In silico analysis, structural modelling and molecular docking of putative *Klebsiella pneumoniae* choline kinase

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by

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DEDICATION

To the pure soul of my beloved brother

Muhammad

I dedicate this effort to you

You've left an impression to my life that time cannot erase, your soul travels with me wherever I go and never abandon me, all times we spent, all moments we shared, still permanently etched in my memories. Whenever thing I feel faint, your words reverberate in my head encouraging me to keep going. You were, and always will be an inspiration to me

I shall keep praying for you till the last

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LIST OF ABBREVIATIONS

USM	Universiti Sains Malaysia
ChoK	Choline kinase
ChoKIs	Choline kinase inhibitors
KpChoK	*Klebsiella pneumoniae* choline kinase
hChoK	Human choline kinase
SpChoK	*Streptococcus pneumoniae* choline kinase
PfChoK	*Plasmodium falciparum* choline kinase
PaChoK	*Pseudomonas aeruginosa* choline kinase
NmChoK	*Neisseria meningitidis* choline kinase
SpChoK	*Streptococcus pneumoniae* choline kinase
CRE	Carbapenem-resistant Enterobacteriaceae
ESBL	Extended Spectrum Beta-lactamase
KPC	*Klebsiella pneumoniae* carbapenemase
HC-3	Hemicholinium-3
AMR	Antimicrobial resistance
CDC	Centers for Disease Control and Prevention
IV Catheter	Intravenous catheter
UTIs	Urinary tract infections
HV	Hypervirulent
LPS	Lipopolysaccharide
OMPs	Outer membrane proteins
ORF	Open reading frame
ATP	Adenosine triphosphate

rRNA	Ribosomal ribonucleic acid
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
CPS	Capsular polysaccharide
C-terminus	Carboxyl terminus
N-terminus	Amino terminus
HDTAB	Hexadecyl Trimethyl Ammonium Bromide
CBPs	Choline-Binding Proteins
NPs	Nanoparticles
NCBI	National Center of Biotechnology Information
FASTA	FAST-All
SIB	Swiss Institute of Bioinformatics
EMBOSS	European Molecular Biology Open Software Suite
Protein-sol	Protein Solubility
Solu Prot	Solubility Protein
RPSP	Recombinant Protein Solubility Prediction
pI	isoelectric point
Mw	molecular weight
3D	Three dimensions
UCSF	University of California, San Francisco
PDB	Protein Data Bank
C	Carbon
H	Hydrogen
S	Sulfur
N	Nitrogen

O	Oxygen
Asp	Aspartic acid
Glu	Glutamic acid
Arg	Arginine
Lys	Lysine
Ext. coefficient	Extinction coefficient
Cys	Cysteine
QMEAN	Qualitative Model Energy Analysis
GMQE	Global Model Quality Estimate
pH	Potential of Hydrogen
kDa	Kilo Dalton
DTT	Dithiothreitol
trxB	Thioredoxin Reductase gene
gor	Glutathione Reductase gene
NMR	Nuclear Magnetic Resonance

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ANALISIS *IN SILICO*, PEMODELAN STRUKTUR DAN PELABUHAN MOLEKUL KOLINA KINASE *KLEBSIELLA PNEUMONIAE* PUTATIF

ABSTRAK

Perkembangan kerintangan antibiotik bakteria (AMR) telah menjadi suatu ancaman yang menakutkan kepada kehidupan. Ancaman ini masih lagi meningkat disebabkan penggunaan antibiotik yang tidak terkawal dan keterlaluan. Oleh itu, adalah penting untuk komuniti saintifik menangani masalah ini dan mencari penyelesaian alternatif dalam memerangi AMR. Pada bakteria Gram-positif, kolina kinase bertanggungjawab mensintesis fosforilkolina, bahan pemula untuk asid lipoteikoik dan asid teikoik pada dinding sel. Pada bakteria Gram-negatif, fosforilkolina merupakan komponen pada lipopolisakarida membran. Perencat kolina kinase (ChoKI) manusia telah diuji pada *Streptococcus pneumonia* dan menunjukkan keputusan yang memberangsangkan apabila menyebabkan penguraian dinding sel bakteria berkenaan. Dalam kajian ini, analisis *in silico* dengan kaedah bioinformatik terhadap kolina kinase *Klebsiella pneumoniae* putative (KpChoK) telah dijalankan dalam proses mencari keadaan terbaik untuk penghasilan KpChoK rekombinan pada masa akan datang. Penilaian potensi ChoKI sebagai perencat KpChoK telah dijalankan dengan kaedah pemodelan struktur dan pelabuhan molekul. Ramalan kelarutan protein menunjukkan kelarutan KpChoK adalah lebih rendah daripada purata kelarutan protein *Escherichia coli*. Pelabuhan molekul KpChoK dengan hemicholinium-3 (HC-3), sejenis ChoKI yang telah terbukti, menunjukkan mod pelekatan yang sesuai pada poket pelekatan kolina. Ini sekali gus memberikan gambaran bahawa HC-3 berupaya berfungsi sebagai perencat bersaing dan seterusnya menyokong penggunaan ChoKI dalam melawan kerintangan antibiotik. Kajian ini telah merintis jalan ke arah penghasilan KpChoK

terlarut untuk diuji dengan ChoKI yang ada pada masa kini dan keputusan kajian juga menunjukkan potensi ChoKI sebagai agen anti-*K. pneumoniae*. Walaupun KpChoK telah diramal mempunyai kelarutan yang rendah dalam sistem ekspresi *E. coli*, beberapa kaedah untuk meningkatkan kelarutan telah dibincangkan. Pada masa akan datang, partikel nano boleh digunakan untuk meningkatkan keberkesanan ChoKI dengan bertindak sebagai sistem penghantaran drug.

IN SILICO ANALYSIS, STRUCTURAL MODELLING AND MOLECULAR DOCKING OF PUTATIVE *KLEBSIELLA PNEUMONIAE* CHOLINE KINASE

ABSTRACT

The frightening development of antimicrobial resistant bacteria (AMR) poses an explicit threat to life. This threat is still in an upward curve due to the indiscriminate and overuse of antimicrobials. Therefore, it is essential for the scientific community to keep pace with this development and search for alternative solutions to stop AMR. Choline kinase (ChoK) is considered as one of the new targets for inhibition to combat AMR. In Gram-positive bacteria, choline kinase is responsible for the synthesis of phosphorylcholine, a precursor of lipoteichoic acid and cell wall teichoic acid. In Gram-negative bacteria, phosphorylcholine is incorporated into the membrane lipopolysaccharides. Human choline kinase inhibitor (ChoKIs) has already been tested on *Streptococcus pneumoniae* with encouraging results by causing degradation of bacterial cell wall. In this study, *in silico* bioinformatics analysis of putative *Klebsiella pneumoniae* choline kinase (KpChoK) was performed to search for the best conditions for the production of recombinant KpChoK in the future. Evaluation of ChoKIs as the potential inhibitors of KpChoK by structural modeling and molecular docking approaches were also carried out. The prediction of protein solubility revealed that the solubility of KpChoK was lower than that of the average soluble *Escherichia coli* proteins. Molecular docking of KpChoK model structures with hemicholinium-3 (HC-3), a well-established ChoKI, showed a suitable binding mode within the choline binding pocket, indicating a promising competitive inhibition by HC-3. These results of molecular docking thus indicate the promising application of ChoKIs to combat antimicrobial resistance. Therefore, this study paved the way towards successful

overexpression of soluble KpChoK to be tested with currently available ChoKIs and reveals the potential of these compounds as novel anti-*K. pneumoniae* agents. Although the KpChoK was predicted to have low solubility in *E. coli* expression system, several methods have been discussed to improve the solubility. In the future, nanoparticles can also be used to enhance the activity of ChoKIs by acting as a drug delivery system.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Carl Friedlander initially characterized *Klebsiella pneumoniae* in 1882 as a bacterium isolated from the lungs of pneumonia patients who had died (Ashurst & Dawson, 2018). *Klebsiella* species may colonize medical equipment and the health care organization, but they can also be observed all around natural environment, including water, soil, and animals. *Klebsiella* species is opportunistic bacteria that colonize mucosal surfaces and cause illnesses. It can spread to other tissues and cause life-threatening infections such as pneumonia, urinary tract infections, bloodstream infections, and sepsis (Podschun & Ullmann, 1998). *Klebsiella pneumoniae* is one of these important clinical pathogens that have community and public concern. It is also a major *Enterobacteriaceae* which is regarded an aggressive infection which induces a large range of illnesses and is increasingly resistant to drugs (Paczosa & Mecsas, 2016).

According to Shiri et al. (2017), the bacterium is responsible for almost one-third of all Gram-negative infections, including urinary infections, cystitis, pneumonia, surgical wound infections, endocarditis, and septicemia. Necrosis pneumonia, pyogenic liver abscesses, and endogenous endophthalmitis are also caused by it. The infections caused by this bacterium are frequently correlated with higher death rates, hospital admission, and massive costs (Effah *et al.*, 2020).

Klebsiella pneumoniae is now one of a few bacteria which currently have a high level of secondary antibiotic resistance due to changes in the organism's essential DNA

(Ashurst & Dawson, 2018). In 1929, the first discovery of beta-lactam resistance in Gram-negative organisms was reported by Alexander Fleming (Kong, Schneper & Mathee, 2010).

Antibiotic resistant *K. pneumoniae* was detected in Europe in 1983 and in the United States in 1989 (Ashurst & Dawson, 2018). It has been widely investigated and revealed that it produces a beta-lactamase inducing beta-lactam ring hydrolysis in antibiotics (Tooke *et al.*, 2019). This beta-lactamase is known as extended spectrum beta-lactamase (ESBL). Oxymine cephalosporins can be hydrolyzed by the ESBLs, which prevents the action of third-generation cephalosporins. As a result of this resistance, carbapenems become a viable therapy option for ESBL. However, *K. pneumoniae* was responsible for about 80% of the 9000 cases reported to the Centers for Disease Control and Prevention (CDC) in 2013 owing to carbapenem-resistant *Enterobacteriaceae* (Ashurst & Dawson, 2018). Carbapenem resistance is associated with the increase in efflux systems, a change in the cell wall, and a rise in the synthesis of ESBL enzymes in the organism.

Although antibiotic resistant *K. pneumoniae* is poorly present in the population, pneumonia treatment should follow standard sanitation and hygiene recommendations. Once *K. pneumoniae* disease is diagnosed, antibiotic treatment must be adapted for the local sensitivities of antibiotics (Paczosa & Mecsas, 2016). Modern regimens for *K. pneumoniae* obtained in a community include a 14-day treatment with either 3rd or 4th-generation cephalosporins as monotherapy or with respiratory quinolones as monotherapy or with an aminoglycoside in one of the previous systems (Cao *et al.*, 2018). A trial of aztreonam or a respiratory quinolone should be carried out if the

patient is allergic to penicillin. Because of its global sensitivity, carbapenem treatment should be carried out as soon as ESBL is detected. When CRE (carbapenem-resistant Enterobacteriaceae) was detected, an infectious disease consultation should be acquired to guide therapy (Rawat & Nair, 2010). Antibiotics from the polymyxin family, tigecycline, fosfomycin, aminoglycosides, and dual treatment carbapenems are among the antibiotics used to treat Influenza. Combination therapy with two or more of the medicines may reduce mortality as contrasted to treatment individually (Coates *et al.*, 2020).

Antibiotic resistance in pathogenic bacteria should be combated with novel therapeutics. In bacteria, choline kinase (ChoK) catalyzes the production of phosphorylcholine, which is to be integrated into the cell wall or outer membrane. Phosphorylcholine is also utilized to generate membrane phosphatidylcholine in certain bacteria (Khalifa, Few & See Too, 2020). Several human ChoK inhibitors (ChoKIs) have been developed and investigated for anticancer activity such as Hemicholinium-3 (HC-3), MN-58b, and RSM-932A (Lacal, 2001; Zimmerman *et al.*, 2013). ChoKIs shown a potential impact by distorting the cell wall and slowing the pathogen's proliferation (Khalifa, Few & See Too, 2020). Researchers further recommend that ChoKIs and nanoparticles be used for targeted delivery to the pathogen and that the human host be protected from probable adverse effects of inhibitors. Additional research should focus on verifying the reported bacterial activity of ChoK and characterizing the active ChoKIs.

Several microorganisms quickly evolved and developed resistance to antibiotics as a result of their evolution. There are currently no effective treatments for the illnesses

produced by these evolved microorganisms (Davies, 1996). To combat these illnesses, a new approach is required. This is especially true in this period, which the Centers for Disease Control and Prevention (CDC) refers to as the "post-antibiotic age" in which antibiotic development has come to a standstill, and the “golden era” of antibiotic discovery was long past, especially because resistance has been developed to any antibiotic which has been administered. The situation has been caused by researchers' struggling to cope with the speed of antibiotic resistance in bacteria, also known as antimicrobial resistance (AMR). Due to the continued use of current antibiotics, this has accelerated the development of AMR. As a result, AMR must be treated immediately and quickly.

Recently, choline kinase (ChoK) in eukaryotes has been a recognized drug target (Lacal, 2015). By inhibiting choline kinases by several ChoKIs such as hematocholinium 3 (HC-3) and other inhibitors, it has shown an antogonistic effect on cancer growth (Janardhan, Srivani & Sastry, 2006). ChoK is overexpressed in human cancers (Granata *et al.*, 2014), as well as in parasites such as *Plasmodium falciparum* (Zimmerman *et al.*, 2013; Serran-Aguilera *et al.*, 2015) and autoimmune diseases (Guma *et al.*, 2015). In prokaryotes, many pathogens possess the putative ChoK gene, such as *S. aureus*, *Bacillus subtilis*, *Clostridium perfringens*, *Clostridium botulinum* and *K. pneumoniae*. Having this gene suggests that it can be inhibited by choline kinase inhibitors just like in eukaryotic organisms. *S. pneumoniae* has been confirmed to possess ChoK activity and its inhibition by ChoKIs resulted in growth inhibition of this pathogen (Wang *et al.*, 2015; Zimmerman & Ibrahim, 2017). This indicates a high potential for application of eukaryotic ChoKIs to inhibit ChoKs in prokaryotes and

used as new generation of antimicrobials. Thus, choline kinase inhibitors (ChoKIs) are one of the most promising new antimicrobial drugs.

1.2 Objectives of the study

1.2.1 General objectives

To perform *in silico* analysis and molecular modelling of *Klebsiella pneumonia* putative choline kinase (KpChoK), molecular modelling of KpChoK protein and molecular docking of potential inhibitors with the modelled KpChoK structure.

1.2.2 Specific objectives

- To analyze the amino acid sequence of KpChoK to determine the basic physicochemical parameters such as molecular weight, isoelectric point, and other related parameters like abundant amino acid, total number of atoms, and general formula.
- To compare the amino acid sequence and align it with choline kinases from other organisms especially from bacteria (including human choline kinase).
- To generate model structure of KpChoK.
- To perform, identify and evaluate the docking site of potential choline kinase inhibitors with the model structure of KpChoK

1.3 The rationale of the Study

There is a strong demand for new antimicrobials to establish the ground-breaking step toward drug discovery and with antimicrobial resistance (AMR) continuing. We need to biologically discover new compounds or repurpose existing ones such as choline kinase inhibitors (ChoKIs). Therefore, the use of an *in silico* approach is a quick way

to identify the potential of these ChoKIs before more tedious and time-consuming methods are carried out in the laboratories.

CHAPTER 2

LITERATURE REVIEW

2.1 *Klebsiella pneumoniae*

Klebsiella pneumoniae belongs to the *Enterobacteriaceae* family and is described as a Gram-negative, encapsulate, and non-motile bacterium (Rønning *et al.*, 2019). It belongs to the normal flora of the human mouth and intestine. But it can be dangerous if they get into other parts of the body. It can turn into "superbugs" that are almost impossible to fight with common antibiotics. The germs can cause pneumonia, infect wound or blood, and cause other serious problems (Jun, 2018). Infection is not common among healthy people due to strong immune systems, but it is more likely among unhealthy people such as those with alcoholism, cancer, diabetes, kidney failure, liver disease, and lung disease.

Taking certain antibiotics for a long time or under other treatments also can raise the chances for a *Klebsiella* infection. These germs don't spread through the air, but through direct contact, such as touching a cut on skin with contaminated hands. Most infections happen in hospitals, nursing homes, and other places with lots of sick people. The germs can also spread when they get on medical devices like Intravenous Catheter (IV catheters), endotracheal tubes, ventilators, and urinary catheters (Jun, 2018).

The symptoms vary according to the location of the infection. In the case of pneumonia, the patient suffers from fever, cough, chest pain, trouble breathing, thick and bloody mucus. *K. pneumoniae* infect other parts of the body. Such as surgical wound, blood (bacteremia or septicemia), brain (meningitis), heart (endocarditis), skin (cellulitis), and

urinary tract (UTIs). Each of these infection sites has its own symptoms (Ashurst & Dawson, 2018).

Klebsiella infections can be dangerous, but starting treatment with antibiotics immediately may reduce its severity and speed up the recovery. Antibiotics such as cephalosporins (cefotaxime and ceftriaxone) and carbapenems (imipenem or cilastatin) are usually used to treat infections, with the need to take the entire course of treatment to prevent the possibility of the infection returning (Paczosa & Mecsas, 2016). Most people who get a *Klebsiella* infection recover. But some cases can be deadly, especially pneumonia in people who are already very sick.

2.2 *Klebsiella pneumonia* virulence factors

K. pneumoniae employs many strategies to grow and protect itself from the host immune response. To date, there are four major classes of virulence factors that have been well characterized in *K. pneumoniae* that can lead to infection and antibiotic resistance as illustrated in Figure 2.1 (Paczosa & Mecsas, 2016). These virulence factors consist of:

- capsule, including the production of hyper-capsule in hyper-virulent (HV) strains. It is the most important virulence factor and allows the bacteria to evade opsonophagocytosis and serum killing by the host organism (Rønning *et al.*, 2019). It is an extracellular polysaccharide matrix that envelops the bacteria. Classical *K. pneumoniae* strains produce a capsule that can be of any of the serotypes K1 to K78; K1 and K2 are associated with increased pathogenicity. To date, 77 different capsular types have been studied, and those *Klebsiella*

species without a capsule tend to be less virulent. HV strains make a hypercapsule, which amplifies the production of capsular material, resulting in a relatively larger capsule, and are predominantly of the K1 serotype, while the remaining strains are of serotype K2 (Podschun & Ullmann, 1998).

- lipopolysaccharide (LPS), an integral part of the outer leaflet of the outer membrane, is produced by both classical and HV *K. pneumoniae* strains and can be of O-antigen serotypes 1 to 9 (O1 to -9) (Effah *et al.*, 2020). It coats the outer surface of Gram-negative bacteria. The sensing of lipopolysaccharides releases an inflammatory cascade in the host organism and has been a major culprit of the sequela in sepsis and septic shock (Podschun & Ullmann, 1998).

- siderophores, enterobactin is made by almost all strains, and yersiniabactin is made by approximately half of classical and almost all HV strains. Salmochelin and aerobactin are rarely produced by classical strains but are typically secreted by HV strains, with aerobactin being the most highly expressed of the siderophores (Russo *et al.*, 2015; Paczosa & Mecsas, 2016). It can acquire iron from the host to allow propagation of the infecting organism.

- Fimbriae, which also known as pili. It allows the organism to attach itself to host cells. Both types of *K. pneumoniae* make membrane-bound adhesive structures, type 1 and type 3 fimbriae, and secrete iron-scavenging siderophores (Stahlhut *et al.*, 2012; Paczosa & Mecsas, 2016).

Several other factors were recently identified as being important for *K. pneumoniae* virulence. However, these factors are not yet thoroughly characterized, and much work remains to be done to fully understand their mechanisms of action and clinical significance. These virulence factors include OMPs, porins, efflux pumps, iron transport systems, and genes involved in allantoin metabolism (Paczosa & Mecsas, 2016). The virulence factors characterized for *K. pneumoniae* play various roles in different types of *K. pneumoniae* infection and in different strains of *K. pneumoniae*. In addition, several recent compelling studies are revealing a number of other factors that play critical roles in mammalian infection. Based on these known virulence factors, the modus operandi of *K. pneumoniae* appears to be defensive rather than offensive in protecting itself against the host immune response (Paczosa & Mecsas, 2016). For example, pathogenic *Yersinia* species use type III secretion systems to inject toxins into attacking immune cells in order to inactivate the phagocytic capability of these cells (Trosky, Liverman & Orth, 2008). In contrast, *K. pneumoniae* appears to evade, rather than actively suppress, phagocytosis by using capsule to make it more difficult for the bacteria to be bound and taken up by phagocytes (Domenico *et al.*, 1994).

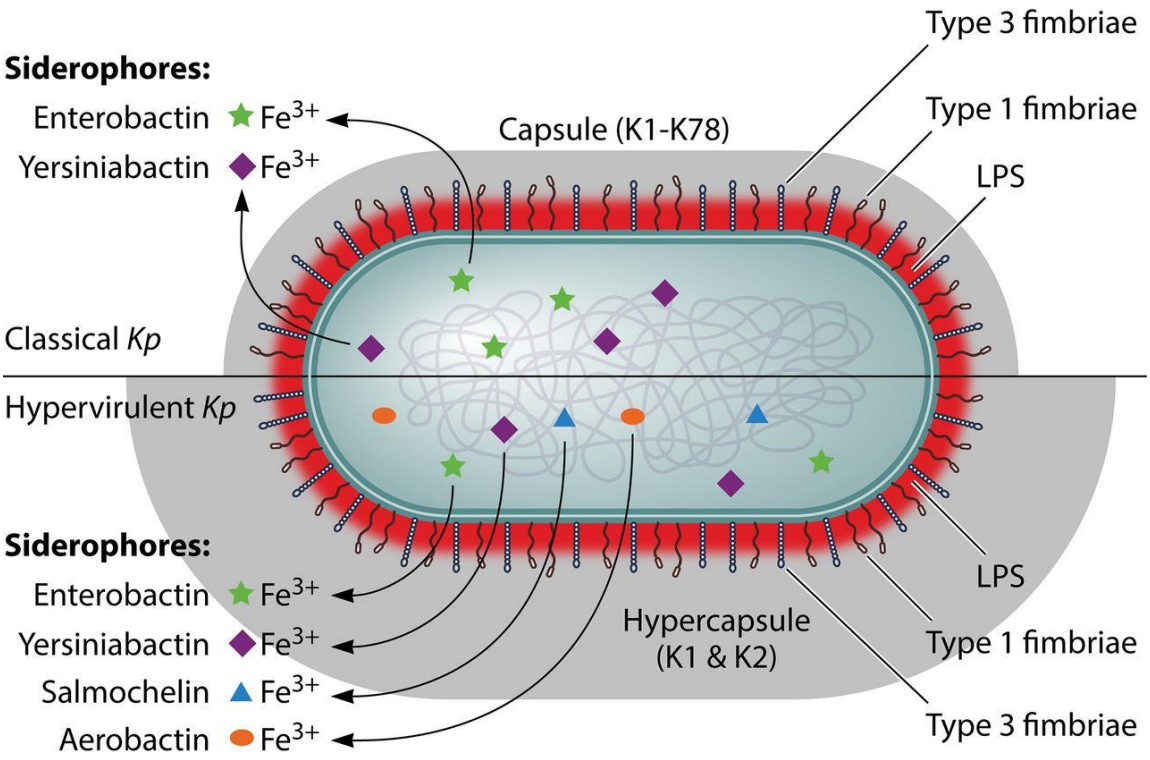

Figure 2.1: Four well-characterized virulence factors in classical and HV *K. pneumoniae* strains. capsule, LPS, fimbriae (type 1 and type 3), and siderophores (Paczosa & Mecsas, 2016).

2.3 *Klebsiella pneumoniae* infection

Not surprisingly, this pathogen has been classified as an "urgent threat to human health" by many organizations. They cause a wide range of diseases including pneumonia, urinary tract infections (UTIs), bloodstream infections, and sepsis. These infections affect millions annually of different age groups, whether new-borns, the elderly, or individuals with immunodeficiency. *Klebsiella* is also responsible for a large number of community-acquired infections. Associated *Klebsiella* strains are highly virulent and have distinctive morbidity and mortality. The increasing isolation of multidrug-resistant strains has narrowed or eliminated treatment options for *Klebsiella* infection in some places. As a result of co-evolution of *Klebsiella* it uses stealth strategies and effectively suppresses innate immune defences to overcome the host immune responses to survive in tissues. A better understanding of *Klebsiella* immune evasion strategies in the context of the host–pathogen interactions is pivotal to develop new therapeutics, which can be based on antagonising the anti-immune strategies of this pathogen (Bengoechea & Sa Pessoa, 2019).

2.4 *Klebsiella pneumonia* epidemiological risk factors and resistant

The chance of exposure to antibiotics in countries with an advanced medical system increases the likelihood of bacterial resistance. *K. pneumoniae* is quickly becoming known for its resistant properties to many of the antibiotics currently used as a cause of many acute infections. It poses a real dilemma, especially in hospitals, if the increasing trends in the isolation rate of *K. pneumoniae* are a major concern. In China, although there is an increasing trend in the rate of isolation, the rates of its resistance over the years have been evident in decreasing trends.

Generally, the decreasing resistant rate of *K. pneumoniae* to most of the antimicrobials in China can be attributed to; (i) the requirement of taking different activities for anticipation of bacterial disease such as isolating the pathogen carriers and requirement of hand sanitization of therapeutic experts by the government through the Nosocomial Contamination Control Committee, (ii) the limitation and control of the utilize of anti-microbials by the Chinese Ministry of Hygiene, which has actualized rules for the sound use of anti-microbials since 2006 (Effah *et al.*, 2020).

The worldwide rise and spread of genes of antimicrobial resistance such as extended spectrum beta-lactamases (ESBL) and carbapenemase genes in *K. pneumoniae* is a threat to public wellbeing. This is because carbapenems have long been considered as the final restorative resort or alternative of anti-microbials utilized to treat infections and diseases caused by multidrug-resistant Gram-negative bacteria. The quick worldwide emergence of *K. pneumoniae* strains, resistant to nearly all β-lactams, counting carbapenems the *K. pneumoniae* strains capacity to respond rapidly to natural immune system changes. The heavy and indiscriminate use of carbapenems is the main reason that led to the development of carbapenemases mediated by plasmid, that is, enzymes that hydrolyze all β-lactams including the last line of carbapenems (Queenan & Bush, 2007).

Diverse genes mediate resistance to antimicrobial drugs in *K. pneumoniae*. The nearness of some carbapenem resistance genes such as blaOXA. blaNDM and blaKPC may well be mostly causative of the high rate of resistance to carbapenems (imipenem and meropenem) (Padilla *et al.*, 2010). In *K. pneumoniae* and other various *Enterococcus*, blaKPC genes are transcendently carried on plasmids that confer resistance to nearly all

β-lactam anti-microbials. Therefore, from an epidemiological viewpoint, the disclosure of carbapenems is critical since they are mediated by the plasmid and can be transmitted horizontally between species diversity of bacteria (Padilla *et al.*, 2010).

The *Klebsiella pneumoniae* carbapenemase (KPC) gene is partially responsible for the resistance of *K. pneumoniae* to cephalosporins (e.g. ceftazidime, ceftriaxone, and cefotaxime), because KPC hydrolyzes extended-spectrum cephalosporins. It can therefore be used to identify KPC-mediated genes and resistance to these cephalosporins (Chew, Lin & Teo, 2017)

As for its resistance to aminoglycosides such as (amikacin and gentamicin), it is attributed to changes in cell permeability due to modifications in the AcrAB-TolC and KpnEF pump systems in addition to the loss of the putative purine, KpnO. Also, disturbances in AcrAB-TolC may increase sensitivity of *K. pneumoniae* to gentamicin (Andrade *et al.*, 2014). The 16S rRNA methylases encoded on plasmids confer resistance to all aminoglycosides. Mutations conferring resistance by targeted modification could also be a possible cause of increased resistance of *K. pneumoniae* to most aminoglycosides (Galimand, Courvalin & Lambert, 2003) . While the low prevalence of polymyxin resistance (Colistin) made a lot of sense due to its limited medical use because of its toxicity, which dates back to between the eighties and the two thousandths of the last century (Falagas & Kasiakou, 2005). Polymyxin displaces cations (Ca2 + / Mg2 +) which disrupt the integrity of the outer membrane, and by binding to negatively charged lipopolysaccharides (LPS) leading to cytolysis. According to a report by Dong et al 2018, species of *K. pneumoniae* will survive in the

barrage of antibiotics used in the treatment of nosocomial infections because they contain determinants of resistance (Dong *et al.*, 2018).

K. pneumoniae employs a set of virulence components for colonization and proliferation within the host cell. These include (a) a surface antigen, particularly a capsular polysaccharide (CPS, K antigen); (b) ferric acid capable for ferric iron-binding secreted by iron-binding proteins within the host; and (c) adherence variants capable for the binding to host cell surfaces, such as type 1 and type 3 fimbriae, and non-fimbrial attachment proteins. *K. pneumoniae* strains use virulent factors in pathogenesis such as adhesive genes, mucosal hyper-phenotype, genes related to mucosal viscosity, genes for lipopolysaccharide biosynthesis, and genes for iron uptake and transport. *K. pneumoniae* acquires iron, an important component and extremely rare in free form in host plasma, predominantly through the secretion of iron carriers which are molecules that have a greater iron effect than host-transport proteins. Erobactin is the most important among the iron carriers secreted by *K. pneumoniae* (Williams *et al.*, 1989), as it causes severe infection by transferring the microbe from the intestine to different tissues as well as entering into the process of reproduction (Russo *et al.*, 2014, 2015). According to Fang et al.2004, the genes carried by the plasmid, rmpA and rmpA2, contribute to the enhancement of capsule production. *K. pneumoniae* strains also use the MagA gene to demonstrate high B resistance to human serum and phagocytosis and thus are a good candidate for new drug targets. The Tis gene can be used as a molecular marker for rapid diagnosis and can also be useful for tracing the roots of emerging infectious diseases caused by *K. pneumoniae* (Fang *et al.*, 2004). According to Stahlhout et al 2009. It has been shown that fimbriae contributes to the development of biofilms and participates in microbial adhesion to medical devices and is considered the

most important virulent agent of *Klebsiella* causing urinary tract diseases (Paczosa & Mecsas, 2016) . Therefore, expression of these genes could improve the adhesive capacity of *pneumococci* on respiratory epithelial cells and also to the surfaces of other medical devices such as ventilators, increasing their ability to cause ventilator-associated disease. It may be a major factor for biofilm-related disease and host entry capabilities of the pulmonary bacteria (Huang, Li & Gregory, 2011; Stahlhut *et al.*, 2012).

2.5 Choline kinase as promising drug target

It is known that pathogens threaten the health of humans, animals and plants, causing many diseases, some of which are difficult to treat due to the resistance of pathogens to antibiotics. With the emergence of many resistant strains, the treatments currently used have limited capacity and diminished over time. Therefore, the optimal solution is to continually develop ways to eradicate pathogens by focusing drug exploration efforts on new targets (Zimmerman, Lacal & Ibrahim, 2019). In particular, it is necessary to focus on the available information regarding the enzymatic pathways that have been identified as drug targets in eukaryotic systems and to explore similar pathways found in prokaryotic systems. This method is promising for discovering new drug targets in prokaryotes, which could help eliminate many pathogens that are resistant to antibiotics. This strategy is effective and will save a lot of time, effort and cost because the same inhibitors in eukaryotic systems that are developed to block these pathways can also be used in prokaryotes. To ensure the effectiveness of the drug recycling strategy which using drugs to treat eukaryotic cell diseases as antibiotics to control bacterial pathogens, the primary and tertiary structures of the target between eukaryotes and prokaryotes

must be preserved. Choline kinase (ChoK) is a potential new target that fits these parameters, whose active site sequences are conserved (Figure 2.2) and whose tertiary structure (Figure 2.3) is maintained (Zimmerman, Lacal & Ibrahim, 2019). ChoK is a putative drug target through its role in the growth and pathogenesis of Gram-positive bacteria. *Streptococcus Pneumonia* and Gram-negative Bacteria *Haemophilus influenzae* (Wang *et al.*, 2015). Reuse of drugs known to inhibit the human isoform of ChoK (hChoK), is a promising strategy to inhibit bacterial cell growth by inhibiting the activity of ChoK that has downstream physiological effects on the cell wall (Zimmerman, Lacal & Ibrahim, 2019).

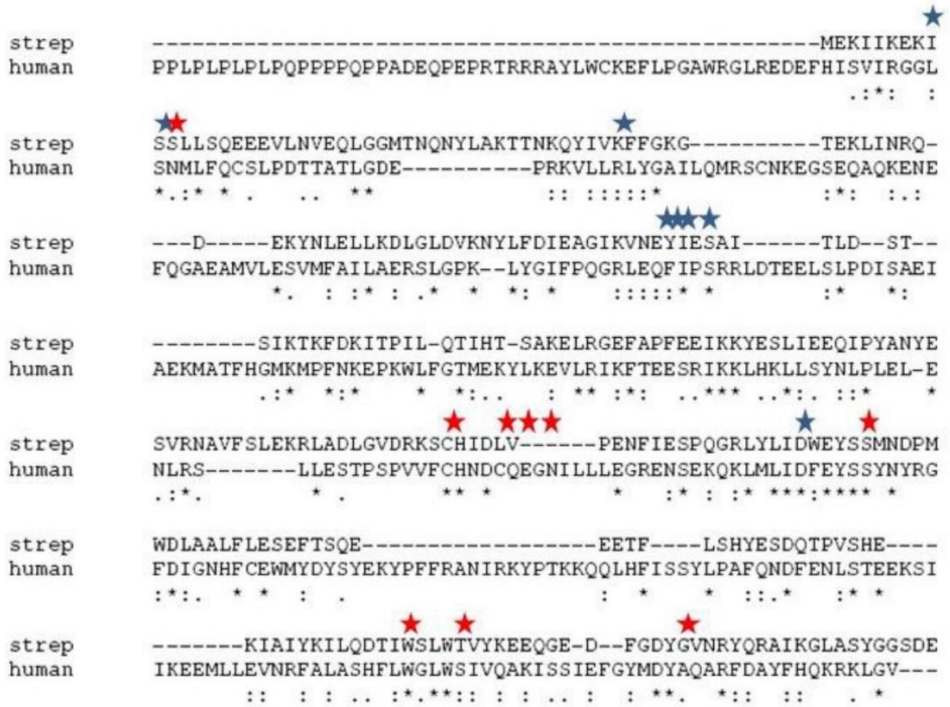

Figure 2.2: Alignment of the primary sequences of bacterial sChok and human hChoK using ClustalW. The positions of the choline kinase binding residues of hChoK are shown in with a red star. The ATP binding site residues are shown with a blue star (Zimmerman, Lacal & Ibrahim, 2019).

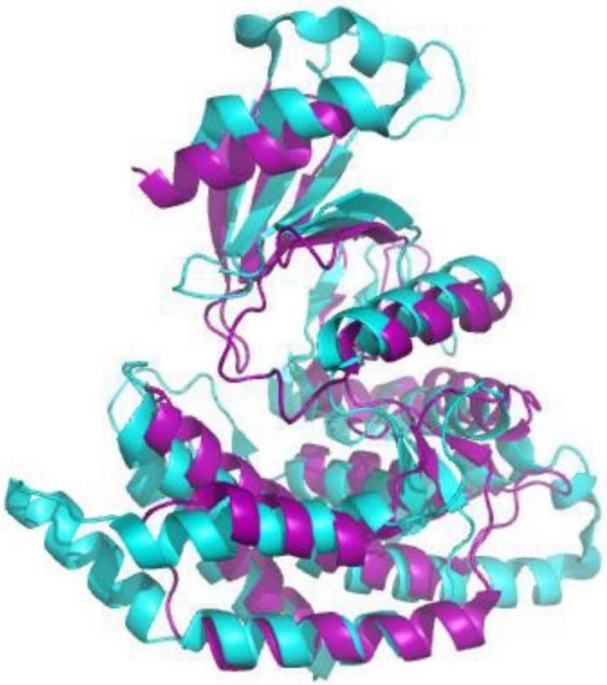

Figure 2.3: Alignment of the crystal structures of bacterial ChoK (purple. RCSB accession 4R77) and human hChok (cyan, RCSB accession #2CKO). The basic N-terminal and C-terminal domains are shown to be generally conserved. Alignment and figure generation carried out with the PyMol Packages(Zimmerman, Lacal & Ibrahim, 2019).

2.5.1 Criteria for prokaryotic ChoKIs:

Successful application of eukaryotic ChoKIs as a novel antimicrobial against antimicrobial resistance requires meeting certain criteria including conserved primary and tertiary structures. An example is the preservation of the tertiary structure between *S. pneumoniae* ChoK (SpChoK) and hChoK (Wang *et al.*, 2015; Zimmerman, Lacal & Ibrahim, 2019) . Although the primary structures are only roughly preserved (Zimmerman et al., 2019), the catalytic site residues have a high level of conservation (Fig. 2.2). Furthermore, ATP and the choline-binding site for hChoK and many pathogens are also conserved (Serran-Aguilera *et al.*, 2015).

The structural homology at the active sites between hChoK and other ChoKs makes it conceivable for ChoKIs to block SpChoK and PfChoK. Hence, making it conceivable to utilize ChoKIs on pathogenic prokaryotes with a high amino acid sequence homology of their ChoK with hChoK at the active site (Khalifa, Few & See Too, 2020). Some ChoKIs could prove to be suitable in inhibiting PfChoK due to their previous application that demonstrated antimalarial activity for example hexadecyl trimethyl ammonium bromide (HDTAB), BR23, and BR25(Serran-Aguilera *et al.*, 2015).

In Brenner's phosphotransferase motif, conserved asparagine residues (Asp-306 and Asn-311) are essential for the catalytic activity as they interact with the phosphate group of phosphorylcholine in the hChoK structure. hChoK is catalyzed by a coordinated magnesium ion through a conserved aspartate residue (Asp-330) which can lose its activity if mutated (Malito *et al.*, 2006). The conservation of the sequence of the active site between different species signifies the probability of the effective antimicrobial appliance of ChoKIs in a different bacterial species (Khalifa, Few & See Too, 2020).

2.5.2 Potential Prokaryotic ChoKIs

Established eukaryotic ChoKIs, such as Hemicholinium-3, MN-58b, and RSM-932A have demonstrated to competitively inhibit ChoK activity (Zimmerman, Lacal & Ibrahim, 2019). For example, HC-3 inhibits *S. pneumoniae* ChoK in addition to damaging the cell wall (Hong *et al.*, 2010; Zimmerman & Ibrahim, 2017). Moreover, it can block some eukaryotic Cho transporters (Dey & Ray, 2018), in addition to inhibiting ChoK in cancer and parasites (Lacal, 2001; Zimmerman, Lacal & Ibrahim, 2019). However, HC-3 has disadvantages that may make us need to look for safer compounds, for example it has neuromuscular toxicity effects (Slone, Kosh & Freeman, 1986) and causes cognitive deficits and impairments in spatial learning impairment (Domino *et al.*, 1973; Slone, Kosh & Freeman, 1986).

Referring to the fact that both MN58b and RSM-932A may block hChoK, it was found that they are more effective and convenient than HC-3 (Zimmerman, Lacal & Ibrahim, 2019). In particular, MN58b, with its limited toxicity, has the ability to prevent cancer. As such, new ChoKIs such as TCD-717, ICL-CCIC-0019, EB-3D and EB-3P could be more suitable (Kall, EJ & A, 2018; Sola-Leyva *et al.*, 2019; Zimmerman, Lacal & Ibrahim, 2019).

Additionally, ChoKIs are capable of inhibiting choline-binding proteins (CBPs) e.g. autolysin LytA (Maestro & Sanz, 2016). HC-3 can block autolysis, but not MN-58b and RSM-932A (Zimmerman & Ibrahim, 2017; Zimmerman, Lacal & Ibrahim, 2019). ChoKIs might also inhibit normal flora (Obanla *et al.*, 2016). Therefore, more studies should be conducted for the identification of the best suited ChoKIs to be used on prokaryotes.

2.6 Bioinformatics tools and alternative therapies against AMR

There are many methods of combating antibiotic-resistant bacteria that have shown promising results. One of these methods is bioinformatics tools that can predict the genetic determinants of antibiotic-resistant bacteria resistance for effective control and treatment of resistant bacteria (McArthur & Wright, 2015; Guitor *et al.*, 2020) . These tools have aided in revolutionary discoveries in the field of AMR by being able to design new potential antibiotics. And with the advent of alternative therapies to antibiotics, such as phage therapy, which can restore the balance to combat against AMR (Lin, Koskella & Lin, 2017; Orzechowska & Mohammed, 2019; Tang *et al.*, 2019) . Explicitly when the AMR defensive workings orchestrated by the bacteria are met with counter-defensive procedures by the phages. In addition, the combination of the two treatments (phages and antibiotics) can be more beneficial and more efficient in reducing the widespread of AMR than using each treatment separately. However, this will make the bacteria helpless to antibiotics in the event that they can create resistance to the sterilants. Taking the essential preventive measures decreases the rate of infection of antibiotic-resistant bacteria, but there's still a critical requirement for successful and radical countermeasures to reduce the hazard of antimicrobial resistance. Each of these areas has the potential to assist increment of the effectiveness of ChoKIs or indeed a combination of ChoKIs and Nanoparticles (NPs). Effective engineering and fabrication of NPs containing miniature amounts of ChoKI and phage (usually phage particular to specific antibiotic-resistant microbes) can be a compelling, targeted, and secure choice to combat antibiotic-resistant bacteria in microbes (Khalifa, Few & See Too, 2020).

2.7 *In silico* approach as a prediction of novel therapeutic targets

Earlier this century, the field of bioinformatics emerged rapidly. From the pioneering laborious mapping and comparison of protein and gene sequences in molecular biology, into a science of its own, which today has reached a high level of maturity and sophistication. Tools in bioinformatics are nowadays used with great success in structural biology, computational chemistry, genetics, molecular biology, pharmaceutical industry, pharmacology and more (Silakari & Singh, 2021).

The term *in silico* stems from the computer component silicium; *in silico* methods, therefore, refer to methods or prediction using computational approaches. *In silico* methods have the advantage that they can make fast predictions for a large set of compounds (Amberg, 2013). Significantly reducing the initial search space by computational prediction of successful targets affects attrition rates in the field of drug discovery. *In silico* approach effectively shows that data linking genes to diseases is sufficient to predict new therapeutic targets. This type of evidence emphasizes the need to formulate and reinforce hypotheses in the target discovery process (Ferrero, Dunham & Sanseau, 2017). *In silico* methods, depends on the structure of the compound for its predictions, even before it is synthesized. They can be used very early in the drug development process, for compounds that are planned to be synthesized, for which no or little compound is available, or also for impurities or degradation products later in the drug development process, for which no synthesis is available. However, a good prediction of the *in silico* method is crucial if the method is to be introduced into the drug development process (Amberg, 2013).

2.7.1 Molecular modeling

Molecular modeling is the science of simulating the behavior of molecular structures and representing them numerically, with the equations of quantum and classical physics (Gu & Li, 2011), describing the generation, manipulation or representation of three dimensional structures of molecules and their associated physicochemical properties (Nadendla, 2004). According to its applications in many research fields, molecular modeling is undergoing rapid growth, and it is now widely used to study the molecular structure of large systems and in physics, chemistry and biology. It is also used to simulate molecular behavior in chemical or biological systems. Accordingly, it is a leading technology that works with a wide range of applications, such as drug design, biomaterials, emerging materials and spectroscopy. Molecular modeling can also describe nucleophilicity, electrophilicity, electrostatic potential, and predict molecular and biological properties to understand structure and activity relationships to provide a rationale for drug design (Saleh, Elhaes & Ibrahim, 2017).

2.7.2 Molecular docking

Molecular docking in a simple definition, is a molecular modeling technique used to predict how a protein (enzyme) will interact with small molecules (ligands). For supramolecular complex formation, the ability of protein (enzyme) and nucleic acid to interact with small molecules plays a major role in protein dynamics, which may enhance or inhibit its biological function (Roy, Kar & Das, 2015). By using molecular docking, the behavior of small molecules in the binding pockets of the target proteins can be described. This method aims to predict the affinity between a ligand and a protein and to determine the correct positions of the ligands in the binding pocket of the protein, based on the types of ligand. It is also often used as a computational and easily

accessible method to propose a binding mode on a protein target. The structural elements responsible for a particular activity can be identified by revealing the points of interaction between the ligand and the target. Binding site and pharmacophore similarities are used to explain the multi-target effects. It also allows simulating the effects of potential synthetic structural modifications to improve desired activities and exclude undesirable targets. Furthermore, docking can also be used to explore different targets to determine the most likely one for a ligand to bind, in virtual target fishing setups. In addition, this technique is often combined with other molecular modeling methods to predict activity and explore binding mechanisms (Temml & Schuster, 2021).

2.8 Advantages of *in silico* against *in vivo* and *in vitro*

Many unique advantages offered by *in silico* methods, mainly nonuse of animals, low cost, and time saving (Ekins, Mestres & Testa, 2007). Obviously, *in silico* models do not require animal experiments but refer to previous experiments, or in some cases *in vitro* data. This means that the quality of the *in vivo* or *in vitro* data is of fundamental importance. And thus, the uncertainty of the *in vivo* data limits the accuracy of the model. Due to its reliance on experimental data, results from *in silico* methods cannot be expected to be better than those used in building the model. If the data is noisy, then the model is poor. For this reason, it is essential to check the quality of the data. On the other hand, since *in silico* methods are statistical, they can handle with errors in toxicity data. Thus, in some cases, unusual values may be detected in a series of similar compounds. Some *in silico* models are more robust than others in noisy data (Benfenati *et al.*, 2010).