

## Phylogenetics Tree of Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and Non-CRKP on Whole-genome Orthologous Analysis Using Nanopore Sequencing

แผนภูมิวิวัฒนาการของเชื้อแบคทีเรียเคลบเซลลา นิวโมเนียที่ดื้อต่อยาคาบาพิเนมและไม่ดื้อต่อ

ยาคาบาพิเนมโดยการทำการวิเคราะห์ยีนที่คล้ายคลึงกันในจีโนมทั้งหมดที่ได้จาก

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### ABSTRACT

The incidence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infection, which has high morbidity and mortality, is rapidly increasing worldwide as one of the most important nosocomial infections. In this study, Nanopore whole-genome sequencing of 40 *Klebsiella pneumoniae* isolates from clinical samples during 2020–2021 in King Chulalongkorn Memorial Hospital were assembled to make a phylogenetic tree on orthologous analysis. Eighteen of 20 CRKP isolates belong to the same clades, while only 1 of 20 non-CRKP isolates is a member of one of those particular clades. Since the phylogenetic tree using the whole genome can distinguish CRKP from non-CRKP, we might be able to use Nanopore real-time sequencing to rapidly detect CRKP by matching the sequence of its DNA fragments to the local bacterial whole genome database without the need to take a long time to directly identify the resistant genes.

### บทคัดย่อ

เชื้อแบคทีเรีย Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) เป็นเชื้อดื้อยาที่เป็นปัญหาสำคัญของการติดเชื้อในโรงพยาบาล ซึ่งเป็นภัยคุกคามทางด้านสาธารณสุขทั่วโลก เนื่องจากการแพร่ระบาดอย่างรวดเร็ว ก่อให้เกิดการเจ็บป่วยและมีอัตราการเสียชีวิตเพิ่มสูงขึ้น ในการศึกษานี้ได้ทำ Nanopore whole-genome sequencing ของเชื้อแบคทีเรีย *Klebsiella pneumoniae* 40 ตัวอย่างที่เก็บตั้งแต่ปี 2020 ถึง 2021 ภายในโรงพยาบาลจุฬาลงกรณ์ สภากาชาดไทย และทำการรวมตัวอย่างเพื่อสร้าง phylogenetic tree ด้วยการวิเคราะห์ทาง orthologous เชื้อแบคทีเรีย CRKP 18 ตัวอย่างจากทั้งหมด 20 ตัวอย่างอยู่ใน clade กลุ่มเดียวกัน ในขณะที่มีเพียงตัวอย่างเดียวของเชื้อแบคทีเรีย non-CRKP ที่แยกออกมาปะปนอยู่ภายใน clade นี้ ดังนั้น phylogenetic tree สามารถแยกเชื้อแบคทีเรีย CRKP จาก non-CRKP ได้ เราอาจจะสามารถตรวจหาเชื้อแบคทีเรีย CRKP ได้อย่างรวดเร็วโดยการหาจับคู่เปรียบเทียบกับลำดับเบสของชิ้นส่วน bacterial DNA ที่ได้จาก Nanopore real-time sequencing กับฐานข้อมูล bacterial whole genome ของโรงพยาบาล โดยไม่ต้องเสียเวลาหาอินดิเคเตอร์ที่มีขนาดเล็กโดยตรง

**Keywords:** Phylogenetics tree, CRE, Nanopore sequencing

**คำสำคัญ:** แผนภูมิวิวัฒนาการ ชีอาร์อี การหาลำดับเบสด้วยนาโนพอร์

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## Introduction

Carbapenem-resistant Enterobacteriaceae (CRE) is one of the most important opportunistic pathogens. These bacteria, mainly *Klebsiella pneumoniae* (KP), can cause nosocomial and community-acquired infections. (Moradigaravand et al., 2017) Outside gastrointestinal tract, they can become pathogens and cause diseases, e.g., urinary tract infection, bacteremia, meningitis, wound infection, etc. (Calfee, 2017) The outbreak of CRE is a serious public health problem. The CDC reports CRE can spread through person-to-person contact or contaminated environment. Paveenkittiporn et al. reported CRE isolates from five regions of Thailand hospitals were found in most hospitals (97%). The most common CRE was *Klebsiella pneumoniae* (72%, n=2660). (Paveenkittiporn et al., 2021) Tunyong et al. also reported 9,567 Enterobacteriaceae isolates, of which 282 (2.95%) were CRE, and 187 (66%) were carbapenem-resistant *Klebsiella pneumoniae* (CRKP). (Tunyong et al., 2021) The decision of antibiotic choices to cover or not cover CRE are crucial. Proper antibiotics must be prescribed as early as possible, but routine antimicrobial-susceptibility tests require overnight culture and usually have a turnaround time of approximately 2-3 days.

Nanopore is the third-generation sequencing, rapidly generating long-read sequences in real-time. A single-molecule DNA or RNA is pulled through the nanometer-sized protein pore, which changes an ion current through the pore. (Bharagava et al., 2019; Niu et al., 2019) Nanopore sequencing was successfully applied to characterize carbapenemase-encoding plasmids in enterobacteria isolated from a wastewater treatment plant. (Ludden et al., 2017) We found that Nanopore sequencing of DNA extracts from urine of patients with urinary tract infection took 12–24 hours to identify the carbapenem-resistant genes (unpublished data). This is much faster than routine antimicrobial susceptibility test but is still not early enough for making decision of the first-dose antibiotics.

Detection of a single resistant gene in a sea of DNA fragments requires some time. It should be much faster to just match those DNA sequences to known bacterial genome database and identify the bacterial lineage/strain/isolate they are closely related to. We can then predict that the phenotypic antimicrobial resistance of bacteria in sample should be similar the matched strain in the database. However, this approach needs that bacterial lineage/strain must be strongly associated with the pattern of phenotypic antimicrobial resistance. Therefore, we tried to build a phylogenetic tree of CRKP and non-CRKP using Nanopore sequencing to find out whether they were separated into different clades or not.

## Objectives of the study

This study aimed to describe the phylogenetic tree of CRKP and non-CRKP.

## Methodology

### Sample collection and Bacterial culture

Forty *Klebsiella pneumoniae* isolates were obtained from 2020 to 2021 in King memorial Chulalongkorn hospital. Twenty of them were Carbapenem-resistant *Klebsiella pneumoniae* (CRKP),

while the rest were non-CRKP (10 extended-spectrum beta-lactamase (ESBL) producing KP and 10 non-ESBL) Bacteria were cultured in 5 ml of LB broth at 37°C overnight.

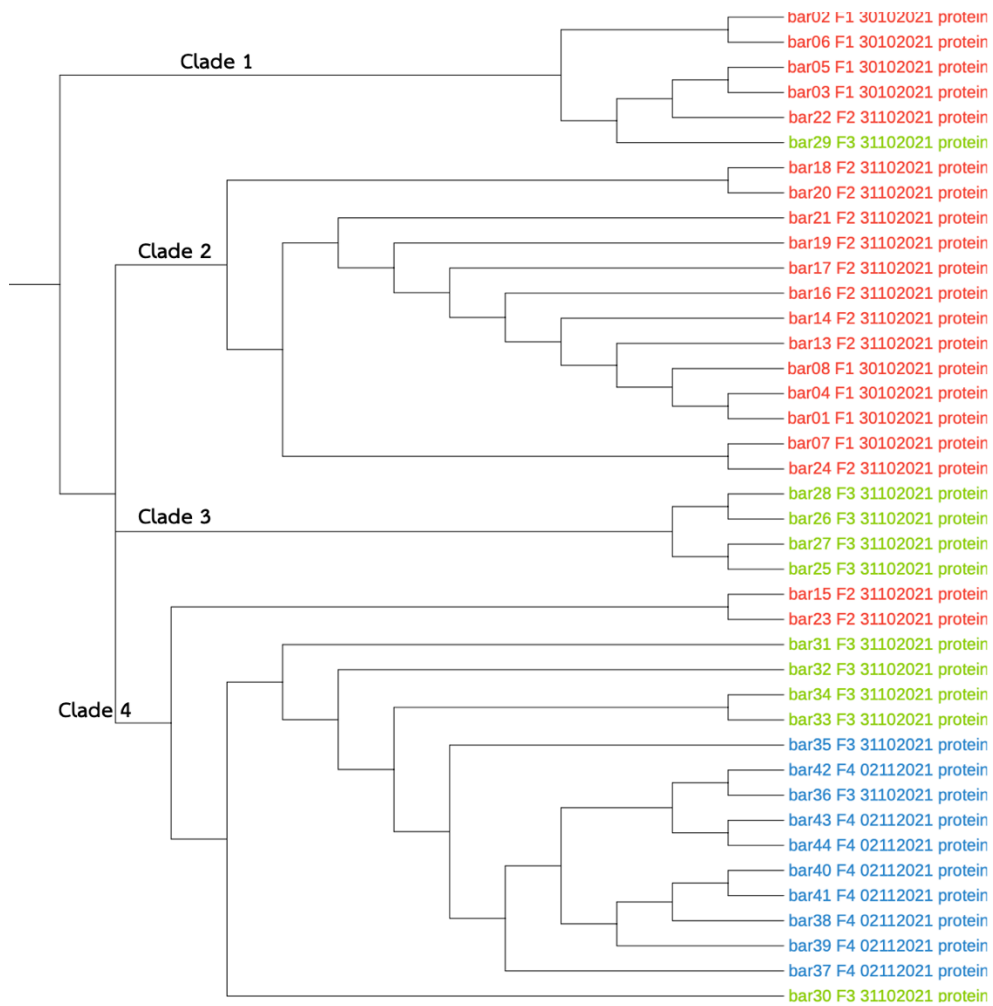
#### **Extraction of DNA bacterial and Nanopore sequencing**

Five ml of LB broth was spun down at 3,000g for 10 minutes and the supernatant was discarded. DNA was extracted with Quick-DNA HMW Magbead kit (Zymo Research), following the manufacturer's instruction. Library was prepared with Rapid Barcoding kit 96 SQK-RBK110.96 (Oxford Nanopore, UK), following the manufacturer's protocol. Sequencing was performed on MinION flowcells (R9.4.1 FLO-MIN106) and the MinKNOW software v.21.05.21 (<https://nanoporetech.com/nanopore-sequencing-data-analysis>).

#### **Nanopore sequencing data analysis**

The first output file in fast5 format was changed into fastq format with Guppy basecaller v.5.0.16. Porechop v.0.2.4 (<https://github.com/rwick/Porechop>) was used to find and remove the adapter from each read. Prinseq v.0.20.4 (<https://github.com/Adrian-Cantu/PRINSEQ-plus-plus>) was used to filter reads with a minimum read length of 200 bp. Whole genome was de novo assembled from each read using the Flye v.2.9. (<https://github.com/fenderglass/Flye>). The whole-genome data were matched with BLASTn to the NCBI database (<https://ftp.ncbi.nlm.nih.gov/blast/db/v4>). Only hits with  $\geq 85\%$  similarity, E-value  $\leq 10^{-6}$ , and with  $\geq 80\%$  coverage were kept (Taxt et al., 2020). Whole genomes were annotated by Prokka v.1.12 (<https://github.com/tseemann/prokka>). Comparative genomes by orthogroups were done with Orthofinder v.2.5.4 (<https://github.com/Davidemms/OrthoFinder>). The phylogenetic tree was visualized using iTOL (<https://itol.embl.de>).

Results



**Figure 1** Phylogenetic tree of 40 isolates of *Klebsiella pneumoniae*. (Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) – Red, Extended-spectrum beta-lactamase (ESBL) – Green, and non-ESBL – Blue), (Bar01 – 24 are CRKP, Bar25 – 34 are ESBL, and Bar35 – 44 are non-ESBL)

Figure 1 shows the phylogenetic tree of 40 KP isolates. Eighteen of 20 CRKP belongs to clade 1 and 2, while 2 of 20 CRKP are in clade 4. Non-CRKP isolates are mainly in clade 3 and 4. Only 1 of 20 non-CRKP is in clade 1. All 10 non-ESBL isolates are exclusively in clade 4, while ESBL isolates can be found in clade 1, 3 and 4.

## Discussion and Conclusions

In this pilot study, we collected 40 *Klebsiella pneumoniae* (KP) clinical isolates from 2021-2022 at King Chulalongkorn Memorial Hospital. The phylogenetic tree from orthologous genes of 40 KP whole genomes clearly separates CRKP and non-CRKP into different clades/groups with only a few misclassifications. We can infer from the tree that phenotypic resistance is associated with the lineage/strain of bacteria. Orthofinder tool also had higher accuracy than the online database method. It is an easy-to-use and rapid tool for the classification of DNA sequences. (Emms, Kelly, 2019). Since we have the bacterial whole genome and their phenotypic resistance database, we can predict that the phenotypic resistance pattern of bacteria in clinical samples should be similar to their best matches (e.g., by BLAST, SNP calling, etc.) in the database.

One limitation of this study is that the sample size is small. The horizontal transfer of plasmids containing antimicrobial-resistant genes between bacterial strains/species may decrease the association between bacterial strains and their phenotypic resistances.

Nanopore sequencer is affordable and small, suitable for point-of-care services. Proper automated analysis of its real-time sequencing may help predict the bacterial phenotypic resistance much earlier so that proper antibiotics can be prescribed in time. Further studies on larger sample size and a database-matching algorithm are required.

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