoxyribonucleic acid

CP = Carbapenemase producing

NDM = New Deli Metallo-β-lactamase

KPC = *Klebsiella pneumoniae* carbapenemase

IMI = Imipenem-hydrolyzing β-lactamase

GES = Guiana extended-spectrum β-lactamase

MBLs = Metallo-β-lactamase

OXA = Oxacillinase

VIM = Verona integron-bone Metallo-β-lactamase

GIM = German imipenemase

SIM = Seoul imipenemase

AmpC = Type C ampicillinase

ESBLs = Extended-spectrum β-lactamase

MHT = Modified-Hodge Test

CHAPTER I

Introduction

1. Background and Rationale

Carbapenem-resistant *Enterobacteriaceae* (CRE) is an important problem in public health. CRE is a nosocomial pathogen that rapidly spread and has high morbidity and mortality. CRE has been rapidly emerging and increasingly reported. (2) Paveenkittiporn et al. reported 3,946 (93%) of 4,296 Enterobacteriaceae isolates were carbapenem-resistant and the most common organism was *Klebsiella pneumoniae* (72%, n=2,660) (3) The risk factors of carbapenem-resistant *K. pneumoniae* (CRKP) include a long length of hospital stays, admission to ICU, prior hospitalization, long day of ICU stay, transplantation recipient, steroid use, central venous catheter use, mechanical ventilation, parenteral nutrition, and previous antibiotic use. (4)

Bacterial culture, identification of bacteria, and conventional antimicrobial susceptibility test (AST) require a turnaround time of 2–3 days. This delays the appropriate antibiotic treatment. Overuse or inappropriate use of antibiotics is a major cause of drug-resistant bacteria, (5) which increases mortality and morbidity. (6) The molecular technique can rapidly identify bacteria and their antimicrobial-resistant (AMR) genes.

Oxford nanopore sequencing is a third-generation sequencing. This technique generates very long reads, which can be analyzed in real time. A single-molecule DNA or RNA fragment passes through the nanometer-sized protein pore from negative to positive charge, partially blocking the electrical current through the pore. The pattern of the electrical current can be deciphered into a specific base sequence. (7) Oxford nanopore sequencing can detect AMR genes quite fast, ranging from 10 minutes to 16 hours. (6, 8) This is much faster than a standard AST but still not early enough for the first-dose antibiotics.

Many molecular techniques have been developed to rapidly identify pathogens and antimicrobial resistance genes (AMR gene). These techniques usually rely on direct detection of AMR genes, either with or without

amplification. The amplification techniques need extra time for amplification and are limited to only known AMR genes that are amplified and detected by specific primers/probes. In contrast, direct sequencing without amplification can detect all AMR genes simultaneously.

However, the size and the copy number of AMR genes in the whole DNA extract determine the speed of AMR gene detection by nanopore real-time metagenomic sequencing. Also, quality of direct clinical samples can often be poor, e.g., low bacterial DNA with high load of human DNA. The real-time detection of small AMR genes (~1kb) in the sea of bacterial genome (~3-6mb) and human genome (~3gb) can be quite slow, up to more than 24 hours in our previous project. Due to these limitations, inability to detect the AMR genes early on does not always imply the absence of drug-resistant bacteria in samples.

Brinda et al. demonstrates antibiotic resistance of *S. pneumoniae* (SP) in clinical samples can be assumed to be the same as their best match in the database of *S. pneumoniae* genomes with known AST patterns. Since the bacterial whole genomes are much larger than each resistant genes, the clade matching by mapping each read from real-time nanopore sequencing is very fast, taking only 10 minutes, early enough to help prescribe 1st-dose antibiotic properly. Though the accuracy in term of identifying resistant bacteria may be inferior to direct detection of AMR genes, identification of non-resistant bacteria by clade matching should be superior to by the inability to directly detect AMR genes.

AMR genes in *S. pneumoniae* usually reside in its chromosome. On the other hands, many AMR genes in *Klebsiella pneumoniae* (KP) are in plasmids. Their clades (determined by chromosome) and AMR association may not be as strong as *S. pneumoniae*. Therefore, if we want to apply the approach of Brenda et al., we need to confirm this association in *K. pneumoniae*.(9)

In this study, we ought to simply visualize this association by construct the phylogenetic tree of *K. pneumoniae* clinical isolates and find out

whether carbapenem-resistant *K. pneumoniae* (CRKP) isolates are grouped together in the same clade(s) or distributed evenly in all clades. The former will represent the association between clade(s) and carbapenem resistance, while the latter will represent no association.

Phylogenetic tree can be constructed from SNP (single nucleotide polymorphism). If there is the association between clades and carbapenem resistance, SNP barcode unique to CRKP may be generated and used for primer/probe design.

SNP or Single Nucleotide Polymorphism is a genome variation that demonstrates a difference in a DNA sequence. (10) SNP barcode is a set of clade-specific SNP that is shared by all and most of the bacteria in that clade. SNP barcode genotyping can identify strains of pathogens. (11)

This study aimed to construct a phylogenetic tree of CRKP and non-CRKP clinical isolates. The process included DNA extraction, library preparation, nanopore sequencing, basecalling, adapter/barcode trimming, filtering, and de novo assembly of the KP whole genome. Phylogenetic trees were then constructed from SNP and from amino acid sequence in orthologous genes (OrthoFinder) of the whole genomes. SNP barcode for CRKP would be generated if unique SNP for CRKP could be identified.

2. Research Questions

- Can a phylogenetic tree and/or SNP barcodes distinguish carbapenem-resistant *Klebsiella pneumoniae* (CRKP) from non-carbapenem-resistant *Klebsiella pneumoniae* (non-CRKP)?

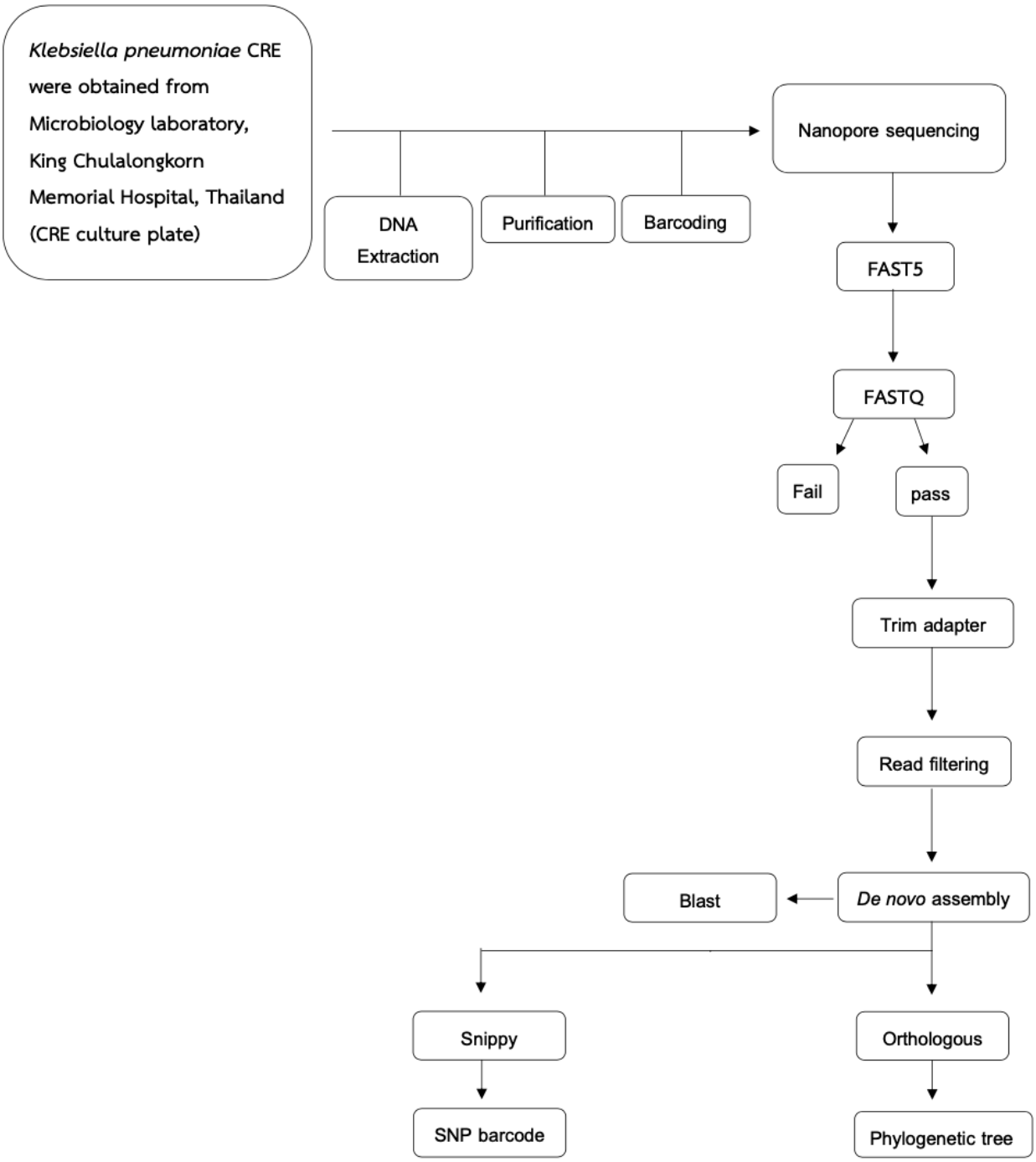
3. Objective

- To construct a phylogenetic tree between CRKP strains and non-CRKP strains.
- To identify specific SNP barcodes associated with CRKP strains.

4. **Research Hypothesis**

   - Carbapenem-resistant phenotypes are associated with KP strains, classified by a phylogenetic tree of the whole bacterial chromosome.

5. **Conceptual Framework**



6. Expected Benefit and Application

- A local database of CRKP and non-CRKP whole genome for future use
- SNP barcodes may identify and predict a carbapenem-resistant phenotype of KP

7. Keyword

Carbapenem-resistant Enterobacteriaceae, Klebsiella pneumoniae, phylogenetic tree, Oxford Nanopore sequencing, SNP barcode

CHAPTER II

Literature review

1. *Klebsiella pneumoniae*

Ecology

Klebsiella pneumoniae (KP) is a gram-negative bacillus. (Figure 1a.) It grows a mucoid lactose fermenter colony on MacConkey agar. (Figure 1b) It's a facultatively anaerobic, non-motile, and encapsulated bacterium. (12) *K. pneumoniae* is one of normal flora in the upper respiratory and gastrointestinal tracts. Thus, the reservoir for transmission of pathogenic *K. pneumoniae* is human feces. (13) Contaminated environment is one source of transmission that can rapidly spread. (14) *K. pneumoniae* has been reported as a pneumonia and bloodstream infection pathogen. (15) Therefore, this bacterium is an important clinical pathogen. (16)

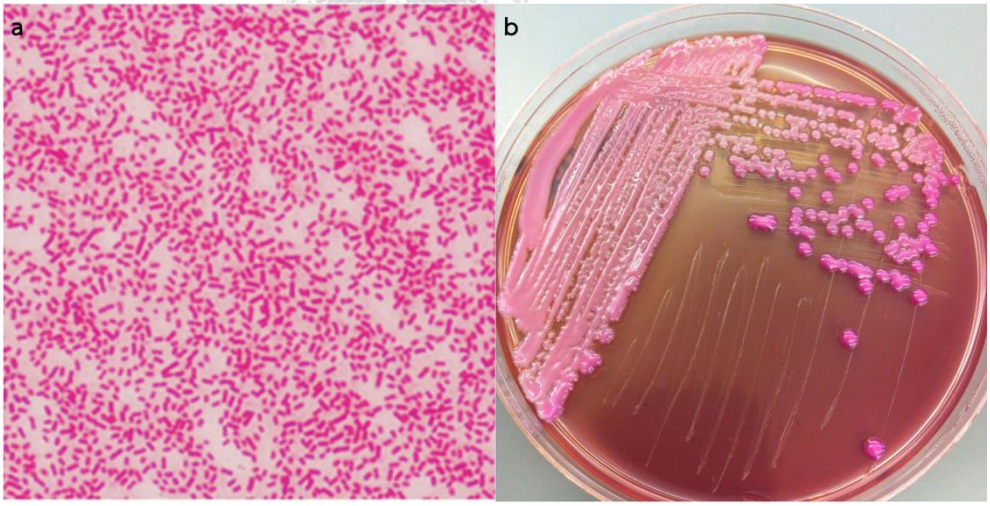


Figure 1. Gram stain of *Klebsiella pneumoniae* (A) and colonies of *Klebsiella pneumoniae* on MacConkey agar (B). (17)

Virulence factors

Virulence factors of *K. pneumoniae* can lead to antibiotic resistance and infection. First, the polysaccharide capsule is a crucial virulence factor to avoid opsonophagocytosis by the hosts. Next, lipopolysaccharide (LPS) which coats the outer surface of *Klebsiella pneumoniae* can induce an inflammatory cascade that causes septic shock and sepsis. Fimbriae of KP help the bacteria attach to host cells. Lastly, *K. pneumoniae* siderophores can bind iron from hosts for propagating themselves. (18) (Figure 2.)

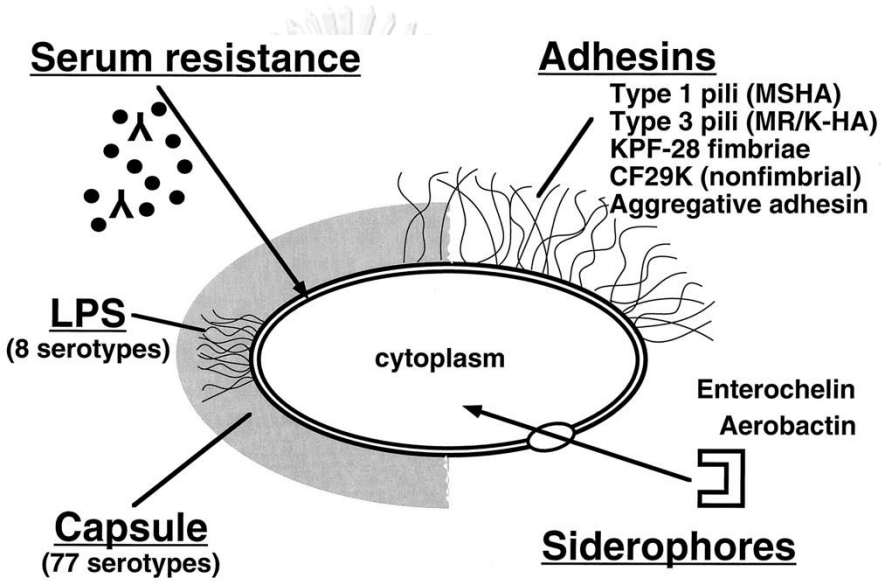


Figure 2. The virulence factors of Klebsiella pneumoniae. (13)

Epidemiology

Klebsiella pneumoniae (KP) is one of the most common nosocomial and opportunistic pathogens in hospitals. (16) The prevalence of bloodstream infections by *K. pneumoniae* is increasing worldwide. (15, 19) *K. pneumoniae* can rapidly spread from person to person by contamination on the hands of hospital personnel. (13) Risks of nosocomial KP infections are long-length hospital stay, catheter use, ventilator use, and poor infection control strategies. (4) The incidence of *K. pneumoniae* bacteremia (640 episodes) in the population of 1.2 millions, from 2000 to 2007) was 7.1 per 100,000 (27%);

174 were nosocomial (27%) and 276 were healthcare-community onset (43%). In 2018, Farida et al., reported 168 isolates of Enterobacteriaceae from 4 Thailand hospitals. Forty-one *K. pneumoniae* isolates (24.4%) were resistant to all carbapenem (Imipenem, doripenem, ertapenem and meropenem). (20)

Diagnosis of *Klebsiella pneumoniae*

Klebsiella pneumoniae can be isolated from specimens on blood agar, chocolate agar, and MacConkey agar at 37°C overnight. On the blood agar, their colonies are mucoid and induce no hemolysis. On MacConkey agar grows mucoid and lactose-fermenter colonies (pink colonies). Next, the bacteria are then identified by biochemical tests. (Table 1.) After knowing the genus or species of bacteria, antimicrobial susceptibility is tested. The bacteria culture and antimicrobial susceptibility test (AST) usually require 2-3 days, too late for the first-dose antibiotic prescription, leading to unnecessary antibiotic use.

Test	Reaction
Indole Production Test	Negative (*K. oxytoca* is Indole positive)
Methyl-Red Test	Negative
Voges-Proskauer Test	Positive
Citrate Utilization Test	Positive
Hydrogen Sulfide Production(TSI)	Negative
Urea Hydrolysis Test	Positive
Lysine Decarboxylase Test	Positive
Arginine Dihydrolase Test	Negative
Ornithine decarboxylase test	Negative
Motility at 36 °C	Non-motile
D-Glucose (acid/gas)	Positive/Positive
D-mannitol fermentation Sucrose fermentation	Positive
Lactose fermentation	Positive
D-sorbitol fermentation	Positive
Cellobiose	Positive
Esculin hydrolysis	Positive
Acetate Utilization Test	Positive
ONPG Test	Positive

Table 1. The biochemical tests for identifying Klebsiella pneumoniae.

2. Carbapenem-resistant Enterobacteriaceae (CRE)

Mechanism of carbapenem-resistance in Enterobacteriaceae

Carbapenem-resistant Enterobacteriaceae (CRE) or Carbapenem-producing Enterobacteriaceae (CPE). CRE infections are hard to be treated and elevate the risks of illness, disease spread, and death. (21) Carbapenems are a type of beta-lactam antibiotics. Being broad-spectrum, they can be used against gram-positive, gram-negative, aerobic bacteria, and anaerobic bacteria. (22) The carbapenems include imipenem, doripenem, meropenem, and ertapenem. Carbapenems bind to penicillin-binding protein and then disrupt the growth and the structure of bacteria cell walls. (23) The three major mechanisms of Enterobacteriaceae resistance to carbapenem antibiotics include efflux pump, enzyme production, and porin mutation. (24) One of the most common resistant mechanisms is enzyme production. Gram-negative bacteria expand the production of β-lactam-hydrolyzing enzymes. (25) They can hydrolyze most cephalosporins, penicillins, carbapenems, monobactams, and β-lactamase inhibitors. (26) The rising prevalence of Enterobacteriaceae with carbapenem resistance is conferred by enzymes such as New Deli Metallo-β-lactamase (NDM) and *Klebsiella pneumoniae* carbapenemase (KPC). (27, 28) CRE is separated into two main subgroups including carbapenemase-producing CRE (CP-CRE) and non-carbapenemase producing CRE (non-CP-CRE). (29)

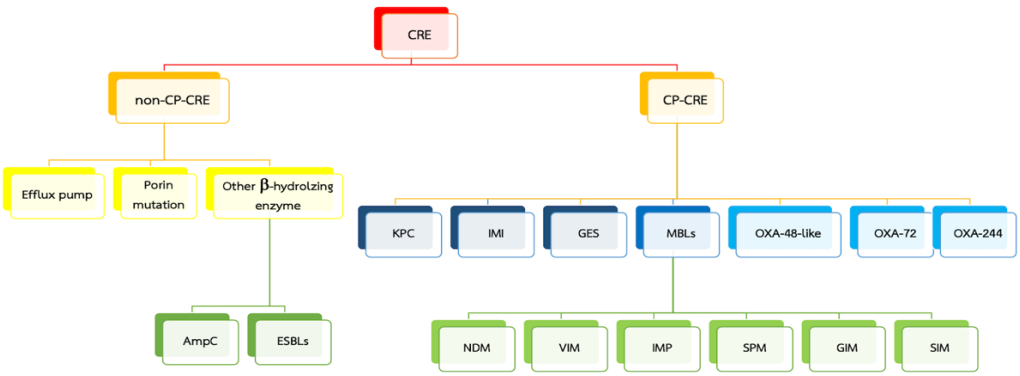


Figure 3. The grouping of the different drug-resistant mechanisms of carbapenem-resistant *Enterobacteriaceae* (Dark blue: Ambler class A, Blue: Ambler class B, and

Light blue: Ambler class D) (CRE: Carbapenem-resistant Enterobacteriaceae, CP: carbapenem producing, KPC: Klebsiella pneumoniae carbapenemase, IMI: Imipenem-hydrolyzing β-lactamase, GES: Guiana extended-spectrum β-lactamase, MBLs: Metallo-β-lactamase, OXA: Oxacillinase, NDM: New Delhi Metallo-β-lactamase, VIM: Verona integron-bone Metallo-β-lactamase, GIM: German imipenemase, SIM: Seoul imipenemase, AmpC: Type C ampicillinase, ESBLs: Extended-spectrum β-lactamase)

Carbapenem-producing CREs have various types of carbapenemases that can be distinguished into three groups—Ambler class A, class B, and class D β-lactamases. (30) The Ambler class A carbapenemase is related to *Klebsiella pneumoniae* carbapenemase (KPC). (31) This plasmid-encoded enzyme that hydrolyzes carbapenem can be inhibited by clavulanic acid. (32) KPC has been found in *Klebsiella pneumoniae* isolates. Furthermore, KPC-producing Klebsiella *oxytoca*, *Escherichia coli, Serratia marcescens, Enterobacter cloacae, Proteus mirabillis, and Salmonella enterica* have been identified. (33) (Figure 3.)

Mobile genetic elements

Mobile genetic element is an important mechanism in bacterial evolution and adaptability against antibiotics in the form of a gene cassette in a bacterial genome. (34, 35) The mobile genetic elements (e.g., plasmids, transposons, or prophages) can move in the host genome or jump across genomes. The mobile genetic elements that insert themselves into the chromosomal genome can be transmitted vertically. They can also be horizontal transferred by 3 processes including transformation, conjugation, and transduction. (36) One type of mobile elements is an integron. It acts as a receptor of antibiotic-resistant gene cassettes and can carry more than 50 antibiotic-resistant gene cassettes. (37) These can confer resistance to beta-lactams, trimethoprim, aminoglycosides, and other antibiotics. (38)

Epidemiology

Carbapenem-resistant Enterobacteriaceae (CRE) is a major and serious public health problem worldwide. (1) CRE transmission usually occurs in a healthcare setting and is caused by bacterial contamination (such as a catheter) or inappropriate antibiotic use in humans and animals. (3, 39) Transmission of CRE from animals to humans can occur through contaminated food chain. (40) In 2020, Yuanyuan Li et al. identified the risk factors and reported the prevalence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) had been increasing year by year over the past 5 years. (Figure 4) The most important risk factors are long hospitalization stay and medical device use. (41)

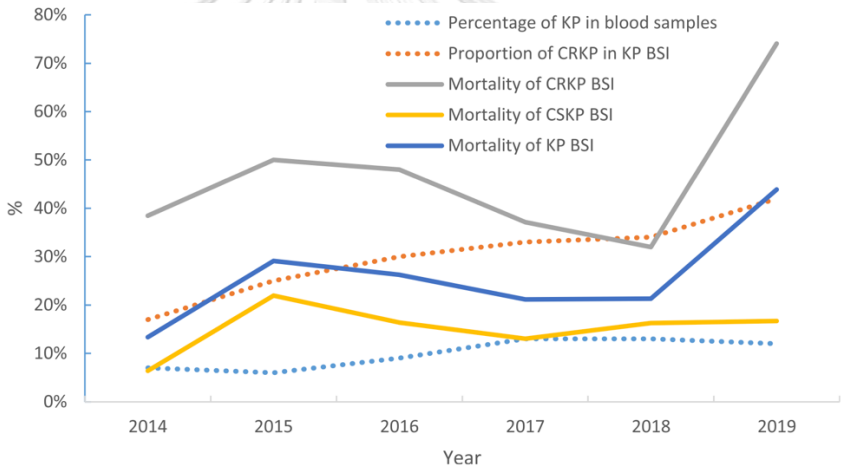


Figure 4. The graph shows trends of prevalence and mortality in CRKP and KP that are increasing year by year over the past 5 years. CRKP: Carbapenem-resistant *Klebsiella pneumoniae*, CSKP: Carbapenem-susceptible *Klebsiella pneumoniae*, KP: *Klebsiella pneumoniae* (41)

Diagnosis of carbapenem-resistant Enterobacteriaceae

Modified-Hodge

The Modified-Hodge test (MHT) detects carbapenemase production. This test has low specificity and sensitivity. This technique is based on the inactivation of carbapenem by the tested isolate which enables a carbapenem-susceptible indicator strain (*Escherichia coli* ATCC 25922) to extend the growth toward a carbapenem disc. Mostly, bacteria with *Klebsiella pneumoniae* carbapenemase (KPC) and Metallo beta-lactamase (MBL) are positive in MHT test. It produces the result in approximately 72 hours. (42)

Carba NP test

The Carba NP test is a biochemical test for rapid identification of carbapenemase-production. (43) This test detects hydrolysis of beta-lactam ring of carbapenem (imipenem) that is detected by changing the color of the pH value (phenol red solution). The color will change from red to orange/yellow which is positive (carbapenemase producer) (44) (Figure 5.) The limitation of the Carba NP test is OXA-48-like carbapenemase producer detection. (45)

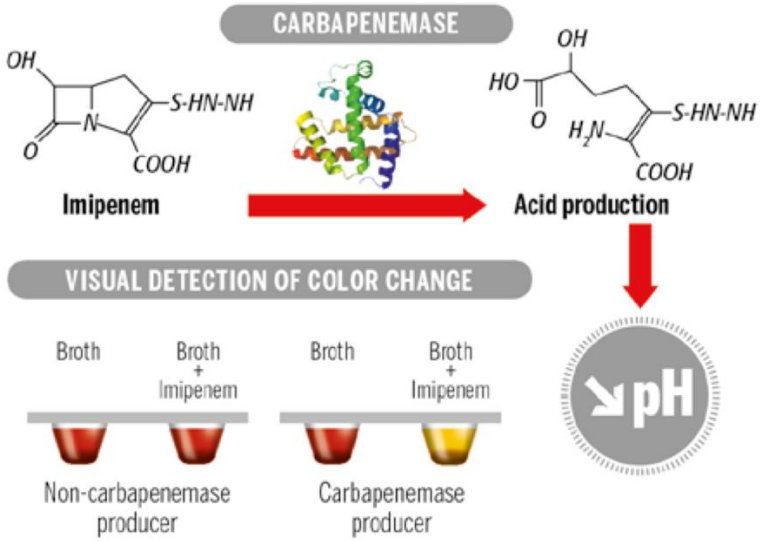


Figure 5. The principle of colorimetric indication by carbapenemase activity (46)

Accuracy and timely are the most important for pathogenic bacterial identification to aid the early prescription of appropriate antibiotics. (47) Routine identification method including bacterial culture, biochemical test, and antimicrobial susceptibility test (AST) has too long turnaround time

3. Nanopore sequencing

Oxford nanopore sequencing is the 4th generation sequencing. (48) It can generate ultralong reads (approximately 10^4-10^6 bases), analyzed data in real-time , and increase thought put. (49) A single DNA or RNA molecule can be sequenced without amplification or chemical labeled by passing through a tiny pore. (Figure 6.) This nanopore is a biological transmembrane protein channel made from α-hemolysin. (Figure 7.) The α-hemolysin is an exotoxin secreted from *Staphylococcus aureus*. (48) Nanopore sequencing measures the electric current fluctuation from a DNA/RNA molecule passing through a nanopore channel at the speed of 450 base per second (R9.4 nanopore). (50)

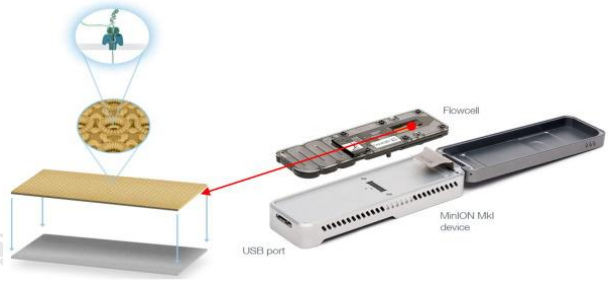


Figure 6. Nanopore sequencing (MinION) device with flowcell. (51)

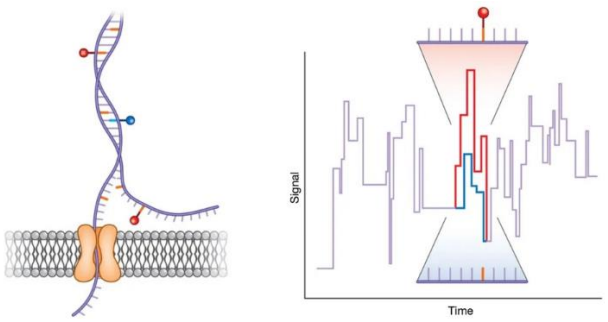


Figure 7. A single strand of DNA/RNA passes through a nanometer-size pore (left), causing a current change (right). (52)

In 2020, Arne M. taxt et al., used the nanopore sequencing technology to identify pathogens and detect AMR genes from positive bloodstream infection samples. Generally, the turnaround time for hemoculture with AST is 1-3 days. The nanopore sequencing can identify pathogens in 10 minutes and detect the AMR gene within 1 hour of sequencing (Figure 8.) (6)

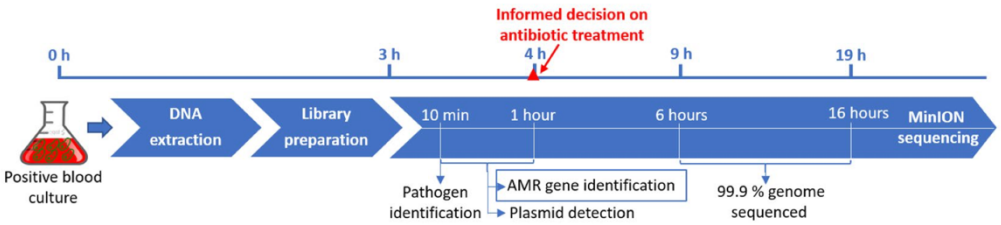


Figure 8. The timeline of nanopore sequencing from positive blood culture. (6)

In 2021, Nan wu et al. used nanopore sequencing to develop a rapid detection and compared nanopore sequencing to different methods. This study collected 83 endotracheal aspirates (ETA) samples from Peking University Third Hospital (PUTH) and the results showed the whole method took 5–6 hours to identify pathogens. Therefore, MinION (nanopore sequencing) provides a new rapid identification of pathogens in clinical samples. (53)

4. SNP barcode

Single nucleotide polymorphism (SNP) or single nucleotide variation (SNV) is a genetic variation at a single nucleotide base in a specific position of DNA molecule among individual organisms. (54) A single nucleotide base modification can be insertion, deletion, transition, or transversion. (55) SNPs are important markers in many previous studies that indicate sequence variation is associated to genotype and may affect the phenotype. (56) SNP barcodes are composed of SNP combined, revealing a unique organism pattern. (57) SNP barcodes are a set of clade-specific SNPs that are shared by all, or most, of the organisms in that clade, and do not present in any other organisms, or only a few, outside that particular clade. (58) SNP barcode typing was used to investigate the epidemiology, identification, and classification. (59) In 2014, the previous study designed a 23 SNP barcode to identify 711 *Plasmodium falciparum* isolates from 14 counties that were highly predictive (92%) (60). In 2020, Abebe A. Fola et al. developed a genetic marker that was a SNP barcode for capturing the diversity and the structure of *Plasmodium vivax* in Papua New Guinea and the result showed that a SNP barcode could be used to map the variation of malaria transmission and this technique was low cost and highly feasible. (61) Furthermore, the SNP barcode is an alternative method to rapidly identify and classify organisms.

CHAPTER III

Material and method

1. Materials

Chemicals

- LB agar, Miller (Luria-Bertani)
- LB broth, Miller (Luria-Bertani)
- Quick-DNA HMW Magbead kit (Zymo Research)
- Rapid Barcoding Kit 96 (Oxford Nanopore)
- AMPure XP (Beckman Coulter)
- Qubit dsDNA HS assay kit (Thermo Fisher scientific)

Equipments

- MInION Mk1C (Oxford Nanopore)
- Centrifuge 5424 (Eppendorf)
- Nanodrop one (Thermo Fisher scientific)
- Qubit 4 fluorometer (Thermo Fisher scientific)
- Magnetic Device (Axygen)
- Analytic balance (Mettler Toledo)
- Heater (Biosan)
- Ice bucket
- Vortex mixer
- Microcentrifuge tube 1.5 ml (Axygen)
- Bottle 1 L
- Plate
- Loop
- Heat box
- Pipette, the pipette tip
- Qubit assay tube (Thermo Fisher scientific)

Bioinformatic tools

- Guppy

- Porechop
- Prinseq
- Flye
- BLAST
- Prokka
- Snippy
- Orthofinder

2. Methods

Research design

Descriptive study

Sample collection and bacterial subculture

Forty *Klebsiella pneumoniae* clinical isolates from 2020-2021 were collected at King Chulalongkorn memorial hospital. Each isolate was recovered on LB agar incubated with a clinical specimen at the microbiology laboratory. The samples were distinguished into 4 groups, including carbapenem-resistant *K. pneumoniae* (CRKP), extended-spectrum beta-lactam (ESBL), and non-CRE/non-ESBL. The bacteria were sub-cultured in 5 ml LB broth. They were grown overnight in an incubator shaker at 37° C.

DNA extraction

Microbial lysis

Five ml LB broth was spun down at 3000g for 10 minutes and supernatant was discarded. The pellet up to 100 mg per sample was used for DNA extraction with the Quick-DNA HMW MagBead kit (Zymo Research). Firstly, added DNA/RNA shield 200 ul to the pellet. Centrifuge at 5,000g for 1 minute and transfer the supernatant to a fresh tube (~180 ul). Keep the supernatant and the pellet. One hundred ul PBS was added to the pellet and gently mixed with the pipette. Centrifuge at 5,000g for 1 minute, then combine the supernatant with the supernatant from a previous step. Add 1 ml. of PBS to the pellet and mix until resuspended. Centrifuge 5,000 x g for 1 minute and

discard the supernatant. One hundred ul TE buffer and 25 ul lysosome were added to the pellet, then pipette mixed and incubated at 55°C for 30 minutes. Combine the previous supernatant (~280 ul.) and the digested samples. (~125 ul). The 20 ul of 10% SDS and 10 ul. Proteinase K were added to digested samples, then gently pipette mixed and incubated at 55°C for 10 minutes. Centrifuge the digested samples at 5,000g for 1 minute and transfer the supernatant to the new microtube. The 800 ul Quick-DNA Mag-binding buffer was added to the samples, then mixed well.

DNA purification

Add 33 ul of magnetic bead to each sample, pipette mix 5 times, and put on rotator or shaker 10 minutes for magnetic bead to attach to the bacterial DNA. Transfer samples to the magnetic stand until the magnetic bead was separated and then remove the supernatant. Remove samples from the magnetic stand. Quick DNA MagBinding bead 500 ul was added into each sample and put them on rotator or shaker for 5 minutes. Transfer the samples to the magnetic stand and remove the supernatant. DNA Pre-wash buffer 500 ul was added on to the pellet and pipette mixed 10 times. The samples were transferred to the magnetic stand to separate the bead from the solution and the supernatant was discarded. The 900 ul gDNA wash buffer was added to the pellet and gently mixed with the pipette 10 times. Transfer all the liquid to the new microtube on the magnetic stand until the magnetic bead was separated and discard the supernatant. Repeat the gDNA wash buffer step. Air dry the pellet for 20 minutes at room temperature. Finally, elute the bacterial DNA with 50 ul Ultrapure water.

Qubit fluorometer assay

We used the Qubit fluorometer for DNA quantification. The Qubit assay is highly sensitive for low concentration of DNA (10pg/ul–100 ng/ul). Prepare the working solutions by diluting the Qubit dsDNA reagent 1:200 with Qubit buffer. (Qubit reagent 1 x n ul, Qubit buffer 199 x n ul.) The final volume of each sample must be 200 ul. The working solution was aliquoted into a Qubit tube (199 ul.) and added 1 ul DNA extract into each Qubit tube, then vortex mixed

2-3 seconds. Incubate for 2 minutes at room temperature. DNA extract was stored shortly at 4°C until library preparation.

Nanopore sequencing

Library preparation

Library preparation was performed using rapid barcoding kit 96 (SQK-RBK10.96) with MinION flow cell (R.9.4.1 FLO-MIN106). First, thaw the kits at room temperature, then vortex mix and spin down. Prepare the samples in nuclease-free water by transferring 50 ng purified DNA per sample into a 1.5 ml LoBind tube, then adjust the volume to 9 ul with nuclease-free water. One ul of each rapid barcode was added. The mixture was incubated at 30°C for 2 minutes and then incubated at 80°C for 2 minutes. After that, put the tube on ice. Then, pool all barcoded samples into a single microcentrifuge tube. Resuspend SPRI beads by vortexing. The pooled sample from the previous step was added and mixed with resuspended SPRI beads in equal volume on Hula mixer for 5 minutes at room temperature. Prepare 80% ethanol in nuclease-free water. Spin down the incubated sample and keep on the magnetic, discarding the supernatant. Add 1.5 ml of 80% ethanol and then discard. Repeat the previous step. Bring the sample to spin down and place back on magnetic. Allow to dry 30 seconds. Remove the tube from magnetic and resuspend the pellet in 15 ul of elution buffer and incubate 10 minutes. Place the sample back on magnetic until being clear and colorless. Remove and retain 15 ul of elution in a 1.5 ml Eppendorf DNA LoBind tube. Transfer 11 ul of the eluted sample into a 1.5 ml Eppendorf DNA LoBind tube and add 1 ul of Rapid Adapter F (RAP F). Gently mix and incubate 5 minutes at room temperature.

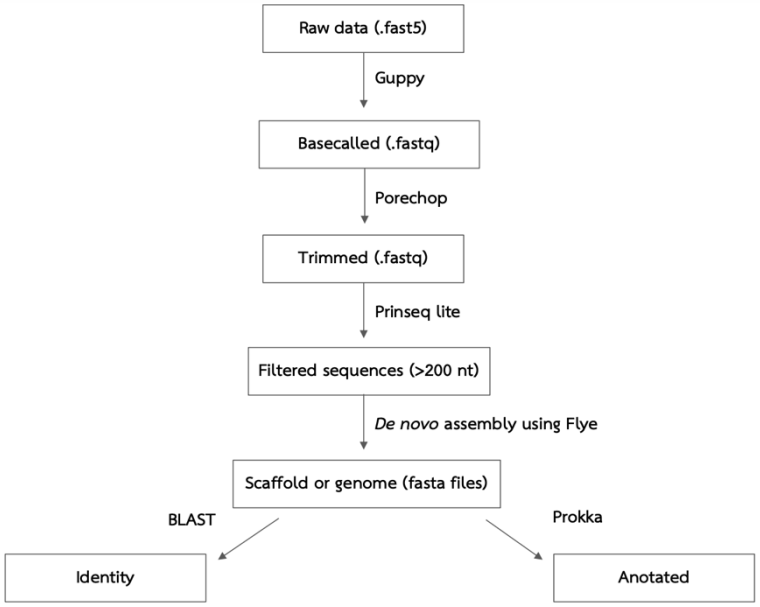
Priming and loading the flow cell

Thaw kit components at room temperature including Sequencing Buffer II (SBII), Loading Buffer II (LBII), Flush Tether (FLT), and Flush buffer (FB) and mix by vortexing. Spin down SBII and FLT. Open the lid of MinION and slide the flow cell under the clip. Then, slide priming port cover clockwise to open.

Check the bubble under the cover and draw back volume to remove bubble in 3 steps, including set a 1000 pipette to 200 ul, insert a tip into priming port, and turn the wheel until show 220-230 ul. Next, we prepared the flow cell priming mix by adding 30 ul of FLT to 1.17 ml of FB and mixing by vortexing at room temperature. Load 800 ul of the priming mix into the flow cell (avoid the bubble). Wait 5 minutes. The library for loading included 37.5 ul Sequencing Buffer II (SBII), 25.5 ul Loading Beads II (LBII) and 12 ul DNA library in a new tube. Lastly, gently lift the port cover and load 200 ul of the priming mix into the flow cell and add 75 ul of sample to the flow cell. Ensure each drop flow into the flow cell and close the priming port and replace the MinION lid.

Bioinformatics analysis

Pipeline



Base-calling

Oxford nanopore sequencer produces a fast5 file (HDF5). A fast5 file is an electrical signal by DNA/RNA strands passing through the nanopore. This process converts the electrical signal to the DNA/RNA sequence (FASTQ). Guppy toolkit (version 5.0.16) is Oxford nanopore's base-calling algorithm.

First, transfer the electrical signal file (FAST5) from the Nanopore sequencer to GPU. Next, the DNA sequence was base-called by the Guppy tool on the server.

Trimming adapter/barcode

In the library preparation step, we must add the adapter and barcodes to the DNA/RNA strands. After base-called, the adapter and barcodes were trimmed and removed from Nanopore reads by Porechop tool version 0.2.4 (https://github.com/rrwick/Porechop). Porechop tool trimmed adapter off from the end or middle of reads. The input is fastq files that are converted files from the Guppy tool output.

Filtering the read

Prinseq is a quality control tool for DNA/RNA sequence data. We used Prinseq tool version 0.20.4 (https://github.com/uwb-linux/prinseq). It is often used to filter, trim, and reformat. After trimming the adapter off and removing the barcode by Porechop tool, we used Prinseq tool to filter reads with a minimum read length of 200 bp.

***De novo* assembly**

De novo assembly is genome reconstruction from DNA fragments for genotyping. DNA sequences were assembled as contigs. We assembled the genomes by Flye tool version 2.9 (https://github.com/fenderglass/Flye). The input is filtered DNA sequence from the result of the Prinseq tool (fastq file). The outputs of Fyle are several files in a fasta format.

Identification of Klebsiella pneumoniae

BLAST or Basic Local Alignment Search Tool helps match regions on nucleotide sequences to biological identity database, i.e., NCBI (National Center of Biotechnology Information). We used the BLASTn version 4 with the results of the Flye tool (Fasta file) as the inputs. The output from BLAST includes E value (the number which describes the posssibility to match just by chance), percent identity (similarity of the query DNA sequences to the target sequences), and query cover (the number describes how much of a query sequence is covered by the target sequence). Only hits with ≥ 85% of

similarity, e-value $\leq 10^{-6}$, and with ≥ 80% coverage were kept.

Gene prediction

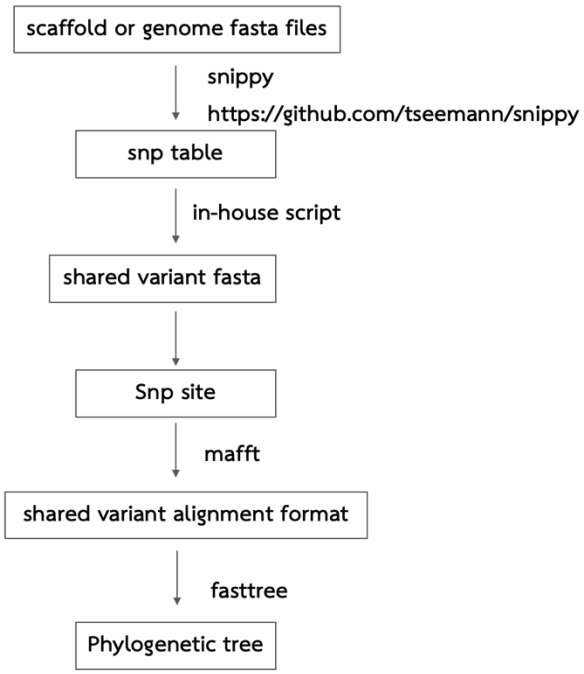
Prokka is a command-line annotation tool, helping reveal the interesting features of genomes. The inputs were the *Klebsiella pneumoniae* assembled genomes from Fyle tool. The outputs of Prokka are several files such as fna file (Fasta file of original input contigs), faa file (protein Fasta file of translate coding gene), ffn file (Nucleotide Fasta file of prediction transcripts), or txt file (annotation summary).

Phylogenetic tree and SNP calling

Orthofinder

Orthofinder is a tool utilized for comparative genome and orthology relationships between the genes using a gene tree. (62) We used the Orthofindertoolversion2.5.0.
(https://github.com/davidemms/OrthoFinder#methods) The inputs were results from the Prokka tool (.faa file). The outputs of Orthofinder are folders such as gene_tree, Orthologous or species_tree. The Newick file in the species_tree folder was utilized to visualize the phylogenetic tree.

Snippy



Snippy is an SNP calling tool version 4.4.0. (https://github.com/tseemann/snippy) The principle of this tool is finding SNP including single polymorphism (SNP) or insertion and deletion (indel) in sequences of interest by comparing to the reference. This tool can determine the variants on the genome. Snippy identifies the differences using fancy Bayesian statistics (FreeBayes). Our inputs included 2 files, the reference genome in a fasta file (Klebsiella pneumoniae subsp. Pneumoniae HS11286, Genbank accession number: CP003200) and the *Klebsiella pneumoniae* assembled genome in fasta file. The outputs are in several file formats such as SNPs VCF file (annotation variants in VCF format) or SNP table (separate summary of variants). Next, we used the SNP table (.txt) to find the shared variants (Fasta file) with an in-house script. Then, we extracted SNP from muti-Fasta alignment by using the SNP-sites tool. The result of the SNP-sites is a multi-fasta alignment. Mafft (version. 7) was utilized for alignment (63). Lastly, we used Fasttree (version 2.1.10) to make a phylogenetic tree (64).

SNP filter

Klebsiella pneumoniae reference genome (*Klebsiella pneumoniae* subsp. Pneumoniae HS11286) were compared to 40 *K. pneumoniae* assembled genomes to identify SNPs. Next, the insertions and deletions were removed (58). Only 631 substitution SNP sites that existed in all 40 KP genomes were selected. Then, we filtered 631 SNP sites to 64 SNP sites by removing the sites that had the same base across all 40 KP genomes. (Example: If SNP site position 2 is A base in all *K. pneumoniae* genomes, discard position 2).

Identification of specific SNP barcode

After we had 64 filtered SNP sites from the previous step, we tried to identify a specific SNP barcode for classifying CRE and non-CRE from 2 SNP tables (Table 1 and 2). The SNP table in Table 1 is sorted in order of 64 SNP phylogenetic tree. Forty *Klebsiella pneumoniae* isolates were barcoding as 1–44 (barcode 9–12 were removed). Barcode 1–24 were carbapenem-resistant *K. pneumoniae* (CRKP), and barcode 25-44 were non-CRKP. We identified a clade-specific SNP set by applying the following criteria. first, SNPs are specific to the clade. Next, SNPs are not found in another clade. Then, the variants are found in all/almost all individuals in the clade.

CHAPTER IV

Results

The whole genome assembly of 40 *Klebsiella pneumoniae* isolates

Table 2. The table shows the results of whole genome assembly of 40 *Klebsiella pneumoniae* isolates. The highlights indicate the contigs that are complete bacterial chromosomes.

Barcodes	Contigs	Bacteria	%Identity	Coverage	Circular	Length
Barcode01	contig_2	Klebsiella pneumoniae strain AR_0152 plasmid tig00000216_u, complete sequence	98.01	153	Yes	107573
	contig_4	Klebsiella pneumoniae strain BA4656 chromosome, complete genome	99.172	82	Yes	5301174
	contig_5	Klebsiella pneumoniae plasmid pIT-01C22, complete sequence	99.669	1899	Yes	122666
	contig_6	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed1, complete sequence	99.864	1692	Yes	114649
Barcode02	contig_1	Klebsiella pneumoniae strain Kp_Goe_821588, complete sequence	99.197	383	Yes	5317430
	contig_10	Klebsiella pneumoniae strain QS17-0161 plasmid pMR0617aac, complete sequence	99.578	675	No	41988
	contig_11	Klebsiella pneumoniae subsp. pneumoniae Kp13 plasmid pKP13c, complete sequence	99.689	118	Yes	4993
	contig_3	Klebsiella pneumoniae strain KP36 plasmid 1, complete sequence	99.512	1269	No	27461
	contig_4	Klebsiella pneumoniae strain QS17-0161 plasmid pMR0617aac, complete sequence	99.815	755	No	131700
	contig_5	Klebsiella pneumoniae strain PIMB15ND2KP27 plasmid pKP27-NDM4, complete sequence	99.933	361	No	4498
	contig_6	Klebsiella pneumoniae strain NH25 plasmid pNH25.2, complete sequence	99.636	1987	No	52891
	contig_8	Klebsiella pneumoniae strain NH25 plasmid pNH25.2, complete sequence	99.812	509	No	5851
	contig_9	Enterobacter hormaechei subsp. xiangfangensis M206 plasmid pM206-NDM1 DNA, complete sequence	99.905	1283	No	59852
Barcode03	contig_1	Klebsiella pneumoniae strain BA33875 plasmid pBA33875_IncFIB, complete sequence	99.891	595	No	3658
	contig_2	Klebsiella pneumoniae strain WCHKP34 plasmid pQnrB_LL34, complete sequence	99.784	637	No	122714
	contig_3	Klebsiella pneumoniae strain 555 chromosome, complete genome	99.195	207	Yes	5397692
	contig_4	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.528	8835	Yes	6112
	contig_5	Klebsiella pneumoniae strain 0773 plasmid pKpn114, complete sequence	99.706	48	No	4167
	contig_7	Klebsiella pneumoniae strain A64477 plasmid pKP64477b, complete sequence	98.382	412	Yes	210593

Barcodes	Contigs	Bacteria	%Identity	Coverage	Circular	Length
Barcode04	contig_2	Escherichia coli strain Ecol_AZ147 plasmid pECAZ147_KPC, complete sequence	99.713	670	Yes	121902
	contig_3	Klebsiella pneumoniae strain CCUG 70747 chromosome	99.461	256	Yes	5376977
	contig_4	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed1, complete sequence	99.84	658	Yes	125132
	contig_5	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.576	411	Yes	12236
	contig_6	Klebsiella pneumoniae strain INF274 plasmid unnamed4, complete sequence	98.594	4648	No	4648
	contig_7	Escherichia coli plasmid pV323-a DNA, contig: V323-a_scaffold_7, strain: V323	99.766	67	Yes	15728
Barcode05	contig_1	Klebsiella pneumoniae strain F93-1 chromosome, complete genome	99.13	410	Yes	5310299
	contig_2	Klebsiella pneumoniae strain CRKP-2297 plasmid pCRKP-2297_3, complete sequence	98.232	247	No	103738
	contig_3	Klebsiella pneumoniae genome assembly, plasmid: 70	99.728	768	Yes	212246
	contig_4	Klebsiella pneumoniae strain Kp_Goe_822917 plasmid pKp_Goe_917-7, complete sequence	99.719	656	Yes	7102
	contig_6	Klebsiella pneumoniae strain AR_0152 plasmid tig00000200, complete sequence	99.9	279	Yes	108025
	contig_7	Klebsiella pneumoniae strain KpvST147B_SE1_1_NDM plasmid pKpvST147B_5, complete sequence	99.859	896	Yes	116405
	contig_8	Klebsiella pneumoniae isolate 91a83dc8-b809-11e8-aae5-3c4a9275d6c8 genome assembly, chromosome: 1	97.58	425	Yes	74487
	contig_9	Klebsiella pneumoniae strain QS17-0029 plasmid pMR0617mcr, complete sequence	99.828	5317	Yes	33801
Barcode06	contig_1	Klebsiella pneumoniae strain KpN01 plasmid pKpN01-SIL, complete sequence	99.805	387	Yes	164657
	contig_2	Klebsiella pneumoniae strain AR_0066 chromosome, complete genome	99.155	261	Yes	5223981
	contig_3	Klebsiella pneumoniae strain FDAARGOS_442 plasmid unnamed4, complete sequence	99.527	153	No	1053
	contig_5	Klebsiella pneumoniae strain KpvST147B_SE1_1_NDM plasmid pKpvST147B_4, complete sequence	99.711	799	No	3109
	contig_6	Klebsiella pneumoniae strain AATZP plasmid pNDM-1fa, complete sequence	99.92	857	No	42698

Barcodes	Contigs	Bacteria	%Identity	Coverage	Circular	Length
Barcode07	contig_1	Klebsiella pneumoniae strain AP8555 chromosome, complete genome	99.207	70	No	685700
	contig_10	Shigella sonnei strain 4303 plasmid pC, complete sequence	99.377	20	No	2272
	contig_11	Klebsiella oxytoca strain 4928STDY7071151 genome assembly, chromosome: 1	97.523	858	Yes	3956
	contig_12	Klebsiella pneumoniae strain CCUG 70742 plasmid pKpn70742_3	99.046	20	Yes	4986
	contig_2	Klebsiella pneumoniae subsp. pneumoniae strain KpvK54 chromosome, complete genome	99.576	67	Yes	4604377
	contig_3	Klebsiella pneumoniae strain JNM10C3 chromosome	99.879	118	No	34783
	contig_5	Klebsiella pneumoniae strain kp757, complete genome	100	113	No	1662
	contig_9	Salmonella enterica subsp. enterica serovar Heidelberg strain SH14-028 plasmid pSH14-028_2, complete sequence	97.883	20	No	2400
Barcode08	contig_1	Escherichia coli strain Ecol_AZ147 plasmid pECAZ147_KPC, complete sequence	99.707	832	Yes	121897
	contig_2	Klebsiella pneumoniae strain QS17-0029 chromosome, complete genome	99.859	368	Yes	5311667
	contig_3	Escherichia coli M216 plasmid pM216_mF DNA, complete sequence	99.854	761	Yes	113275
	contig_4	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.527	514	Yes	12226
Barcode13	contig_1	Escherichia coli strain Ecol_AZ147 plasmid pECAZ147_KPC, complete sequence	99.732	432	Yes	121939
	contig_2	Klebsiella pneumoniae strain BA4656 chromosome, complete genome	99.167	196	Yes	5375915
	contig_4	Escherichia coli strain 4/1-1 plasmid p4_1_1.1, complete sequence	99.856	412	Yes	125157
	contig_5	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.717	413	Yes	12244
	contig_6	Klebsiella pneumoniae strain INF274 plasmid unnamed4, complete sequence	98.767	505	Yes	9309
Barcode14	contig_1	Klebsiella pneumoniae strain 2N3 chromosome, complete genome	99.258	128	Yes	5316537
	contig_2	Escherichia coli strain Ecol_AZ147 plasmid pECAZ147_KPC, complete sequence	99.74	295	Yes	121943
	contig_3	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.674	3539	Yes	6122
	contig_4	Escherichia coli M216 plasmid pM216_mF DNA, complete sequence	99.865	269	Yes	113309

Barcodes	Contigs	Bacteria	%Identity	Coverage	Circular	Length
Barcode15	contig_1	Klebsiella pneumoniae strain AR_0160 chromosome, complete genome	99.041	98	Yes	5309737
	contig_2	Klebsiella pneumoniae strain FDAARGOS_629 plasmid unnamed3, complete sequence	99.861	247	Yes	109322
	contig_3	Klebsiella pneumoniae subsp. pneumoniae strain ARLG-3135 plasmid p1, complete sequence	99.939	172	Yes	92985
	contig_4	Klebsiella pneumoniae strain N201205880 plasmid p205880-NR2, complete sequence	98.863	172	Yes	95294
	contig_5	Escherichia coli strain ST648 plasmid pEC648_5, complete sequence	99.806	868	No	2082
	contig_6	Escherichia coli strain EcoI_AZ155 plasmid pECAZ155_3, complete sequence	93.459	548	Yes	6503
	contig_7	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.731	21	Yes	18389
	contig_8	Klebsiella pneumoniae subsp. pneumoniae strain ARLG-3135 plasmid p6, complete sequence	99.8	23	Yes	23938
	contig_9	Escherichia coli strain EC25 plasmid pEC25-4, complete sequence	99.94	22	Yes	19936
Barcode16	contig_1	Klebsiella pneumoniae strain INF274 plasmid unnamed4, complete sequence	99.349	500	Yes	9364
	contig_2	Klebsiella pneumoniae strain FDAARGOS_440 chromosome, complete genome	99.91	235	Yes	5378238
	contig_3	Escherichia coli strain EcoI_AZ147 plasmid pECAZ147_KPC, complete sequence	99.734	543	Yes	121934
	contig_4	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.574	396	Yes	12240
	contig_5	Escherichia coli plasmid pV323-a DNA, contig: V323-a_scaffold_7, strain: V323	99.45	400	Yes	10478
	contig_6	Escherichia coli M216 plasmid pM216_mF DNA, complete sequence	99.852	493	Yes	104896
Barcode17	contig_1	Klebsiella pneumoniae strain SWU01, complete genome	99.324	213	Yes	5384133
	contig_2	Escherichia coli strain EcoI_AZ147 plasmid pECAZ147_KPC, complete sequence	99.709	717	Yes	121924
	contig_3	Klebsiella pneumoniae strain INF274 plasmid unnamed4, complete sequence	98.644	517	Yes	9326
	contig_4	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.716	551	Yes	12248
	contig_5	Escherichia coli strain 4/1-1 plasmid p4_1_1.1, complete sequence	99.828	692	Yes	119762

Barcodes	Contigs	Bacteria	%Identity	Coverage	Circular	Length
Barcode18	contig_1	Escherichia coli strain CREC-A6 plasmid pCREC-A6-NDM, complete sequence	99.938	263	No	3224
	contig_10	Klebsiella pneumoniae strain 1220 plasmid p1220-CTXM, complete sequence	98.822	709	Yes	189502
	contig_2	Klebsiella pneumoniae subsp. pneumoniae strain BR7 plasmid unnamed1, complete sequence	100	85	No	1726
	contig_3	Klebsiella pneumoniae strain AR_0158 plasmid tig00000727, complete sequence	99.736	656	No	32182
	contig_5	Raoultella planticola strain GODA plasmid unnamed1, complete sequence	100	123	No	1910
	contig_6	Escherichia coli strain Ecol_422 plasmid pEC422_1, complete sequence	99.228	779	No	71470
	contig_7	Klebsiella pneumoniae strain AR_0109 chromosome, complete genome	99.498	346	Yes	5300090
	contig_8	Escherichia coli strain CREC-591 plasmid pCREC-591_4, complete sequence	99.838	1141	No	41193
	contig_9	Klebsiella pneumoniae strain KP617 plasmid KP-p1, complete sequence	98.722	714	No	161348
Barcode19	contig_1	Klebsiella pneumoniae strain NCTC9157 genome assembly, chromosome: 1	99.184	299	Yes	5385693
	contig_2	Escherichia coli strain Ecol_AZ147 plasmid pECAZ147_KPC, complete sequence	99.721	748	Yes	122704
	contig_3	Escherichia coli strain 4/1-1 plasmid p4_1_1.1, complete sequence	99.848	730	Yes	125158
	contig_5	Escherichia coli plasmid pV323-a DNA, contig: V323-a_scaffold_7, strain: V323	99.693	523	Yes	10479
	contig_6	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.727	465	Yes	12251
Barcode20	contig_1	Klebsiella pneumoniae strain BA6740 plasmid pBA6740_1, complete sequence	99.81	542	No	28539
	contig_10	Klebsiella pneumoniae JM45 plasmid p2, complete sequence	95.038	895	Yes	10031
	contig_11	Klebsiella pneumoniae strain WCHKP7E2 plasmid p3_085072, complete sequence	99.526	498	Yes	11775
	contig_2	Klebsiella pneumoniae subsp. pneumoniae strain AUSMDU00008079 plasmid pAUSMDU8079-1, complete sequence	99.683	707	Yes	239721
	contig_5	Klebsiella pneumoniae genome assembly, plasmid: 70	99.791	801	Yes	139798
	contig_6	Proteus mirabilis isolate Pm37THOMI tRNA modification GTPase gene, complete cds; Salmonella genomic island 1 Pm37THOMI mobile element, complete sequence; and membrane protein gene, complete cds	99.852	172	Yes	9166
	contig_7	Klebsiella pneumoniae subsp. pneumoniae strain 234-12, complete genome	98.657	417	Yes	5387216

Barcodes	Contigs	Bacteria	%Identity	Coverage	Circular	Length
Barcode21	contig_1	Klebsiella pneumoniae strain SWU01, complete genome	99.32	277	Yes	5402819
	contig_2	Escherichia coli plasmid pV323-a DNA, contig: V323-a_scaffold_7, strain: V323	99.675	486	Yes	10469
	contig_3	Klebsiella pneumoniae strain INF274 plasmid unnamed4, complete sequence	98.386	886	Yes	4669
	contig_4	Escherichia coli strain 4/1-1 plasmid p4_1_1.1, complete sequence	99.836	749	Yes	114888
	contig_5	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.479	4415	Yes	6113
Barcode22	contig_1	Klebsiella pneumoniae strain NH54 chromosome, complete genome	99.924	143	Yes	5341431
	contig_2	Klebsiella pneumoniae strain WCHKP34 plasmid pQnrB_LL34, complete sequence	99.842	322	Yes	122743
	contig_3	Klebsiella pneumoniae strain 0773 plasmid pKpn114, complete sequence	99.779	491	Yes	15649
	contig_4	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.707	777	Yes	6120
Barcode23	contig_1	Klebsiella sp. PO552 plasmid p6, complete sequence	91.495	775	Yes	5834
	contig_10	Escherichia coli strain ECOR 10 genome assembly, plasmid: RCS83_pI	88.997	611	Yes	7920
	contig_11	Klebsiella pneumoniae strain FDAARGOS_440 plasmid unnamed3, complete sequence	99.788	17	Yes	6130
	contig_12	Klebsiella pneumoniae strain A1731 plasmid pA1731-KPC, complete sequence	95.791	763	Yes	7340
	contig_13	Klebsiella pneumoniae strain NKU_KlebA1 plasmid pKlebA1, complete sequence	98.922	830	Yes	119969
	contig_14	Klebsiella sp. PO552 plasmid p2, complete sequence	98.556	904	Yes	111200
	contig_2	Acinetobacter sp. WCHAc010005 plasmid pOXA58_010005, complete sequence	99.946	145	No	1864
	contig_3	Klebsiella pneumoniae JM45 plasmid p2, complete sequence	93.686	5	No	12218
	contig_4	Klebsiella pneumoniae strain 1050 chromosome, complete genome	94.646	3	No	6743
	contig_5	Klebsiella pneumoniae strain AR_0107, complete genome	99.265	121	Yes	5245387
	contig_6	Klebsiella pneumoniae strain KSB2_1B plasmid unnamed4, complete sequence	96.394	912	Yes	202429
	contig_7	Escherichia coli strain WCHEC005237 plasmid pNDM5_005237, complete sequence	99.828	2894	No	47144
	contig_8	Salmonella enterica strain GTA-FD-2016-MI-02533-1 plasmid pGTAFD2016-MI-0253.2, complete sequence	99.766	868	Yes	4003
	contig_9	Klebsiella pneumoniae strain INF274 plasmid unnamed4, complete sequence	98.582	17	Yes	9338

Barcodes	Contigs	Bacteria	%Identity	Coverage	Circular	Length
Barcode24	contig_1	Klebsiella pneumoniae strain 203 chromosome, complete genome	99.322	214	Yes	5275368
	contig_2	Klebsiella oxytoca strain 4928STDY7071151 genome assembly, chromosome: 1	97.326	501	Yes	7917
Barcode25	contig_1	Escherichia coli strain YPE12 plasmid pYPE12-101k-tetX4, complete sequence	100	242	No	2722
	contig_10	Serratia marcescens strain E28 plasmid pE28_001, complete sequence	98.642	1029	No	142376
	contig_2	Enterobacter hormaechei strain S6 plasmid pIncHI2-1502264, complete sequence	99.915	115	No	3538
	contig_3	Acinetobacter baumannii strain XH860, complete genome	100	121	No	1844
	contig_4	Escherichia coli strain 2019XSD11-TC2 plasmid p2019XSD11-TC2-284, complete sequence	99.885	175	No	11292
	contig_5	Klebsiella pneumoniae strain WCHKP3 chromosome, complete genome	99.24	357	Yes	5330758
	contig_7	Escherichia coli plasmid pV423-b DNA, contig: V423-b_scaffold_1, strain: V423	99.847	855	Yes	5430
	contig_8	Klebsiella pneumoniae strain S12 plasmid p1502320-1	93.986	946	Yes	112910
	contig_9	Klebsiella pneumoniae strain FC1 plasmid pMET-1 FC1 multiresistance plasmid, complete sequence	95.946	271	Yes	54638
Barcode26	contig_1	Klebsiella pneumoniae subsp. pneumoniae strain AUSMDU00008079 plasmid pAUSMDU8079-1, complete sequence	99.725	818	Yes	236390
	contig_2	Klebsiella pneumoniae strain KP36, complete genome	99.851	263	Yes	5196696
	contig_3	Klebsiella pneumoniae subsp. pneumoniae MGH 78578 plasmid pKPN7, complete sequence	97.216	605	Yes	6933
	contig_4	Klebsiella pneumoniae strain INF235-sc-2280127 plasmid unnamed5, complete sequence	99.727	540	Yes	6587
Barcode27	contig_1	Klebsiella pneumoniae strain 2N3 chromosome, complete genome	99.45	302	Yes	5214222
	contig_2	Klebsiella pneumoniae strain AR_0160 plasmid unnamed1, complete sequence	99.807	499	Yes	181716
Barcode28	contig_1	Klebsiella pneumoniae isolate Kp_Goe_154414, complete genome	99.09	212	Yes	5345897
	contig_2	Klebsiella pneumoniae strain FDAARGOS_444 plasmid unnamed1, complete sequence	99.455	351	Yes	215350
	contig_3	Klebsiella pneumoniae strain Kp_Goe_822917 plasmid pKp_Goe_917-7, complete sequence	99.831	593	Yes	7108
	contig_4	Escherichia coli strain BR43-DEC chromosome	99.693	386	Yes	13272
	contig_5	Klebsiella aerogenes strain NCTC9644 genome assembly, plasmid: 2	99.443	484	Yes	74590
	contig_6	Klebsiella pneumoniae subsp. pneumoniae strain KpvST15_NDM plasmid unnamed4, complete sequence	97.984	438	Yes	22071
	contig_7	Escherichia coli plasmid PN25, complete sequence	99.923	150	Yes	67376

Barcodes	Contigs	Bacteria	%Identity	Coverage	Circular	Length
Barcode29	contig_1	Klebsiella pneumoniae strain KP30835 chromosome, complete genome	99.156	283	Yes	5289536
	contig_2	Raoultella planticola strain FDAARGOS_429 plasmid unnamed1, complete sequence	98.052	10475	Yes	4157
	contig_3	Escherichia coli strain WCHEC4533 plasmid pNDM4_000533, complete sequence	99.534	751	Yes	44368
	contig_4	Salmonella enterica subsp. enterica serovar Lomita strain SL131 plasmid pSL131_IncA/C-IncX3, complete sequence	99.605	677	Yes	160382
	contig_5	Klebsiella pneumoniae strain AR_0087 plasmid unnamed1, complete sequence	99.836	735	Yes	138386
	contig_6	Uncultured prokaryote from Rat gut metagenome metamobilome, plasmid pRGRH0369	93.312	585	Yes	9792
Barcode30	contig_1	Klebsiella variicola strain X39 plasmid pX39-6, complete sequence	93.736	5	No	5364
	contig_10	Klebsiella pneumoniae strain 4743 plasmid unnamed2, complete sequence	99.892	1215	Yes	185700
	contig_11	Enterobacter kobei strain WCHEK045523 plasmid p2_045523, complete sequence	99.321	22	Yes	29236
	contig_12	Escherichia coli strain YPE10 plasmid pYPE10-78k, complete sequence	99.77	16	Yes	5438
	contig_13	Klebsiella pneumoniae strain 002SK2 plasmid p002SK2_B, complete sequence	99.913	20	No	47357
	contig_2	Escherichia coli strain Ecol_542 plasmid pEC542_KPC, complete sequence	99.782	836	Yes	4812
	contig_3	Klebsiella pneumoniae strain 121 plasmid pKP121-4, complete sequence	99.198	7	No	2663
	contig_4	Enterobacter asburiae strain CAV1043 plasmid pCAV1043-10, complete sequence	93.831	7	No	3644
	contig_5	Klebsiella pneumoniae MH16-390M plasmid pMH16-390M_1 DNA, complete genome	98.4	899	No	37019
	contig_6	Klebsiella pneumoniae strain 002SK2 plasmid p002SK2_B, complete sequence	99.848	24	Yes	48180
	contig_7	Klebsiella pneumoniae strain XH209, complete genome	99.162	570	Yes	5392341
	contig_8	Klebsiella oxytoca strain 4928STDY7071151 genome assembly, chromosome: 1	98.335	3	No	4719
	contig_9	Klebsiella aerogenes strain NCTC9793 genome assembly, chromosome: 1	96.465	129	No	5104
Barcode31	contig_1	Klebsiella pneumoniae strain CR-HvKP1 chromosome, complete genome	99.256	300	Yes	5350407
	contig_2	Klebsiella pneumoniae subsp. pneumoniae strain AUSMDU00008079 plasmid pAUSMDU8079-1, complete sequence	99.723	403	Yes	155615
Barcode32	contig_1	Klebsiella pneumoniae subsp. pneumoniae strain KPCTRSRTH07 chromosome, complete genome	99.251	338	Yes	5298862
	contig_2	Serratia marcescens strain E28 plasmid pE28_001, complete sequence	98.654	677	Yes	157731

Barcodes	Contigs	Bacteria	%Identity	Coverage	Circular	Length
Barcode33	contig_1	Klebsiella pneumoniae strain KSB1_1I-sc-2280289 chromosome, complete genome	99.085	208	Yes	5124717
	contig_2	Klebsiella pneumoniae strain KPN1344 chromosome	99.017	424	Yes	194304
	contig_3	Uncultured prokaryote from Rat gut metagenome metamobilome, isolate RGRH0148	75.671	846	Yes	5525
Barcode34	contig_1	Klebsiella pneumoniae strain R50 chromosome, complete genome	99.128	155	Yes	5193513
	contig_2	Klebsiella pneumoniae strain WCHKP7E2 plasmid p3_085072, complete sequence	97.755	802	Yes	4428
	contig_3	Klebsiella oxytoca strain 4928STDY7071151 genome assembly, chromosome: 1	99.514	649	Yes	9590
	contig_4	Klebsiella pneumoniae plasmid pKPN_CZ, complete sequence	99.662	364	Yes	176008
	contig_5	Escherichia coli strain AR_0011 plasmid tig00001069_pilon, complete sequence	99.43	415	Yes	82984
Barcode35	contig_1	Klebsiella pneumoniae subsp. pneumoniae strain KC-PI-HB1 chromosome, complete genome	99.885	318	Yes	5485910
	contig_2	Klebsiella variicola strain WCHKP19 plasmid p4_020019, complete sequence	99.438	891	Yes	4662
Barcode36	contig_1	Klebsiella pneumoniae strain NUHL30457 chromosome, complete genome	94.279	156	Yes	5630220
	contig_2	Klebsiella variicola strain FDAARGOS_627 plasmid unnamed1, complete sequence	96.187	283	Yes	272228
Barcode37	contig_1	Acinetobacter baumannii strain XH860, complete genome	96.868	3	No	26136
	contig_2	Klebsiella pneumoniae strain JNM10C3 chromosome	99.234	559	Yes	5188036
	contig_3	Klebsiella pneumoniae strain INF235-sc-2280127 plasmid unnamed2, complete sequence	95.04	9	Yes	5481
	contig_4	Acinetobacter baumannii strain KAB01, complete genome	96.818	3	No	34917
	contig_5	Acinetobacter baumannii strain AB34299, complete genome	96.734	3	No	27381
	contig_6	Acinetobacter baumannii strain XH860, complete genome	97.914	3	No	22746
	contig_7	Acinetobacter baumannii strain AB34299 plasmid unnamed2, complete sequence	99.954	16	Yes	8730
	contig_8	Klebsiella pneumoniae strain INF116-sc-2279924 plasmid unnamed1, complete sequence	98.364	1371	Yes	218654
Barcode38	contig_1	Escherichia coli strain AMA1416 plasmid pAMA1416, complete sequence	99.851	493	Yes	216342
	contig_2	Klebsiella pneumoniae strain KP14003 chromosome, complete genome	99.864	238	Yes	5254918

Barcodes	Contigs	Bacteria	%Identity	Coverage	Circular	Length
Barcode39	contig_1	Klebsiella pneumoniae strain KSB2_1B chromosome, complete genome	99.265	273	Yes	5340618
	contig_3	Klebsiella pneumoniae isolate Kp_Goe_154414 plasmid pKp_Goe_414-2, complete sequence	99.392	463	No	223454
Barcode40	contig_1	Escherichia coli strain FAM21805 plasmid, complete sequence	99.909	48	No	5516
	contig_2	Klebsiella pneumoniae strain FDAARGOS_566 plasmid unnamed2	99.924	338	Yes	223801
	contig_3	Klebsiella pneumoniae strain CR-HvKP1 chromosome, complete genome	99.249	147	Yes	5244617
Barcode41	contig_1	Klebsiella pneumoniae strain S12 plasmid pIncHI1B-1502320, complete sequence	98.925	495	Yes	223776
	contig_2	Klebsiella pneumoniae subsp. pneumoniae NTUH-K2044 DNA, complete genome	98.629	290	Yes	5242989
Barcode42	contig_1	Klebsiella pneumoniae strain KpvST101_OXA-48 chromosome, complete genome	93.022	187	Yes	5276202
	contig_2	Klebsiella variicola strain LMG 23571 plasmid, complete sequence	99.429	416	Yes	137376
Barcode43	contig_1	Klebsiella pneumoniae strain KSB2_1B chromosome, complete genome	99.262	265	Yes	5431936
	contig_2	Klebsiella pneumoniae strain L39_2 plasmid p2_L39, complete sequence	99.876	449	Yes	230168
Barcode44	contig_1	Klebsiella pneumoniae strain CR-HvKP1 chromosome, complete genome	99.237	280	Yes	5214771
	contig_4	Klebsiella pneumoniae strain DA12090 plasmid pDA12090.1, complete sequence	99.421	523	Yes	142225

The table indicates the 40 assembled whole genomes of *Klebsiella pneumoniae.* Barcode 01 – 24 are carbapenem-resistant *K. pneumoniae* (CRKP) and barcode 25 – 44 are non-CRKP, including barcode 25 – 34 (Extended spectrum beta-lactamase producing KP, ESBL) and barcode 35 – 44 (non-ESBL). The highlighted contigs are bacterial chromosome, while non-highlighted contigs are bacterial plasmid. We used the chromosome contigs to construct a phylogenetic tree.

The phylogenetic tree from the Snippy tool

The phylogenetic tree from the 64 SNP sites by Snippy tool shows 40 *Klebsiella pneumoniae* isolates, including 20 carbapenem-resistant *K. pneumoniae* (CRKP) and 20 non-CRKP (10 Extended-spectrum beta-lactamases (ESBL) and 10 non-ESBL producing isolates) from 2020-2021 at King Chulalongkorn Memorial Hospital. The phylogenetic tree shows fair separation of CRKP from non-CRKP. Clade 1–2 and clade 3 members are mainly non-CRKP and CRKP, respectively. However, there are 5 CRKPs in clade 1 and 2, while clade 3 has 5 non-CRKP.

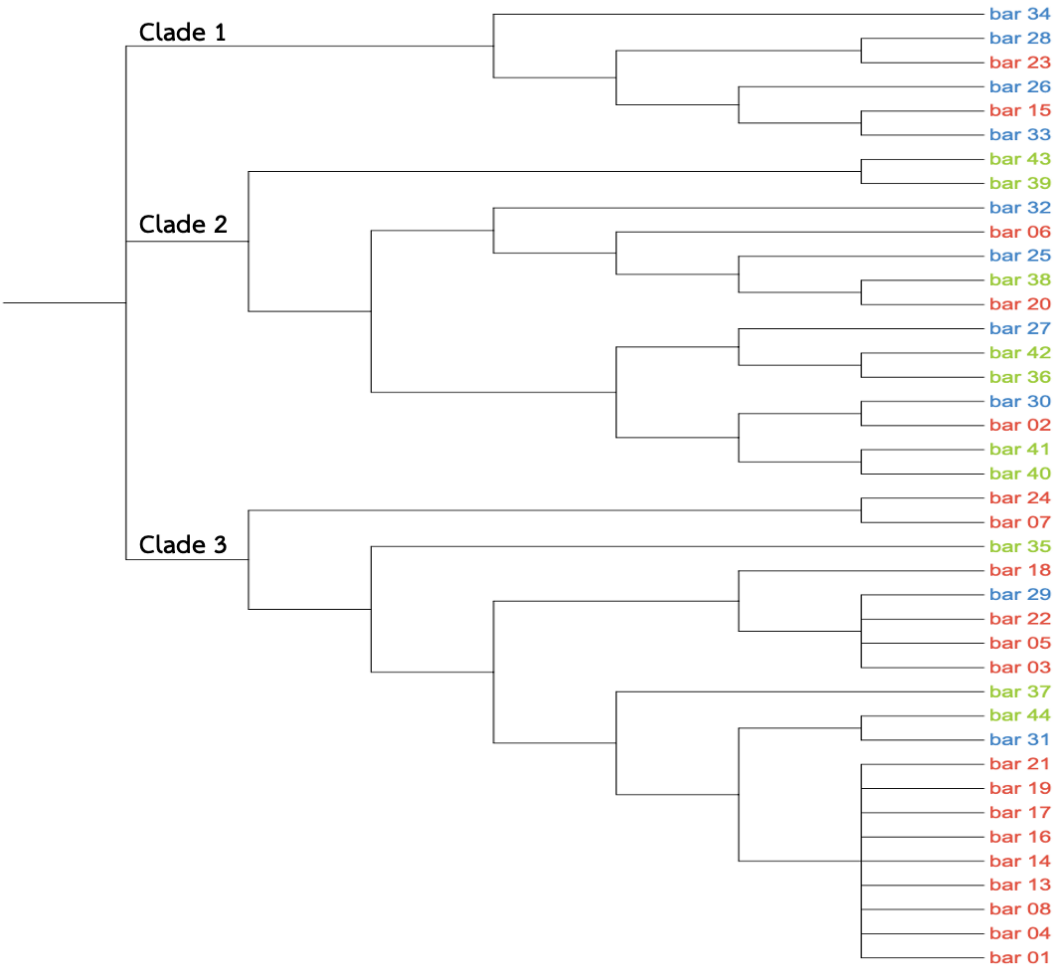


Figure 9. Phylogenetic tree of 40 *K. pneumoniae* isolates built from Snippy tool 64 SNP sites. Red: carbapenem-resistant, Blue: extended spectrum beta-lactamase (ESBL), Green: non-ESBL

The phylogenetic tree from the OrthoFinder tool

The phylogenetic tree from OrthoFinder shows 4 clades that can distinguish CRKP from non-CRKP better than the phylogenetic tree of 64 SNPs from Snippy tool. Clade 1 is mostly carbapenem-resistant *K. pneumoniae* CRKP with only 1 non-CRKP in this clade. All 13 members of Clade 2 are CRKP. Clade 3 has 4 non-CRKP. Clade 4 is mostly non-CRKP (15 non-CRKP and 2 CRKP). Since this OrthoFinder phylogenetic tree was built on amino acid sequences, DNA sequence could not be retrieved for SNP calling or making SNP barcode.

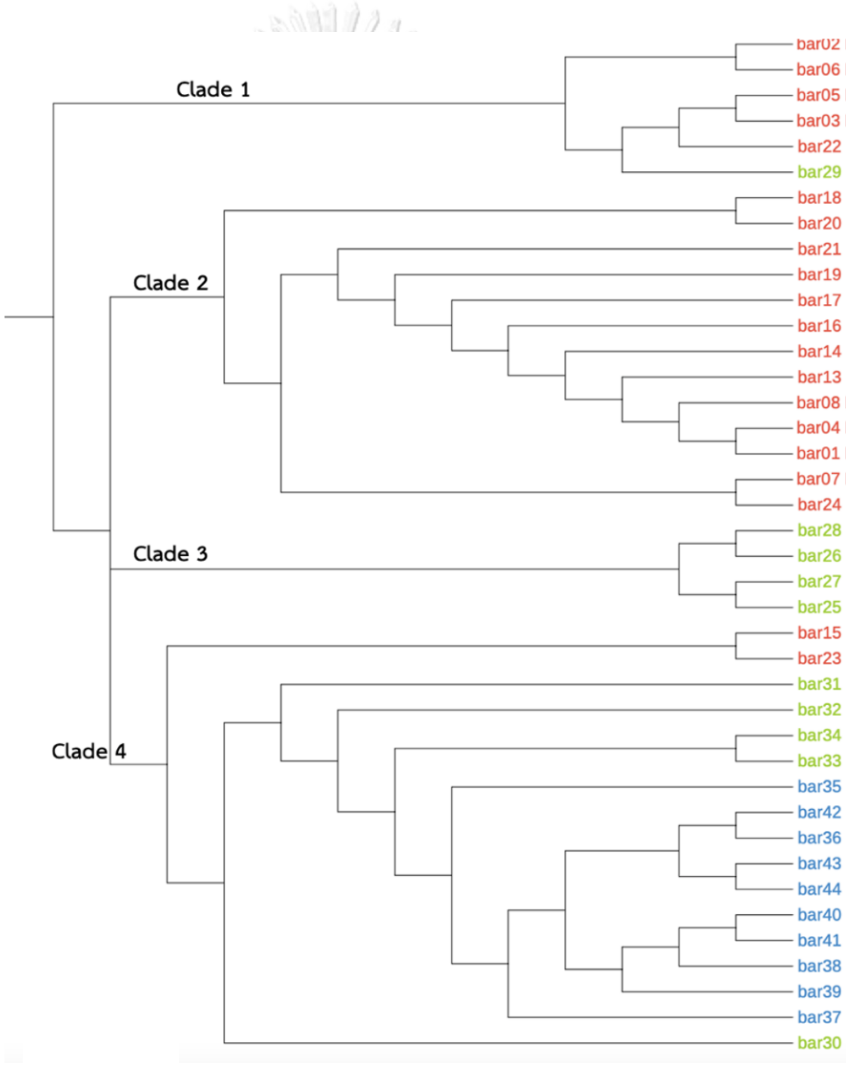


Figure 10. Phylogenetic tree made of 40 KP isolates from the OrthoFinder tool. Red: carbapenem-resistant, Blue: Extended spectrum beta-lactamase, Green: non-E

The SNP barcodes

Figures 6, 7 and 8 show 64 SNP sites of clade 1, 2, and 3, respectively, classified by phylogenetic tree of the Snippy tool. In clade 1, nucleotide C on position 9 and nucleotide A on position 61 are specific SNPs of this clade. Position 9 of other clades is nucleotide G, and position 61 of other clades is nucleotide G. Nucleotide A on position 45 is a specific SNP of clade 2, while other clades have nucleotide G in this position. In clade 3, nucleotide T in position 10 is a specific SNP of this clade, while other clades have nucleotide C. Unfortunately, none of these specific SNPs fulfills the criteria for SNP barcodes mentioned in our methodology. Therefore, if these SNPs are used as barcodes to identify KP clades, the accuracy will be low. Also, a SNP barcode specific to CRKP is not available, since CRKP isolates can be found in all clades of the phylogenetic tree built from the Snippy tool.

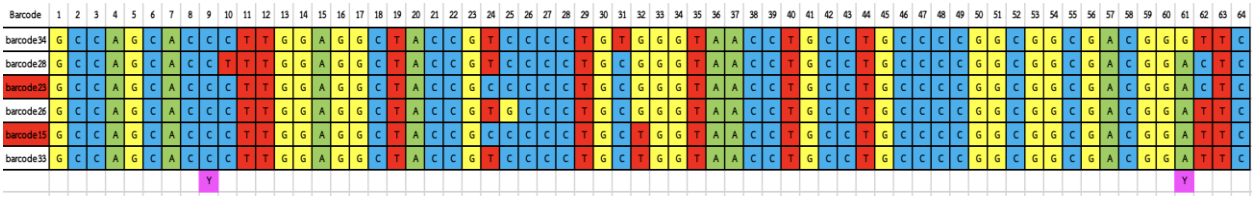


Figure 11. SNPs in clade 1. Position 9 and position 61 (Y - pink) are the selected positions. The barcodes highlighted in red are carbapenem-resistant K. pneumoniae (CRKP)

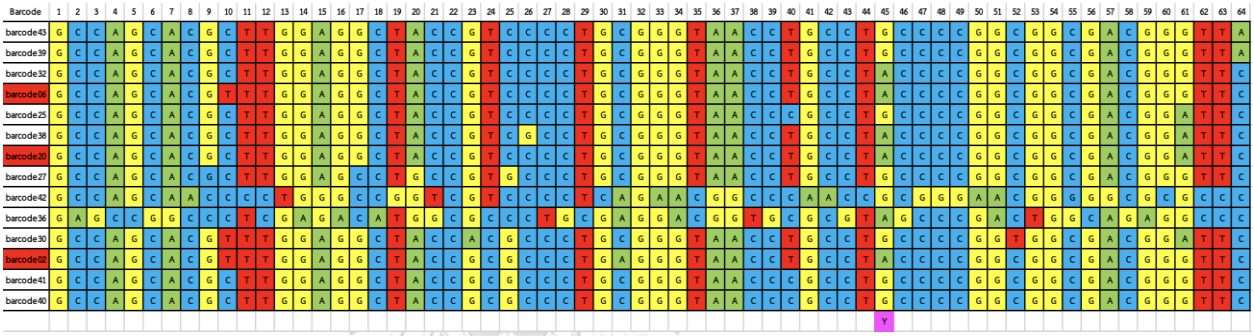


Figure 12. SNPs in clade 2. Position 45 (Y - pink) is the selected positions. The barcodes highlighted in red are carbapenem-resistant K. pneumoniae (CRKP).

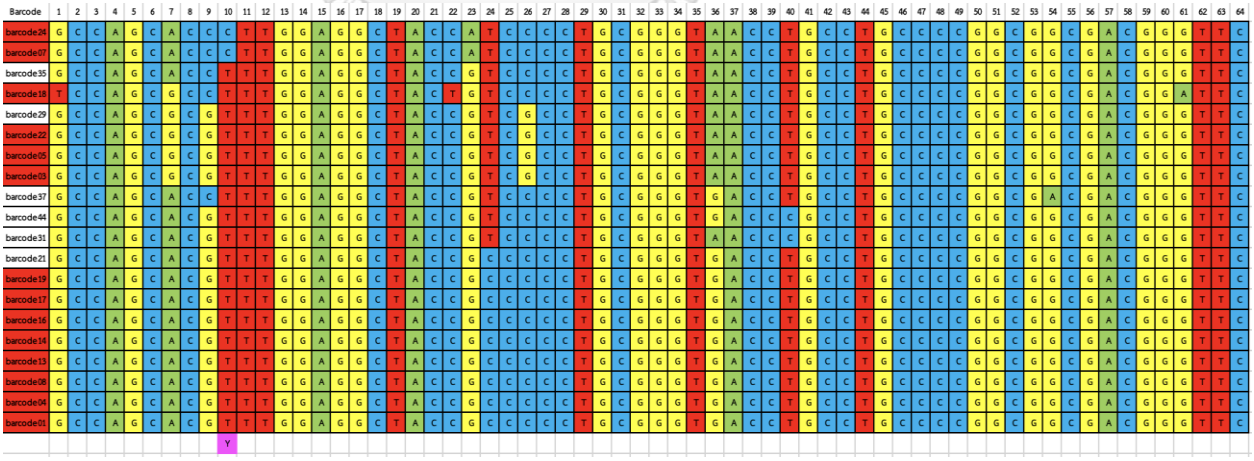


Figure 13. SNPs in clade 3. Position 10 (Y - pink) is the selected positions. The barcodes highlighted in red are carbapenem-resistant K. pneumoniae (CRKP).

CHAPTER V

Discussion

SNP barcodes can be used as a genotypic marker for rapid identification of genus, species, AMR genes, etc. Gary Napier et al., utilized 62 SNP barcodes to rapidly identify *Mycobacterium tuberculosis* complex (MTBC), and the results could accurately reconstruct the clades. (65) Though we could classify 40 KP into roughly 3 clades by 64 SNPs (Snippy), we could not generate unique SNP barcodes for each *K. pneumoniae* clade or for CRKP. CRKPs were mostly found in clade 3, but few of them were still found in clade 1 and 2. To make it simple, we excluded indels and this might diminish the differentiation ability by SNP. Other tools for SNP calling such as SAMtools/BCFtools (SAMTOOLS) and GATK should be further explored. (66)

Unlike MTBC, *K. pneumoniae* has many accessory genes that have not been shared among all KP and may be unique to some KP clades. Therefore, SNP calling from only core genome of bacterial chromosome (discard the accessory genes) might not represent each clinical isolate well. For KP, AMR genes may reside in plasmid or other mobile genetic elements. This further complicated the association between bacterial chromosome and phenotypic resistance. Some AMR genes may also be horizontally transferred to different bacterial strains or just evolved recently within the same strain, jeopardizing the link between bacterial clade/strain typing from bacterial chromosome and AMR genes.

Depth of coverage is the number of unique reads that include a given nucleotide in the reconstructed sequence. Generally, 30x coverage of whole genome sequencing is acceptable, but 100x should be preferred in our study because we would like to find a variation in genomes, where accuracy is a must.

The phylogenetic tree of the OrthoFinder tool could separate CRKP from non-CRKP better than the phylogenetic tree of 64 SNPs. The previous study reported the Orthofinder tool as the most accurate ortholog inference method. (62) In our case,

SNPs were called from the large whole genomes, which were prone to misalignment, leading to errors in SNP calling. On the other hand, OrthoFinder compared the same/similar genes (orthologs), limiting the chance of misalignment.

One limitation of assembled genome by nanopore sequencing is an error in homopolymer that can lead to frame shifts, which may affect the phylogenetic tree built on amino acid sequence using OrthoFinder. Polishing tools, such as Racon or Medaka, may correct this problem.

Multilocus typing (MLST) has sufficient resolution to detect disease outbreak strains (67) and is another possible approach. However, multiplex PCR plus sequencing of 7–8 PCR product takes too long time to be useful in clinical practice. Nanopore sequencing is simple and affordable. Its results can be analyzed in real-time, suitable for point-of-care diagnostic services, e.g., rapid identification of pathogens and antimicrobial susceptibility. (6) Nanopore sequencing data, which are long reads of nucleotide sequences, can be assembled to construct the whole genome to construct a database easier than short reads.

Our study shows the possibility of rapid prediction of antimicrobial susceptibility indirectly by finding the best match between real-time Nanopore reads from clinical specimens and KP strains with known susceptibility in the local KP whole genome database instead of direct detection of antimicrobial-resistant genes. Due to the locations of antimicrobial resistance genes (AMR genes) of K. pneumoniae on the plasmids, a phylogenetic tree based on plasmid sequences might be a better approach to distinguish CRKP from non-CRKP.

CHAPTER VI

Conclusion

In this study, we constructed the phylogenetic trees of 40 *K. pneumoniae* clinical isolates to classify carbapenem-resistant *K. pneumoniae* (CRKP) and non-CRKP. The phylogenetic tree generated from amino acid sequence of orthologous genes (OrthoFinder) was better than from DNA sequence SNP (Snippy) on distinguishing CRKP from non-CRKP. We could not generate SNP barcodes because none of the SNPs was specific to CRKP or each clade.

APPENDIX

Barcode	No.	Nanodrop(ก่อนAMPure)					Nanodrop(หลังAMPure)					Qubit(ng/uLX1:100)	Qubit(ng/uL)	total 200ng (7.5 ul)	add water (total 7.5ul)	total volume 9 ul (addd water)
		ng/uL	A260/A280	A260/A230	A260	A280	ug/uL	A260/A280	A260/A230	A260	A230					
Barcode 01	CRE115	118.6	1.89	2.43	2.37	1.25	125	1.94	2.6	2.5	1.29	1.05	105	1.90	5.60	7.10
Barcode 02	CRE117	156.6	1.9	2.28	3.13	1.65	186.9	1.93	2.45	3.74	1.94	2.02	202	0.99	6.51	8.01
Barcode 03	CRE217	187.9	1.89	2.2	3.76	1.99	98	1.97	2.74	1.96	1	1.65	165	1.21	6.29	7.79
Barcode 04	CRE259	173	1.89	2.23	3.46	1.83	107.4	1.93	2.83	2.15	1.12	1.52	152	1.32	6.18	7.68
Barcode 05	CRE260	165.5	1.88	2.14	3.31	1.76	104.3	1.93	2.36	2.09	1.08	1.03	103	1.94	5.56	7.06
Barcode 06	CRE288	82.5	1.89	1.98	1.65	0.88	55.6	1.9	1.43	1.11	0.59	1.46	146	1.37	6.13	7.63
Barcode 07	CRE289	35.9	1.77	2.29	0.72	0.41	31.7	1.89	2.92	0.63	0.34	0.386	38.6	5.18	2.32	3.82
Barcode 08	CRE290	308.6	1.9	2.18	6.17	3.26	180.7	1.94	2.49	3.61	1.86	2	200	1.00	6.50	8.00
Barcode 13	CRE293	44.2	1.9	1.75	0.88	0.46	30.3	1.97	2.41	0.61	0.31	0.794	79.4	2.52	4.98	6.48
Barcode 14	CRE296	121.8	1.87	1.57	2.44	1.3	59.8	1.93	3.37	1.2	0.62	1.38	138	1.45	6.05	7.55
Barcode 15	CRE297	110.8	1.9	2.08	2.22	1.17	90.7	1.94	2.76	1.81	0.94	1.37	137	1.46	6.04	7.54
Barcode 16	CRE298	157.4	1.89	2.08	3.15	1.67	136.2	1.96	2.32	2.72	1.39	1.85	185	1.08	6.42	7.92
Barcode 17	CRE299	208.2	1.92	2.25	4.16	2.17	215.7	2.02	2.48	4.31	2.14	2.26	226	0.88	6.62	8.12
Barcode 18	CRE300	230.6	1.92	2.16	4.61	2.17	190.7	1.93	1.96	3.81	1.98	1.76	176	1.14	6.36	7.86
Barcode 19	CRE301	85	1.95	2.26	1.7	0.87	69.8	2.04	2.64	1.4	0.68	0.824	82.4	2.43	5.07	6.57
Barcode 20	CRE302	75.6	1.89	2.07	1.51	0.8	58.5	1.93	2.42	1.17	0.61	0.862	86.2	2.32	5.18	6.68
Barcode 21	CRE303	174.9	1.93	2.26	3.5	1.81	160.9	2.03	2.42	3.22	1.59	0.93	93	2.15	5.35	6.85
Barcode 22	CRE305	191.4	1.92	2.13	3.83	2	184.4	2	2.41	3.69	1.85	1.57	157	1.27	6.23	7.73
Barcode 23	CRE306	276.7	1.9	1.97	5.53	2.91	162.8	1.97	2.21	3.26	1.65	0.91	91	2.20	5.30	6.80
Barcode 24	CRE308	198.6	1.82	1.67	3.97	2.18	133.3	1.87	1.75	2.67	1.43	0.87	87	2.30	5.20	6.70

Table 3. The table indicates the result of DNA extraction and purification in barcode 01 – 24

Barcode	No.	Nanodrop(ก่อนAMPure)					Nanodrop(หลังAMPure)					Qubit(ng/uL)(1:100)	Qubit(ng/uL)	total 200ng (7.5 ul)	add water (total 7.5ul)	total volume 9 ul (addd water)
		ng/uL	A260/A280	A260/A230	A260	A280	ug/uL	A260/A280	A260/A230	A260	A230					
Barcode 25	ESBL008	290.4	1.86	1.94	5.81	3.12	129.3	1.9	2.15	2.59	1.36	0.966	96.6	2.07	5.43	6.93
Barcode 26	ESBL088	171	1.83	1.73	3.42	1.87	131	1.9	1.94	2.62	1.38	0.732	73.2	2.73	4.77	6.27
Barcode 27	ESBL089	470.9	1.88	1.99	9.42	5.02	459.9	1.99	2.35	9.2	4.62	4.24	424	0.47	7.03	8.53
Barcode 28	ESBL090	323.9	1.88	2.04	6.48	3.45	278.1	1.97	2.16	5.56	2.83	1.96	196	1.02	6.48	7.98
Barcode 29	ESBL107	232.6	1.8	1.44	4.65	2.59	136.7	1.94	1.89	2.73	1.41	0.736	73.6	2.72	4.78	6.28
Barcode 30	ESBL124	370.7	1.87	1.96	7.41	3.96	306.5	1.89	2	6.13	3.24	2.64	264	0.76	6.74	8.24
Barcode 31	ESBL134	316.5	1.89	1.96	6.33	3.35	219.1	1.98	2.25	4.38	2.21	2	200	1.00	6.5	8.00
Barcode 32	ESBL136	553.5	1.84	1.81	11.07	6	401	1.98	2.3	8.02	4.06	2.02	202	0.99	6.51	8.01
Barcode 33	ESBL137	489.9	1.88	2.03	9.8	5.2	390.8	1.99	2.31	7.82	3.93	2.4	240	0.83	6.67	8.17
Barcode 34	ESBL140	330.5	1.88	1.98	6.61	3.52	221.4	1.95	2.29	4.43	2.27	0.35	35	5.71	1.79	3.29
Barcode 35	non-ESBL01	228.7	1.79	1.12	4.57	2.56	181.8	1.93	2.45	3.64	1.88	1.42	142	1.41	6.09	7.59
Barcode 36	non-ESBL02	241	1.79	1.16	4.82	2.69	334.8	1.93	2.18	6.7	3.47	2.88	288	0.69	6.81	8.31
Barcode 37	non-ESBL03	288.2	1.88	1.92	5.76	3.07	237.1	1.96	2.36	4.74	2.42	1.39	139	1.44	6.06	7.56
Barcode 38	non-ESBL04	361.1	1.88	1.85	7.22	3.84	291.9	1.97	2.35	5.84	2.97	2.02	202	0.99	6.51	8.01
Barcode 39	non-ESBL05	66.2	1.88	1.74	1.32	0.7	53.5	1.88	2.78	1.07	0.57	0.728	72.8	2.75	4.75	6.25
Barcode 40	non-ESBL06	424	1.86	2.05	8.48	4.57	467.7	2.06	2.42	9.35	4.54	1.76	176	1.14	6.36	7.86
Barcode 41	non-ESBL09	294.2	1.89	1.87	5.88	3.12	209.8	1.93	2.27	4.2	2.17	2.34	234	0.85	6.65	8.15
Barcode 42	non-ESBL10	297.3	1.88	1.97	5.95	3.16	256.5	1.97	2.26	5.13	2.61	1.53	153	1.31	6.19	7.69
Barcode 43	non-ESBL11	171.2	1.9	2	3.42	1.81	141.5	1.92	1.91	2.83	1.48	1.4	140	1.43	6.07	7.57
Barcode 44	non-ESBL12	231.1	1.91	2.16	4.62	2.42	242.2	1.94	2.31	4.84	2.5	2.4	240	0.83	6.67	8.17

Table 4.The table indicates the result of DNA extraction and purification in barcode 25 – 44

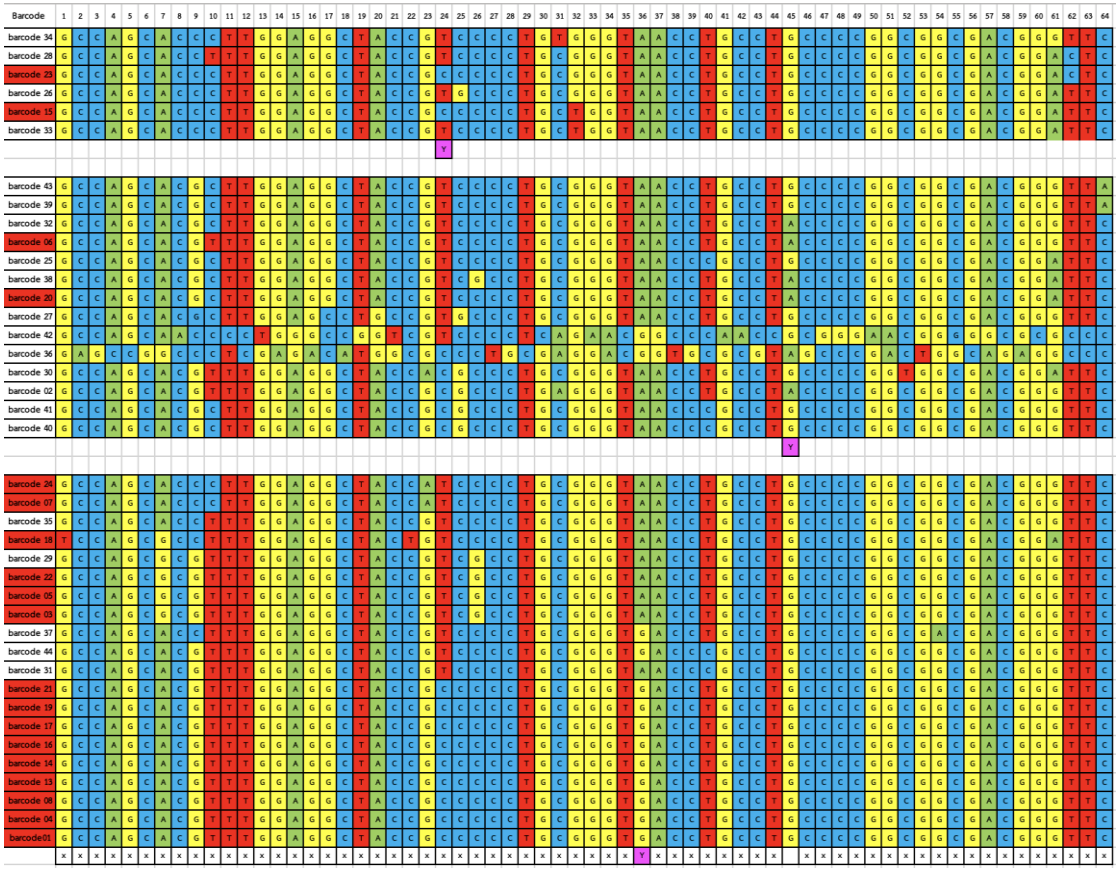


Table 5.The table indicates 64 SNP sites of 40 Klebsiella pneumoniae isolates sorted in order of 64 SNPs phylogenetic tree. Barcode 1-24 (red): carbapenem-resistant K. pneumoniae (CRKP), Barcode 25-44 (white): non-CRKP. Green: base A, Yellow: base G, Blue: base C, and Red: T. Y (pink): selected SNPs from the applied criteria.

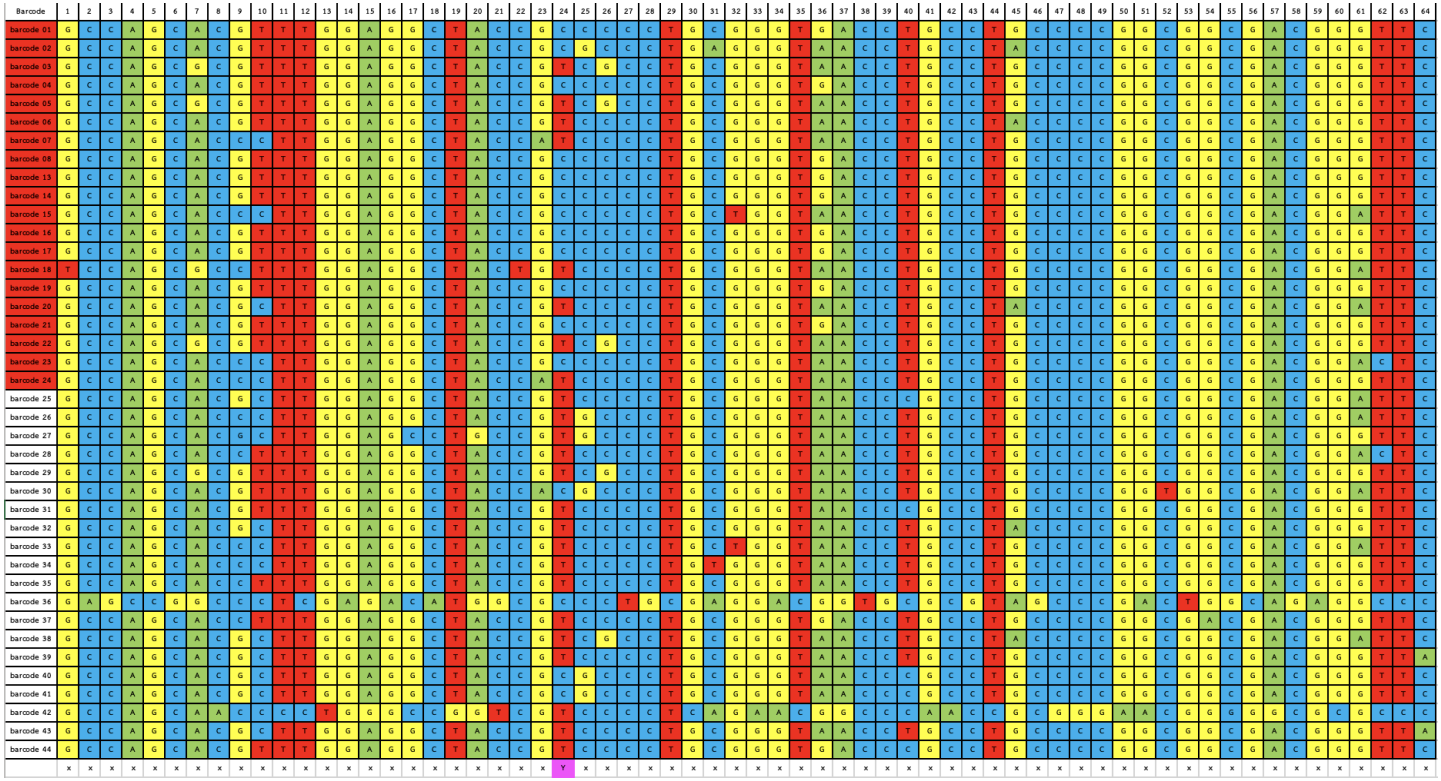


Table 6.The table indicates 64 SNP sites of 40 Klebsiella pneumoniae isolates. Barcode 1-24 (red): carbapenem-resistant K. pneumoniae (CRKP), Barcode 25-44 (white): non-CRKP. Green: base A, Yellow: base G, Blue: base C, and Red: T. Y (pink): selected SNPs from the applied criteria.

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VITA

NAME	Nicha Sangpiromapichai
DATE OF BIRTH	31 March 1996
PLACE OF BIRTH	Bangkok
INSTITUTIONS ATTENDED	2002 - 2007 Elementary School, Anubanrayong school, Rayong, Thailand 2008 - 2013 Science-math program, High school, Rayongwittayakom school, Rayong, Thailand 2014 - 2019 Bachelor of Science (B. Sc.), Faculty of Medical technology, Rangsit university, Patumthani, Thailand 2019 - 2022 Master of Science (Medical Sciences), Chulalongkorn University, Thailand
HOME ADDRESS	52/13 Village No.4 Rayong Road, Nam-kok Sub-district, Muang District, Rayong, 21000