PREVALENCE AND SEROTYPE COMPARISON OF ESBL AND NON-ESBL *Klebsiella pneumoniae* IN MALAYSIAN PUBLIC HOSPITALS

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman : Nurshahira binti Sulaiman, PhD
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Introduction*: Klebsiella pneumoniae* is a common nosocomial pathogen that can be found in the hospital setting and is often implicated in hospital outbreaks. A more virulent strain of *K. pneumoniae*, known as hypervirulent *K. pneumoniae* (hvKp), is frequently found in K1 and K2 capsular serotypes. The emergence and spread of these strains are considered a major public health issue as it compromises the management and treatment of infections caused by this pathogen. This condition is further worsened by the presence of co-resistance in extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* strains. Infections caused by hypervirulent ESBL-producing *K. pneumoniae* strains have been extensively reported to be associated with higher mortality rates, clinical complications, prolonged and costly hospitalizations due to limited treatment options. **Objective**: This study aims to determine the distribution and molecular characteristics of ESBL-producing *K. pneumoniae* clinical isolates in Hospital Sultanah Aminah (HSA), Johor Bahru and Hospital Pengajar Universiti Putra Malaysia (HPUPM), Serdang, Selangor. **Methodology**: A total of 192 *K. pneumoniae* isolates were collected from HSAJB and HPUPM. Antibiotic disc diffusion test was then carried out on all isolates to detect ESBL-producing phenotype among them. Subsequently, hypermucoviscosity test (string test) was performed to observe the formation of viscous string of hypervirulent *K. pneumoniae* strain with more than 5 mm in length. Multiplex PCR was then conducted to detect isolates possessing K1 and K2 capsule-associated genes, *magA* and *K2A* genes, respectively. Isolates found to be positive for ESBL phenotype and K1 or K2 serotypes were selected for DNA sequencing and multilocus sequence typing (MLST). Finally, phylogenetic analysis was performed to characterise the genetic linkage between the K1 and K2 serotypes among ESBL-producing isolates. **Result**: A total of 87 out of 192 (46%) ESBL-producing *K. pneumoniae* were detected in the clinical isolates collected from HSAJB and HPUPM. They showed a higher resistance rate towards aztreonam, ceftriaxone, ceftazidime and cefotaxime compared to non-ESBL producing

isolates. Among the ESBL isolates, only 12 were positive for K1 (n= 4) and K2 (n= 8) capsular serotypes, respectively. Statistical analysis showed that K1 and K2 capsular serotypes were not significantly associated with ESBL phenotype (*P*= 0.196). However, they were significantly associated with hypermucoviscosity, as demonstrated by the positive string test (*P*<.001). MLST analysis identified seven different sequence types (ST) in this study, with ST23 predominantly associated with the K1 serotype. Meanwhile, the K2 serotype is widespread among different sequence types (ST14, ST65, ST86, ST628, ST657 and ST792). Phylogenetic analysis of K1 and K2 serotypes revealed two distinct clusters. Cluster A consists of all K1 serotypes, and cluster B consists of all K2 serotypes. **Conclusion**: A high prevalence of 44.2% and 50% of the *K. pneumoniae* isolates collected from HSAJB and HPUPM respectively, were ESBL producers. However, only 8.3% and 10.9% of the total isolates were detected to carry K1- and K2-serotype associated genes respectively. In the current study, seven different sequence types were identified by MLST analysis, with ST23 being predominantly associated with the K1 serotype. Meanwhile, the K2 serotype is widespread among ST14, ST65, ST86, ST628, ST657 and ST792.

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KELAZIMAN DAN PERBANDINGAN SEROTYPE ESBL DAN BUKAN ESBL *Klebsiella pneumoniae* DI HOSPITAL AWAM MALAYSIA

Oleh

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Pengenalan: *Klebsiella pneumoniae* adalah patogen nosokomial penting yang boleh menyebabkan infeksi kepada manusia di dalam komuniti mahupun hospital. Strain *K. pneumoniae* yang boleh menyebabkan masalah kesihatan yang lebih serius, dikenali sebagai hipervirulen *K. pneumoniae* (hvKp) sering dijumpai dalam serotip kapsul K1 dan K2. Kemunculan dan penyebaran strain ini dianggap sebagai masalah kesihatan awam yang serius kerana ia membahayakan pengurusan dan rawatan jangkitan yang disebabkan oleh patogen ini. Keadaan ini lebih membebankan dengan kehadiran strain *K. pneumoniae* yang berupaya menghasilkan β-laktamase spektrum lanjutan (ESBL) dan seterusnya menghasilkan bakteria pelbagai tahan. Jangkitan yang disebabkan oleh strain hipervirulen *K. pneumoniae* yang menghasilkan ESBL telah dilaporkan secara meluas dan sering dikaitkan dengan kadar kematian yang tinggi, komplikasi klinikal, kemasukan ke hospital yang berpanjangan dan kos rawatan perubatan yang tinggi yang disebabkan oleh pilihan rawatan yang terhad. **Objektif**: Oleh itu, kajian ini bertujuan untuk menentukan taburan dan ciri molekul isolat klinikal *K. pneumoniae* yang menghasilkan ESBL di Hospital Sultanah Aminah Johor Bahru (HSAJB) dan Hospital Pengajar Universiti Putra Malaysia (HPUPM), Serdang, Selangor. **Metodologi**: Sebanyak 192 isolat *K. pneumoniae* diperolehi dari HSAJB dan HPUPM. Ujian penyebaran cakera antibiotik untuk mengesan isolat dengan fenotip yang menghasilkan ESBL telah dijalankan ke atas semua isolat. Seterusnya, ujian hipermucoviscosity (string test) dilakukan untuk memerhatikan pembentukan rentetan strain hipervirulen *K. pneumoniae* dengan panjang lebih dari 5 mm diikuti dengan multiplex PCR dilakukan untuk mengesan isolat yang memiliki gen yang berkaitan dengan kapsul K1 (*magA*) dan K2 (*K2A*). Isolat yang didapati positif untuk fenotip ESBL dan serotip K1 / K2 dipilih untuk penjujukan DNA dan urutan multilokus (MLST). Akhirnya, analisis filogenetik dilakukan untuk mencirikan hubungan genetik antara serotip K1 dan K2 di antara isolat yang menghasilkan ESBL. **Keputusan**: 87 daripada 192 (46%) *K. pneumoniae* yang menghasilkan ESBL dikesan dalam

isolat klinikal yang dikumpulkan dari HSAJB dan HPUPM. Kesemua isolat ESBL menunjukkan kadar rintangan yang lebih tinggi terhadap aztreonam, ceftriaxone, ceftazidime dan cefotaxime berbanding isolat bukan ESBL. Di antara isolat ESBL, hanya 12 yang positif untuk K1 (n= 4) dan K2 (n= 8). Analisis statistik menunjukkan bahawa serotip kapsul K1 dan K2 tidak mempunya kaitan yg ketara dengan fenotip ESBL (*P*= 0.196). Walau bagaimanapun, mereka didapati berkaitan secara signifikan dengan hipermukoviskositi seperti yang ditunjukkan oleh ujian rentetan positif (*P*< .001). Dalam kajian ini, analisis MLST mengenal pasti tujuh jenis urutan yang berbeza (ST), dengan ST23 dikaitkan sepenuhnya dengan serotip K1. Sementara itu, serotip K2 tersebar luas di antara pelbagai jenis urutan (ST14, ST65, ST86, ST628, ST657 dan ST792). Analisis filogenetik serotip K1 dan K2 menunjukkan dua kelompok yang berbeza. Kluster A terdiri daripada semua serotip K1 dan kluster B terdiri daripada semua serotip K2. **Kesimpulan**: Prevalensi tinggi 44.2% (HSAJB) dan 50% (HPUPM) isolat *K. pneumoniae* yang dikumpulkan, adalah pengeluar ESBL. Walau bagaimanapun, hanya 8.3% dan 10.9% daripada jumlah isolat yang dikesan membawa gen yang berkaitan dengan K1 dan K2. Tujuh jenis urutan yang berbeza dikenal pasti oleh analisis MLST dengan ST23 dikaitkan dengan serotip K1. Sementara itu, serotip K2 tersebar luas di antara ST14, ST65, ST86, ST628, ST657 dan ST792.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree celsius
µL	Microliter
µm	Micrometre
A_{600nm}	Optical density at wavelength 600 nanometer
ATCC	American Type Culture Collection
ATM	Aztreonam
ATP	Adenosine triphosphate
bp	Base pair
$CaCl_2$	Calcium chloride
cKp	Classical *Klebsiella pneumoniae*
CLA	Clavulanate
CLSI	Clinical and Laboratory Standards Institutes
CRO	Ceftriaxone
CTX	Cefotaxime
CTZ	Ceftazidime
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylene-diamine-tetraacetic acid
ESBL	Extended Spectrum Beta Lactamase
FEP	Cepedime
gapA	Glyceraldehyde 3-phosphate dehydrogenase
HPUPM	Hospital Pengajar Universiti Putra Malaysia
HSAJB	Hospital Sultanah Aminah Johor Bahru
hvKp	Hypervirulent *Klebsiella pneumoniae*
IBC	Intracellular bacterial communities
infB	Translation initiation factor 2
K. pneumoniae	*Klebsiella pneumoniae*
K2A	K2-associated protein A
L	Litre
LB	Luria-Bertani
magA	Mucoviscosity-Associated Gene A

mdh	Malate dehydrogenase
mg	Milligram
MLST	Multi-locus Sequence Typing
NMRR	National Medical Research Registration
NT	Non-typeable
PCR	Polymerase chain reaction
pgi	Phospoglucose isomerase
phoE	Phosporine E
R	Resistance
ROS	Reactive Oxygen Species
rpoB	Beta-subunit of RNA polymerase
rRNA	Ribosomal ribonucleic acid
SPSS	Statistical package for the social science
ST	Sequence Type
TLR4	Toll-like Receptor 4
tonB	Periplasmic energy transducer
α	Alpha
β	Beta

CHAPTER 1

INTRODUCTION

1.1 Background

Klebsiella pneumonia is responsible for many severe nosocomial infections including pneumonia, urinary tract infections, bacteremia, meningitis, liver abscesses and endophthalmitis (Shon et al., 2013). Over the past few decades, these bacteria have been reported globally to display an increasing resistance profile towards β-lactams antibiotics through the production of β-lactamase, such as extended-spectrum β-lactamase (ESBL) (Ben-Ami et al., 2009). Extended-spectrum β-lactamase is a plasmid-mediated enzyme conferring the ability to hydrolyze the common β-lactam bond of β-lactam antibiotics, rendering them inactive (Bush & Bradford, 2016). Recent studies reported ESBL-producing *K. pneumoniae* to be resistant against the first-, second-, third- and fourth-generation cephalosporins, aztreonam and aminopenicillins but are inhibited by clavulanate (ur Rahman et al., 2018; Magiorakos et al., 2012). This poses a major therapeutic challenge in infection management as the range of treatment options becomes limited.

It is well established that strains belonging to capsular serotypes K1 and K2 exhibit the highest virulent activity in humans compared to their other serotypes' counterparts (Shon et al., 2013). Sporadic cases of severe invasive infections have been reported to be caused by *K. pneumoniae* strains exhibiting hypervirulence that were commonly associated with the K1 or K2 capsular serotype (Lee et al., 2006). Several studies conducted in Taiwan, Europe, North America and Japan found that high mortality rates observed in cases of liver abscess with bacteremia were caused by *K. pneumoniae* of capsular serotype K1 and K2 (Wang et al., 1998; Okano et al., 2002, Rahimian et al., 2004; Fang et al., 2005). Since *magA* and *K2A* genes were restricted to K1 and K2 capsular serotypes respectively (Doud et al., 2009), they serve as useful markers to identify the two capsular serotypes.

Information on the prevalence of K1 and K2 capsular serotypes among *K. pneumoniae* infection in Malaysia may give researchers and physicians a detailed understanding and insight into the situation caused by this strain for proper infection management. Therefore, this study aims to investigate the occurrence of K1 and K2 capsular serotype and ESBL-producing *K. pneumoniae* clinical isolates in Malaysia and their genetic diversity, as well as to evaluate the association between the two factors.

1.2 Problem Statement

Infections caused by the hypervirulent variant of *Klebsiella pneumoniae* are increasing at an alarming rate (Li et al., 2013). The defining clinical features that differentiate it from its "classical" counterpart are the ability to infect younger, healthy individuals from the community and cause serious, life-threatening diseases such as pneumonia, liver abscess, endophthalmitis and meningitis (Russo & Marr, 2019). In addition, this variant also possesses an unusual property for Gram-negative bacilli, in which it can spread metastatically in non-immunocompromised hosts (Shon et al., 2013). This condition causes treatment complications, leading to higher morbidity and mortality rates (Tang et al., 2020). Numerous studies have attributed K1 and K2 capsular serotypes as one of the defining traits of hvKp strain as they were widely known to cause the vast majority of hypervirulent infections (Cubero et al., 2019; Shon et al., 2013). This is further worsened by the coexistence of ESBL- producing ability in hvKp strains, which limits the number of clinically available antibiotics for treatment (Mansouri & Abbasi , 2015). Since the prevalence of *K. pneumoniae* clinical isolates that possess both K1 or K2 capsular serotype and ESBL varies between different geographical regions (Russo & Marr, 2019), there is a need for extensive studies to detect the trend and threat of this pathogen in Malaysian hospitals for better infection control management since they require specific management considerations.

1.3 General Objective

This study aims to characterize a collection of *K. pneumoniae* clinical isolates from HSAJB and HPUPM for serotypes, antibiotic susceptibility patterns and genotypic properties.

1.4 Specific Objectives

1. To determine the antimicrobial susceptibility patterns and their distribution in *K. pneumoniae* clinical isolates.
2. To determine the serotypes and their distribution in the ESBL and non-ESBL *K. pneumoniae* clinical isolates using multiplex PCR.
3. To determine the association between hypermucoviscous phenotype and K1 and K2 serotypes.
4. To determine the sequence type and genetic diversity between K1 and K2 serotypes among ESBL-producing isolates through MLST.

CHAPTER 2

LITERATURE REVIEW

2.1 Klebsiella pneumoniae

In 1882, *Klebsiella pneumoniae* was first described as a non-motile, encapsulated gram-negative bacillus (Ashurst & Dawson, 2021). Due to the capsule property, it produces mucoid phenotype when grown on a nutritive medium (Struve & Krogfelt, 2003). This bacteria is primarily found in the environment, including water surfaces, soil and plant, which likely act as a reservoir for human infections (Bagley, 1985). They can also survive in the hospital setting and can be found on medical devices, therefore are readily transmitted among patients through person-to-person contact before colonizing human skin and mucosal surfaces of the gastrointestinal tract and oropharynx (Pessoa-Silva et al., 2003 & Boll et al., 2012).

Environmental *K. pneumoniae* have been reported through several studies to share similarities with clinically isolated strains in regard to their pathogenicity, virulence and susceptibility patterns (Struve and Krogfelt, 2004; Huang et al., 2016; Dantur et al., 2018). However, they displayed a significantly higher susceptibility to antimicrobial intervention than clinically isolated *K. pneumoniae*, suggesting the presence of selective pressure in the clinical setting (Matsen et al., 1974). Upon entry into the human body, they can proceed to enter other tissues and display high degrees of virulence (Ashurst & Dawson, 2021). Over the last few decades, *K. pneumoniae* has been widely observed among severe infection cases in hospitals around the world, with liver abscess being the most common presentation (Ko, 2002; Siu et al., 2012; Shen et al., 2013). To date, *K. pneumoniae* ranks among the leading causes of nosocomial infections such as ventilator-associated pneumonia, liver abscesses septicaemia, bacterial meningitis, urinary tract and catheter-related infections (Tumbarello et al., 2006). There is also an increasing trend of other invasive infections such as necrotizing fascitis being reported around the world (Prokesch et al., 2016).

2.2 β-lactam antibiotics

β-lactam antibiotics are one of the three largest classes of antibodies, which accounts for approximately 65% (US$15 billion) of annual sales of the total antibiotics in the international market (Thakuria and Lahon, 2013). In addition, β-lactam antibiotics are one of the most abundantly prescribed antibiotics as they provide broad antibacterial actions with minimal side effects (Ruotsalainen et al., 2020). They have been highly effective in antibacterial therapy against various bacterial species for decades. β-lactams were also known to be the most prescribed drugs worldwide due to their potency, safety, tolerability, and low cost

(Alfei & Schito, 2022). For this reason, β-lactam antibiotics were once considered a potent bactericidal agent against susceptible isolates (Peterson, 2008). The first β-lactams antibiotic that was introduced clinically was Penicillin G (benzylpenicillin) (Rammelkamp and Keefer, 1943). However, over the years, more classes of β-lactams antibiotics have been discovered, which include cephalosporins, penicillin, monobactams and carbapenems (Bush & Bradford, 2016).

The major antibacterial activity of β-lactam antibiotics is through the inhibition of bacterial cell wall biosynthesis by binding and inhibiting the carboxypeptidases and transpeptidases in the cell wall of bacteria, as illustrated in Figure 2.1 (Peterson, 2008). The enzymes are responsible for replacing the original D-alaine-D-alanine substrate from penicillin-binding proteins (PBP) that carries the responsibility of cross-linking or interconnecting the peptidoglycan components of the bacterial cell wall (Peterson, 2008). Due to the similarity of the ring of β-lactam antibiotics to the pentapeptide's D-alaine-D-alanine of N-acetylmuramic acid (NAM), the PBP uses β-lactam instead of NAM Pentapeptide as the building blocks for cell wall synthesis (Zapun et al., 2008). As a result, the enzyme PBP are acylated. The transpeptidation activity will subsequently be evaded, leading to the cell wall's disintegration and eventually cell lysis (Fisher & Mobashery, 2009).

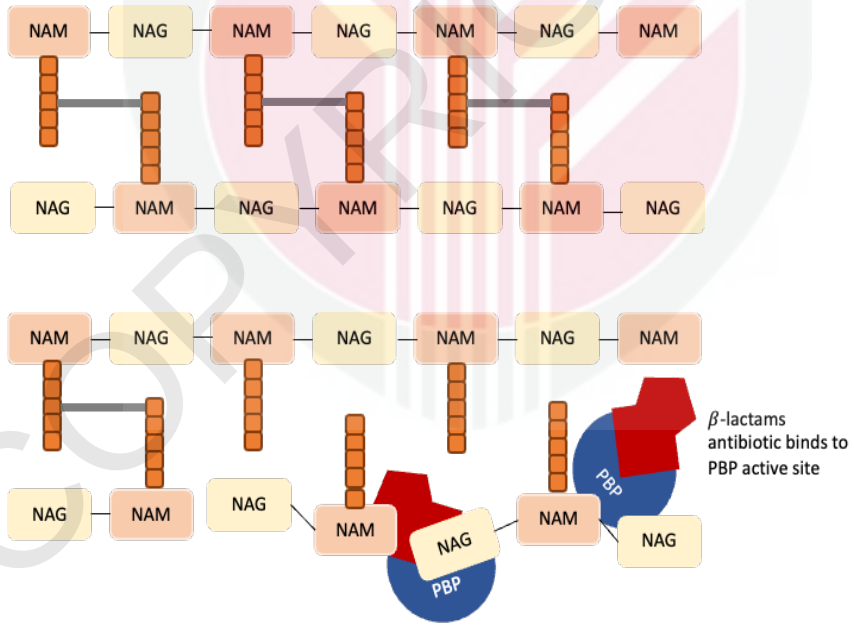

Figure 2.1: Mechanism of action of β-lactam (Adapted from Zango et al., 2019)

2.3 Mechanism of β-lactam resistance

Bacteria including *K. pneumoniae* are constantly evolving in their efforts to develop resistance mechanisms that can survive the detrimental effect of antibiotics. A report published by Çöl et al. (2016) suggested that the evolution of bacterial metabolic function is the main driving force behind the emergence of resistant strains. More importantly, this condition was attributed mainly to the rapid global consumption of antibiotics (Çöl et al., 2016). The development of resistance means lesser antibiotic options will be available to treat infections caused by these bacteria. Therefore, it is crucial to understand the molecular biological mechanisms that contribute to antibiotic resistance for proper evaluation of the effect of intervention strategies (Jiang et al., 2020).

Even before penicillin was commercialised for use, the discovery of a naturally occurring β-lactamase in *E. coli* was reported (Abraham and Chain, 1940). Soon after that, as more β-lactam antibiotics were more clinically used, the resistance evolved and became a selected trait as a consequence of natural selection where susceptible bacteria were killed, and resistant ones were allowed to survive (Martínez & Rojo, 2011). Consequently, other variants of β-lactamase such as bla-TEM and bla-SHV-1 were reported (Abraham and Chain, 1940 & Bradford, 2001). These enzymes share the ability to hydrolyze narrow-spectrum penicillins and cephalosporins (Bush & Fisher, 2011). However, each time new β-lactam agents were introduced and became more frequently utilized, new β-lactamase families began to emerge (Medeiros & Crellin, 1997 & Jacoby & Munoz-Price, 2005).

The emergence of extended-spectrum beta-lactamase (ESBLs) that have been widely linked to frequent outbreaks of hospital-associated infections is also reported through previous study to be caused by multiple drug resistance mechanisms (Rastogi et al., 2010). ESBL-producing bacteria are characterized by specific resistance genes that are responsible for resistance towards most β-lactam antibiotics (Obeid Charrouf et al., 2014; Wragg et al., 2017).

2.4 Emergence of Extended Spectrum Beta-Lactamase (ESBL)-producing *K. pneumoniae*

Over the last decades, the treatment of *K. pneumoniae* infections has been complicated by alterations in the core genome of the organism that has potentiated resistance against a vast range of available antibiotics (Ashurst & Dawson, 2021). The resistance determinants are commonly encoded and carried on bacterial plasmids, chromosomes, transposons and integrons (Kocsis and Sazbo, 2013). Extended and overuse of antibiotics have also promoted the selection of resistant clones in *K. pneumoniae* (Agodi et al., 2015). This condition is further worsened by the slow discovery and development of novel antibiotics to combat the infections caused by this pathogen (World Health Organization

[WHO], 2020). Additionally, the bacteria were reported through previous studies to acquire additional genetic traits and resistant determinants such as ESBL enzyme production, which enables them to better evade the host immunity response (Paterson & Bonomo, 2005; Tenover, 2006; Wang et al., 2020). A study by Gharrah et al., 2017 suggested a correlation between the expression of ESBL and some virulence factors.

Extended-spectrum beta-lactamase (ESBL) is a group of diverse and complex plasmid-mediated enzymes which confer multi-resistance against extended-spectrum penicillin, cephalosporin and monobactam (Paterson & Bonomo, 2005). Bacteria containing these plasmids are known to be multidrug resistant. These plasmids, which can be transmitted between different species of Gram-negative bacteria in vivo, can also carry additional resistance genes to antibiotics (Sirot et al., 1991). In general, β-lactamases are classified by Ambler molecular classification and Jacoby-Medeiros functional classification (Ambler et al., 1991 & Bush et al., 1995). The Ambler scheme divides them into four specific classes (A, B, C and D) depending on their amino acid similarity. Class A, C and D are serine β-lactamases while class B are metallo β-lactamases (Rawat & Nair, 2010). On the other hand, the Bush-Jacoby-Medeiros scheme divides β-lactamases depending on their functional similarities. More importantly, this classification scheme is known to consider clinically relevant β-lactamase and β-lactam substrates (Bush et al., 1995).

To date, more than 350 variants of ESBL have been discovered, which were further classified according to their amino acid sequence into nine different structural and evolutionary families (SHV, TEM, CTX-M, OXA, VEB, PER, GES, TLA and BES) (Bajpai et al., 2017). Among these nine evolutionary groups, the largest group of ESBL came from the TEM and SHV β-lactamase group, which contributes more than 150 members (Rawat & Nair, 2010). ‘TEM’ is a shortened form of Temonniera, the name of the patient from whom the resistant strain was isolated (Jacoby, 1997). At the same time, ‘SHV' is short for variable sulfhydryl and describes the biochemical properties of this β-lactamase. Interestingly, the SHV type is more commonly found among clinical isolates (Jacoby, 1997).

Another significant group of ESBL type after TEM and SHV is CTX-M enzyme, which is β-lactamases known for their potent hydrolytic activity against cefotaxime (Bonnet, 2004). Therefore, bacteria producing this type of ESBL are usually resistant to cefotaxime. However, they may also be able to hydrolyze ceftazidime and confer resistance to this antibiotic (Poirel, 2002). Interestingly, unlike the other ESBLs, CTX-M-type ESBLs were found to have a 10-fold susceptibility against tazobactam compared to clavulanic acid (Bush et al., 1993) stop here Furthermore, they were found to be the leading cause of Enterobacteriaceae urinary tract infections and have displayed alarmingly increasing prevalence (Mazzariol et al., 2017).

After its first discovery in Europe in 1983, several outbreaks caused by ESBL-producing *K. pneumoniae* have been widely reported with a substantial increase throughout the world (Peña et al., 1998 & Haller et al., 2015). In general, β-lactamase is described an enzyme produced by bacteria in its effort to evade destruction by antibiotics by hydrolyzing the bond between β-lactam antibiotics (Abraham & Chain, 1940). However, extended-spectrum β-lactamases (ESBLs) genes expression enables the bacteria to produce a more powerful β-lactamase which is known to have a higher hydrolytic activity toward oxyimino cephalosporins of third-generation cephalosporins and aztreonam (Paterson & Bonomo, 2005). β-lactamase produced by these isolates, however, can be inhibited by clavulanic acid (Bush et al., 1995). Nevertheless, since the family of β-lactam antibiotics is huge, being infected by ESBL-producing organisms means the range of effective agents left for treatment is very limited, as most of the antibiotics used in therapy will be hydrolyzed in vivo by ESBLs. Furthermore, due to the rapid evolution of ESBL, they were also reported in studies to acquire additional resistance towards aminoglycosides and fluoroquinolones (Wener et al., 2010). This condition grew a massive concern as clinically available treatment is no longer effective in treating infections.

2.5 National Antibiotic Resistance Report of *K. pneumoniae* in Malaysia in 2020

In 2020, a total of 30,894 clinical isolates of *K. pneumoniae* were isolated mainly from sputum (23%), urine (17.7%), and blood (16.8%). This is similar to the distribution of *K. pneumoniae* clinical isolates of the previous year, where the majority of them were also from the three specimens where sputum (26%), urine (16.1%) and blood (14.8%) (National Antibiotic Resistance Surveillance Report [NSAR], 2020).

Resistance towards the majority of antibiotics tested for *K. pneumoniae* has also been observed with increasing frequency for the past few years. In 2019, the resistance of *K. pneumoniae* clinical isolates towards cefotaxime and ceftazidime was 22.2% and 19.8% respectively (NSAR, 2020). However, an increasing trend of resistance towards these two antibiotics was noted in the following year, 2020, where the resistance was 22.6% for cefotaxime and 20.4% for ceftazidime. A similar increasing pattern was also observed for imipenem and meropenem in 2019 (1.7% and 2.1% respectively) and 2020 (2.4% and 2.8% respectively). The resistance rates for other antibiotics tested against *K. pneumoniae* were as tabulated in Table 2.1. Even though the resistance rate for imipenem and meropenem was less than 5%, the increasing trend raises concern as these two agents are among the most effective antibiotics available clinically. It is also noteworthy that *K. pneumoniae* isolates from blood expressed higher resistance rates towards the majority of antibiotics tested compared to isolates from other clinical specimens (NSAR, 2020).

Table 2.1: Resistance rate of *K. pneumoniae* clinical isolates in Malaysia towards available antibiotics

Antibiotics	% R in 2015	% R in 2016	% R in 2017	% R in 2018	% R in 2019	% R in 2020
Amikacin	3.9	3.9	4.6	3.1	1.8	2
Amoxicillin/clavulanate	21.8	22.3	22.2	23.3	20.1	20.4
Ampicillin/sulbactam	31.8	30.3	NA	NA	25.8	26.5
Cefepime	21.9	21.7	21.7	18.2	16.4	17.1
Ceftazidime	25.1	23.7	24.6	23.5	19.8	20.4
Cefotaxime	27.7	26	26.9	26.1	22.2	22.6
Cefuroxime	28.2	27.3	28.4	27.2	25.1	25.7
Ciprofloxacin	13.5	11.7	12.2	10.8	11.6	14
Cefoperazone/sulbactam	14.3	12.8	NA	NA	13.3	8.7
Gentamicin	14	12.8	12.4	10.8	8.5	9.4
Imipenem	2.4	2.3	2.7	1.9	1.7	2.4
Meropenem	2.8	2.6	2.9	2.3	2.1	2.8
Co-trimoxazole	28.1	27.2	NA	NA	24.9	25.3

(NSAR, 2020)

2.6 Virulence Factors of *Klebsiella pneumoniae*

Evolution has divided *K. pneumoniae* into two different pathotypes, which are hypervirulent (hvKp) and classical (cKp) *Klebsiella pneumoniae* (Catalán-Nájera et al., 2017). These two pathotypes differ genetically in which hvKp were widely known for their ability to acquire a cluster of plasmids and other mobile chromosome-integrated genetics elements that encode virulence factors. cKp mainly affects patients with comorbidity residing in hospitals, while hvKp is known for its ability to cause life-threatening infections in even healthy individuals (Russo & Marr, 2019). A hallmark of hvKp is the appearance of hypermucoid colonies on agar plates. Therefore, hvKp is alternatively known as hypermucoviscous *K. pneumoniae*. Hypermucoviscosity, which can be ruled through a positive string test, is defined by the formation of viscous strings with a length of >5mm when a loop is stretched from the colony on an agar plate (Fang et al., 2004). The prevalence of hvKp amongst *K. pneumoniae* strains varies largely between 12-45%, depending on the study location (Fang et al., 2005; Nadasy et al., 2007: Siu et al., 2011: Pomakova et al., 2011).

Klebsiella pneumoniae is composed of four key cellular structures which contribute to hypervirulence including capsule, lipopolysaccharides, fimbriae and siderophores, as described in Table 2.2 and Figure 2.2 (Parrott et al., 2021). In *K. pneumoniae*, these factors are commonly known to determine the severity

level of infections (Vuotto et al., 2014). The condition of antibiotic resistance in *K. pneumoniae* is also further worsened when these multitudes of virulence factors work in harmony. Genes harbouring specific virulence factors are often recognized through genomic studies (Martin & Bachman, 2018).

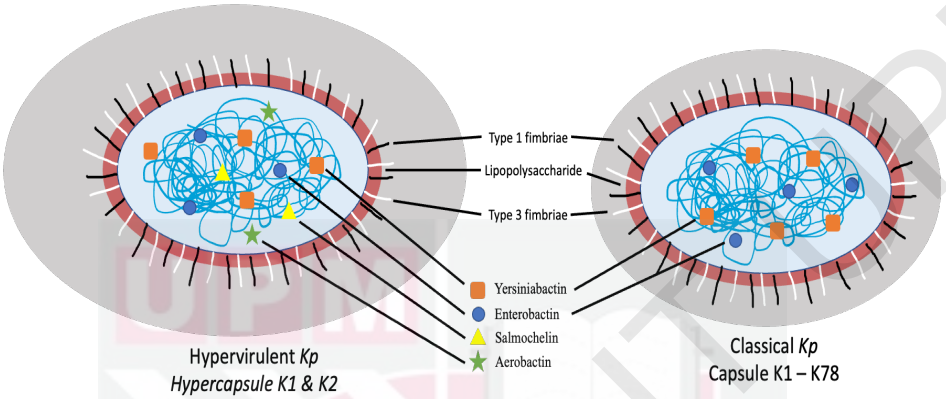

Figure 2.2: The difference between the capsule of classical Kp (K1 – K78) and hypervirulent Kp (K1 & K2) (Adapted from Paczosa & Mecsas, 2016)

Table 2.2: The virulence factors of *K. pneumoniae* and their respective role in pathogenesis

Virulence Factors	Role in Pathogenesis
Capsule	Evade phagocytosis by host cells, inhibit lysis by antimicrobial peptides and complement and prevent early immune response activation (Alvarez et al., 2000; Cortés et al., 2002; Regueiro et al., 2006).
Lipopolysaccharide	Protects against humoral defences by triggering cytokines and chemokines release (Branger et al., 2004, Cai et al., 2009; Standiford et al., 2012).
Siderophores	Increases iron-chelating affinity and scavenges iron molecules for the survival of the bacteria (Miethke & Marahiel, 2007)
Type 1 Fimbriae	Invade bladder cells, form biofilm in the bladder and on abiotic surfaces, increase neutrophils recruitment and promote lactinophagocytosis (Gbarah et al., 1991; Malaviya et al., 1996; Rosen et al., 2008; Struve et al., 2009)
Type 3 Fimbriae	Increase biofilm and the production of neutrophil reactive oxygen species (ROS), binding to extracellular matrix protein and abiotic surface (Przondo-Mordarska et al., 1991; Struve et al., 2009, Schroll et al., 2010).

(Paczosa & Mecsas, 2016)

2.6.1 Capsule

A capsule is a layer of polysaccharide matrix that coats the bacteria and produces an acidic external layer to increase survival in the host environment (Schembri et al., 2005). This is proven through previous studies which reported *K. pneumoniae* strains that are acapsular are much less virulent compared to their capsular counterpart, which can be seen through lower bacterial loads in the lungs, lower mortality rates of the mouse as well as lower systemic spread of bacteria (Yoshida et al., 2000: Lawlor et al., 2005: Cortes, 2002: Lawlor et al., 2006). Capsules also help prevent immune cell-mediated internationalization by inhibiting bacterial binding, therefore producing a much lower robust immune response induction (Evrard et al., 2009). Over the years, *K. pneumoniae* has been classified according to its capsule type, which to date, consists of 78 serotypes (Choby et al., 2020).

2.6.2 Lipopolysaccharide

As for lipopolysaccharide, it constitutes of oligosaccharide core, Lipid A and O antigen, that occupies the external cell membrane of all Gram-negative bacteria, both cKp and hvKp (Bertani & Ruiz, 2018). The outermost subunit of lipopolysaccharide is made up of a long-chain polysaccharide (O antigen) which is known to limit complement-mediated killing by the host's innate immune system through the prevention of pore-formation into the bacterial membrane by binding to a complement component C3b (Shankar-Sinha et al., 2004). Nine O serotypes have been estimated to date, and O1 serotypes are the most common serotypes identified among *K. pneumoniae* clinical isolates (Hansen et al., 1999 & Follador et al., 2016). Despite their benefits during infection, they can also be a hindrance as they can stimulate strong immunity. Lipid A is a potent ligand of a pattern recognition receptor, TLR4, which upon stimulation will produce chemokines and cytokines that activate cellular responses such as macrophages and neutrophils (Branger et al., 2004 & Standiford et al., 2012)

2.6.3 Fimbriae

Fimbriae, namely type 1 and type 3, is another cellular structure of *K. pneumoniae* (Murphy et al., 2013). Type 1 fimbriae are composed of repeating major fimbrial unit, type-1 fimbrial protein A chain (FimA) terminated by a specific adhesion molecule (FimH) which facilitate bacterial attachment to host epithelial cells through interaction with mannose-containing glycoconjugates (Rosen et al., 2008). Unlike type 1 fimbriae which are mannose-sensitive, type 3 fimbriae are mannose-resistant (Klemm & Schembri, 2000). They are able to mediate attachment to a wide range of host cells, including epithelial and endothelial cells of the urinary and respiratory tracts (Hornick et al., 1991: Hornick et al., 1992: Hornick et al., 1995: Tarkkanen et al., 1997). They were also reported through previous study to be able to adhere and trigger biofilm formation on inert surfaces

(Di Martino et al., 2003). Therefore, these fimbriae are the main adhesive structures that facilitate the production of intracellular bacterial communities (IBCs) and subsequently host internal colonisation (Murphy et al., 2013).

2.6.4 Siderophores

K. pneumoniae also secrete molecules called siderophores, which are small, high-affinity chelating compounds of iron produced by a broad range of Gram-negative bacteria (Paczosa & Mecsas, 2016). Siderophores secreted by *K. pneumoniae* strains include yersinibactin, enterobactin, aerobactin and salmochelin (Fung et al., 2012). However, aerobactin and salmochelin are typically produced in hvKp and rarely in cKp. A published report also suggested that aerobactin might be the predominant siderophore expressed in hvKp, causing lung infection (Russo et al., 2015). The receptors will recognize the formation of iron-siderophore on the outer membrane, and they will be transported to the periplasm. In the periplasm, they will be further transported to the inner membrane by the periplasm proteins upon binding with siderophores (Brown and Holden, 2002). Finally, through ABC transporter-mediated passage, iron enters the bacterial cytoplasm, where ferrous iron will be formed, which will be accessible to bacteria (Khan et al., 2018). These molecules are crucial for bacteria to survive and replicate in the host as they compete with host iron-transporting protein and scavenge iron molecules from the host environment (Holden et al., 2016).

2.7 String test in the classification of hypervirulent determinant

After its first discovery in the mid-1980s in Taiwan, hypervirulent variant of *K. pneumoniae* has raised global health threats due to its ability to cause invasive infections even in apparently healthy individuals (Fung et al., 2002). Furthermore, they were reported through previous studies to be able to form abscesses, especially in the liver, and metastasize from the primary sites of infection to the eyes and brain (Lee et al., 2006, Kawai, 2006, Pomakova et al., 2011). This metastatic feature is rare in Gram-negative bacilli (unless in the presence of hose compromise) but very common in specific Gram-positive pathogens (Cheng, 1991). Therefore, accurate classification of hvKp strain is essential using rapid diagnosis methods for epidemiological analysis and proper treatment caused by hvKp strains.

Apart from these important clinical features that differentiate this variant from the classical ones, hvKp variants can be phenotypically classified through their hypermucoviscousity (Li et al., 2013; Zhan et al., 2017). This phenotype can be detected through a test known as the string test, where the hvKp variants will form viscous strings measuring >5 mm in length when a loop is used to stretch the colony grown on an agar plate (Shon et al., 2013). This has been proven through a study conducted by Kumabe & Kenzaka (2014), which revealed that

most strains causing pyogenic infection produce positive string tests. Another study was also conducted where 21 out of 44 (47.73%) of *K. pneumoniae* isolates involved in this study that tested positive for string test had higher resistance rates compared to those that tested negative (Kawser & Shamsuzzaman, 2022). Therefore, these findings have demonstrated the ability of string test as a marker to help in the early diagnosis of infection caused by hypervirulent strain among *K. pneumoniae* isolates. Besides being simple to perform in the laboratory, it is also readily available, produces a rapid result, and is cheaper than other diagnostic methods.

2.8 Capsular serotypes of *K. pneumoniae* that are commonly associated with hypervirulent

Polysaccharide capsules are among the earliest and most commonly reported virulence factors that enable *K. pneumoniae* to evade destruction by the host organism's defence mechanism (Struve et al., 2005). The capsular polysaccharides are vital in protecting the bacteria from opsonophagocytosis, antibiotics permeability and adverse environmental conditions (Struve et al., 2005 & Burmølle et al., 2008). It also aids in the attachment of bacteria to mucosal and epithelial surfaces (Hammerschmidt et al., 2005).

To date, more than 78 serotypes have been identified (Choby et al., 2020). Among these, K1 and K2 serotypes are widely known to be the predominant strains found in invasive infections and demonstrated significantly higher virulence compared to the other capsular serotypes (Turton et al., 2008). Therefore, the presence of K1 and K2 serotypes in clinical isolates of *K. pneumoniae* may serve as an indication of more severe infections. These serotypes were also believed to be a major cause of liver abscesses (Chung et al., 2007 & Shen et al., 2013). The study by Chung et al. (2007) revealed that 59.4% of *K. pneumoniae* liver abscess cases between 2004 and 2005 belonged to the K1 serotype. Similarly, the study by Shen et al. (2013) in Beijing which analysed 101 cases of *K. pneumoniae* liver abscess, reported that 79% of their total isolates were of K1 or K2 serotype.

In a study carried out by Fung et al. and Tsay in 2002, it was reported that K1 or K2 serotypes were significantly more prevalent in *K. pneumoniae* liver abscesses compared to bacteremia. Several other studies have also documented the presence of K1-associated genes in 98.1% (Fang et al., 2004) and 83.3% (Chuang et al., 2006) of *K. pneumoniae* liver abscesses. Identification of K1 and K2 serotypes can be done through Polymerase Chain Reaction (PCR) analysis by detecting the genes associated with respective serotypes (Al-Jailawi et al., 2014).

2.9 Extended-spectrum β-lactamase detection among *Klebsiella pneumoniae* isolates

Early detection of ESBL-producing strains may result in appropriate isolation procedures being implemented, which can help prevent cross-infection with other patients. Therefore, the detection tests must accurately distinguish between ESBL-producing strains and strains with other mechanisms of acquired resistance to β-lactam antibiotics. The double disc synergy test (DDST) was the first-ever detection test designed to observe ESBL production in the Enterobacteriaceae family (Jarlier et al., 1988). This test was initially designed to distinguish between cefotaxime-resistant strains that produce ESBLs and those that overproduce cephalosporinase. The test included the use of 30 µg disc containing third-generation cephalosporins (cefotaxime and/or aztreonam and/or ceftazidime and/or ceftriaxone) separated at a 30 mm distance from another disc containing amoxicillin plus 10 µg clavulanate (Drieux et al., 2008). The test sensitivity can be significantly improved by decreasing the distance between the two discs (Tzelepi et al., 2000). Positive ESBL production will be interpreted when there is a marked increase in inhibition towards disc containing clavulanic acid in front of ceftazidime and/or cefotaxime discs (Drieux et al., 2008).

Another significant test designed to detect ESBL production is ESBL Etests, which were developed to quantify the synergistic effects of extended-spectrum cephalosporins and clavulanic acid. This test consists of double-sided strips containing gradients of ceftazidime (CTZ) or cefotaxime (CTX) or cefepime (FEP) either alone or in combination with 4 mg/L clavulanic acids (Drieux et al., 2008). Isolates were considered ESBL-producing when there were more than three doubling dilution steps reduction around the combination disc (Cormican et al., 1996). The presence of a phantom zone just below the lowest concentration of the CTZ, CTX or FEP gradient and the ellipse deformation of the CTZ, CTX or FEP at the pointy end can also indicate ESBL production (Drieux et al., 2008). It has been reported through a previous study that the interpretation of the inhibition ellipse of ESBL Etests strips might be done incorrectly by laboratories in approximately 30% of cases; therefore, proper training is necessary (Leverstein-van Hall et al., 2002).

ESBL-producing strains can also be detected using the combination disk method. This test was easy to perform, and its interpretation is also straightforward. A study conducted by Linscott and Brown (2005) reported that the sensitivity and specificity for this method were 96% and 100%, respectively. This method is based on measuring the diameters of the zone of inhibition around the cephalosporins-containing disk alone and in combination with clavulanate. Any increase of □5 mm in length or 50% zone expansion between the two diameters indicates ESBL production (M'Zali et al., 2000).

Additionally, ESBL detection can be performed using automated methods such as the VITEK 2 ESBL test (bioMérieux, Marcy l'Etoile, France). The principle of this method is to simultaneously assess the antibacterial activity of 0.5 mg/L of cefotaxime and ceftazidime and 1.0 mg/L of cefepime either alone or plus 4 or 10 mg/L of clavulanic acid, respectively (Drieux et al., 2008). Following the inoculation of card wells containing the respective antibiotics, the cards will be introduced into the VITEK 2 machine. The turbidity will then be measured at regular intervals for each antibiotic tested. The result of growth in wells containing the cephalosporins alone and in combination with clavulanic acid will be interpreted as positive or negative ESBL through a computerised expert system also known as Advanced Expert System (Drieux et al., 2008). Another automated test used to detect ESBL is the Phoenix ESBL test (Becton Dickinson, Sparks, MD, USA). Similarly, this method is based on the growth response to certain expanded-spectrum cephalosporins alone or combined with clavulanates such as ceftazidime, cefpodoxime, ceftazidime/clavulanate, cefotaxime/clavulanate and ceftriaxone/clavulanate. Result interpretation will also be carried out through a computerised system.

2.10 Prevalence of ESBL *Klebsiella pneumoniae* in Southeast Asia

Overall, the prevalence of ESBL in *K. pneumoniae* varies greatly between distinct geographic regions (Bradford, 2001 & Reinert et al., 2007). Southeast Asia is considered the epicentre of ESBL-producing *Enterobacteriaceae* in which high rates of this phenotype, particularly among *K. pneumoniae* isolates, were seen over countries in this region (Hsu et al., 2007; Dat et al., 2017; Fox-Lewis et al., 2018).

Since the prevalence of ESBL strains varies geographically, different regions reported considerably different prevalence and resistance patterns of ESBL-producing *K. pneumoniae*. The characteristics of reviewed publications on ESBL-producing *K. pneumoniae* isolates in Southeast Asian countries are listed in Table 2.3. Previous studies conducted in several countries in Southeast Asia have reported that ESBL production in *K. pneumoniae* varies from 15.3% to 66.2%. In Malaysia, ESBL-producing *K. pneumoniae* ranged from 27.8% to 47%. The percentage is lowest in a tertiary hospital as reported by Mobasseri et al., where 27 (27.8%) ESBL producers among 97 *K. pneumoniae* isolates were identified. On the other hand, the study conducted by Lim et al. in 2009, involving isolates from five Malaysian public hospitals reported the highest percentage (47%) among *K. pneumoniae* isolates. This could be due to different patient population served attributable to different hospitals location.

It is also noteworthy that the study conducted by Mobasseri et al (2020) revealed that all of the ESBL-producing isolates in this study harbored plasmids. 19 strains possessed conjugative plasmids while the majority of the ESBL genes were plasmid-encoded. These findings supported earlier Malaysian studies that demonstrated the transmissibility of ESBL genes found on plasmids (Lim et al.,

2009). Since plasmid has been reported as the primary means of horizontal transmission of ESBL genes, it is considered as the major driver of their global spread (Brolund & Sandegren, 2015).

In Thailand, the prevalence of ESBL reported by two studies is widely different, with a reported yield of 27.4% and 48% respectively (Sethaphanich et al., 2016 & Sawatwong et al., 2019). Meanwhile, *K. pneumoniae* isolates from two studies conducted in Indonesia recorded the highest ESBL detection rate in this review (52.98% and 66.2%). In contrast, the study conducted in Laos reported the lowest ESBL proportion among *K. pneumoniae* clinical isolates compared to other countries (15.3% vs 27.4-66.2%).

The differences between studies might be attributed to differences in ESBL screening methods, patient characteristics, sampling design, antibiotic use guidelines and management of infections (Nur Fitri et al., 2015). Several important factors including antibiotic policy, overcrowded environment in hospitals and the carriage rate among healthcare workers, have also been considered responsible for the emerging phase of resistance in certain institutions (Ananthan & Subha, 2005). Even though the number and type of samples vary between studies, a higher prevalence trend was observed in respiratory samples compared to other specimens. This is expected since *K. pneumoniae* is widely established as one of the most common causes of nosocomial pneumoniae.

Table 2.3: Characteristics of reviewed publications on ESBL-producing *K. pneumoniae* clinical isolates in Southeast Asian countries

Country	Year of sampling	Year of publication	No. of *K. pneumoniae* isolates	Prevalence of ESBL positive (%)	Source of *K. pneumoniae* isolates	ESBL detection method	Author (Reference)
Malaysia	2014	2020	97	27.8%	Blood (25) Bronchoscopic aspirate (24) Swab sample (10) Wound tissue (9) Urine (8) Sputum (8) Pus (6) Poc (3) Fluid (2) Slough (2) Bone (1)	Double disk diffusion	Mobasseri et al., 2020
Malaysia	2014	2016	110	35.5%	Urine (56) Blood (42) Swabs (12)	Double disk diffusion	Mahdi et al., 2016
Malaysia	2004	2009	51	47%	Tracheal aspirates (21) Swab samples (3) Urine (2) Catherer tips (2) Sputum (1) Blood (1)	Double disk diffusion	Lim et al., 2009

Table 2.3: Continued

Country	Year of sampling	Year of publication	No. of *K. pneumoniae* isolates	Prevalence of ESBL positive (%)	Source of *K. pneumoniae* isolates	ESBL detection method	Author (Reference)
					Pus (1) Unknown (20)		
Thailand	2012-2013	2016	306	48%	Not specified	Double disk diffusion	Sethaphanich et al., 2016
Thailand	2008-2014	2019	1059	27.4%	Blood (1059)	Double disk diffusion	Sawatwong et al., 2019
Laos	2010-2014	2020	111	15.3%	Blood (111)	Double disk diffusion	Chang et al., 2020
Indonesia	2017-2018	2020	168	52.98%	Sputum (76) Pus (27) Blood (20) Urine (12) Bronchial washings (9) Faeces (6) Wound swab (5) Sterile site (7) Others (6)	Double disk diffusion	Sinanjung et al., 2020
Indonesia	2015	2018	228	66.2%	Sputum (95) Pus (83) Blood (21) Urine (16) Others (13)	Vitek 2 compact	Anggraini et al., 2018

2.11 Antimicrobial resistance pattern among *Klebsiella pneumoniae* clinical isolates in Malaysia

The variation in ESBL-producing *K. pneumoniae* susceptibility among institutions might be attributed to the paucity of good microbiological diagnostic capacity. The level of awareness of ESBL-producing organisms and the importance of their detection might still be low among clinical microbiology laboratories. Therefore, physicians face severe problems in prescribing appropriate antimicrobials to treat ESBL infections. Uncontrolled and inappropriate use of antibiotics could be a leading contributing factor to resistance acquisition among *K. pneumoniae* (Prestinaci et al., 2015). As a result, the risk of treatment failure will increase and potentially, the spread of infection (Friedman et al., 2016).

In Malaysian hospitals, ESBLs existed in 27.8-47% of the *K. pneumoniae* clinical isolates (Lim et al., 2009; Mahdi et al., 2016; Mobasseri et al., 2020). More importantly, they showed relatively excellent resistance to most, if not all, clinically available antibiotics. The percentage of isolates showing non-susceptibility to cephalosporins was also considerably high in all studies. Although certain antibiotics used in one study are not included in others, the susceptibility of ESBL-producing *K. pneumoniae* to carbapenem, amikacin and piperacillin/tazobactam was consistently good in all Malaysian studies included in this review. A good agreement was also shown between all three studies, where very high resistance rates against ampicillin were observed. This is not uncommon, as *Klebsiella* is widely established to have intrinsic resistance to ampicillin via the SHV-1 penicillinase (Cooney at al., 2014; Wand et al., 2015).

In contrast, very low resistance rates towards carbapenem (imipenem, meropenem & ertapenem), which is also known as “antibiotics of last resort”, were reported, implying the likelihood of successful and effective treatment for infections caused by these strains when treated with these agents. This concurs with the report released by the Malaysian National Surveillance on Antibiotic Resistance Report in 2019, which stated a resistance rate of 1.7% and 2.1% towards imipenem and meropenem respectively.

The study conducted in a hospital in Johor Bahru, Malaysia, analyzed 97 *K. pneumoniae* isolates from inpatients admitted from September to December 2014. The resistance rates varied from 1% (colistin), 2% (imipenem), 6.1% (meropenem), 7.2% (ciprofloxacin), 8.2% (sulbactam cefoperazone), 11.3% (gentamycin), 11.3% (tazobactam), 19.5% (amoxicillin-clavulanate), 25.7% (ceftazidime), 27.8% (aztreonam), 27.8% (cefoperazone), 29.8% (cefixime), 30.9% tetracycline, 31.9% (cefotaxime) and 83.5% (ampicillin). However, this study also reported an increasing trend of resistance against imipenem, from 0% to 2% compared to the previous year (Mobasseri et al., 2020).

Meanwhile, in a separate study conducted in a hospital in Pahang, Malaysia, from May to September 2014, considerably high resistance rates were recorded for piperacillin (43.6%), cefuroxime (35.5%), cefotaxime (35.5%), ceftazidime (34.5%), sulbactam/ampicillin (32.7%), amoxicillin/clavulanic acid (30.9%), cefepime (28.2%), piperacillin/tazobactam (26.4%), gentamicin (25.5%) and ciprofloxacin (20%). In contrast, carbapenem (meropenem, imipenem & ertapenem) and amikacin demonstrated very low resistance rates of <5% (Mahdi et al., 2016).

Another study was conducted by Lim et al. (2009) on sporadic cases from five different hospitals in Peninsular Malaysia. The percentage of resistance was reported as follows: 0% (imipenem), 10% (cefepime), 10% (amoxicillin+clavulanic acid), 12% (amikacin), 12% (ciprofloxacin), 22% (tetracycline), 25% (chloramphenicol), 29% (trimethoprim+sulfamethoxazole), 29% (streptomycin), 29% (cefoperazone), 31% (gentamicin), 35% (nalidixic acid), 35% (kanamycin), 35% (ceftriaxone), 41% (aztreonam), 41% (ceftazidime), 65% (piperacillin) and 96% (ampicillin).

2.12 Antimicrobial resistance pattern among *Klebsiella pneumoniae* clinical isolates in Southeast Asian countries

For the studies conducted by Sethaphanich et al. and Sawatwong et al. in Thailand, the susceptibility rate of *K. pneumoniae* isolates towards piperacillin/tazobactam differs significantly. The studies by Sawatwong et al. showed >85% susceptibility, while the study by Sethaphanich et al. showed only 60% susceptibility. However, both studies reported a similar trend of >85% susceptibility rates towards carbapenem.

In the study carried out in a university hospital in Bangkok, Thailand by Sethaphanich et al. involving 306 *K. pneumoniae*, all isolates were found to be susceptible to imipenem. In addition, >90% showed sensitivity to carbapenems and amikacin, while only around 60% were towards piperacillin/tazobactam. Notably, only 20% were susceptible to cephalosporins (ceftazidime and cefepime). In comparison, 18% of them showed resistance to all oral antibiotics, including ciprofloxacin, levofloxacin, amoxicillin/clavulanate and cotrimoxazole (Sethaphanich et al., 2016).

Meanwhile, in the study conducted by Sawatwong et al., community-onset ESBL-producing *K. pneumoniae* reported susceptibility of 46.2% for amoxicillin, 36.4% for ceftazidime, 28.0% for cefotaxime, 54.6% for gentamycin, 44.4% for ciprofloxacin and 37.0% for trimethoprim/sulfamethoxazole. >85% of hospital-onset ESBL-producing *K. pneumoniae* displayed susceptibility to piperacillin/tazobactam, amikacin, and carbapenems (Sawatwong et al., 2019). More importantly, the 7-year bloodstream infection surveillance conducted in 20

Thailand hospitals by Sawatwong et al. reported no apparent trend in the prevalence of ESBL-producing *K. pneumoniae* over time (*P*= 0.09).

All *K. pneumoniae* isolates from the Mahosot Hospital, Vientiane, Laos PDR expressed susceptibility to imipenem and/or meropenem. They were also sensitive to amikacin except for one case of resistance from a patient with biliary sepsis in 2014. The proportion susceptible to other groups of antibiotics otherwise used in this study was relatively stable, with no apparent trend during the study period (Chang et al., 2020).

In Indonesia, >80% of the ESBL-producing *K. pneumoniae* clinical samples isolated from a general hospital in Central Java displayed high percentage susceptibility against meropenem, amikacin and piperacillin-tazobactam. In contrast, susceptibility to cefepime and ceftriaxone was 0 (Sinanjung et al., 2020). A similar trend of >80% susceptibility towards carbapenem and amikacin was observed in this study and the study conducted by Dr Arifin Achmad General Hospital, Pekanbaru in 2015. The susceptibility of ESBL-producing *K. pneumoniae* towards other antibiotics was as follows: tigecycline (76.8%), piperacillin/tazobactam (40%), gentamycin (35.1%), ciprofloxacin (32.5%), trimethoprim/sulfamethoxazole (25.2%), nitrofurantoin (16.5%). As for the study conducted by Anggraini et al., 2018, all ESBL-producing *K. pneumoniae* isolates recorded no sensitivity towards ampicillin, cefazolin, ceftriaxone, ceftazidime, cefepime and aztreonam.

2.13 Multiplex PCR for the detection of serotype-associated genes in *Klebsiella pneumoniae*

Over the past few decades, hypervirulent strains of *K. pneumoniae*, predominantly K1 and K2, have emerged as the leading cause of deadly and invasive community-acquired infections such as pneumonia, meningitis and liver abscess in previously healthy individuals (Zhu et al., 2021). It is also noteworthy that in many countries, these strains were found to be responsible for most community-acquired primary liver abscesses (Fang et al., 2007; Yang et al., 2009 & Shon et al., 2013). The hypermucoid phenotype and invasive nature are the major virulence factors that limit the therapy option and increase the mortality rate (Jun, 2018). Thus, massive attention is focused on the polysaccharide capsular serotype as the prime virulent determinant, with K1 and K2 serotypes exhibiting most abundantly in humans.

Detecting these serotypes is plausible by identifying the serotype-specific genes restricted to that particular serotype. This can be done through numerous methods, such as PCR-restriction fragment length polymorphism, PCR-single-strand conformation polymorphism, oligotyping and ligase chain reaction (Hernández et al., 2005). However, they can only detect a single gene at a time. Therefore, multiplex PCR are designed to overcome this limitation by being able

to simultaneously amplify multiple genes in one test (Hernández et al., 2005). For example, the mucoviscosity-associated gene A (*magA*) is specified to the K1 serotype, while the K2 capsule-associated gene A (*K2A*) is designated to the K2 serotype (Fang et al., 2004; Turton et al., 2008). In a study by (Fang et al. 2004), the frequent prevalence of *magA* gene was reported in the hypervirulent K1 strains of *K. pneumoniae.* On the other hand, the *K2A* gene was commonly utilised as the diagnostic intervention to identify the presence of K2 serotypes.

The increasing trend seen in ESBL-producing *K. pneumoniae* with serotypes K1 and K2 isolated clinically calls for the need to understand the organism factors that contribute to this condition. Therefore, studies on the prevalence and resistance profiles of *K. pneumoniae* clinical isolates must be extensively carried out. As shown in Table 2.4, the prevalence of K1 and K2 genes from a study conducted by Mobasseri et al., 2020 in Malaysia reported that 38.1% of *K. pneumoniae* isolates were positive for K1 gene. However, no K2 gene was detected in this study. On the other hand, Liu et al., 2018 reported 34.4% and 20.8% occurrence of K1 and K2 genes respectively among *K. pneumoniae* hypervirulent strain. More importantly, the study conducted in Taiwan by Yu et al., 2007 recorded a relatively higher prevalence of K1 serotypes (45.3%). This might be attributed to the inclusion criteria, which includes only *K. pneumoniae* isolates causing primary liver abscesses and other abscesses (Yu et al., 2007).

Table 2.4: Prevalence of K1 and K2 serotypes among *K. pneumoniae* strains based on studies reported in Malaysia, China and Taiwan

Country	Prevalence of K1 serotype (%)	Prevalence of K2 serotype (%)	Reference
Malaysia	37 (38.1)	0	Mobasseri et al., 2020
China	34.4	20.8	Liu et al., 2018
Taiwan	28 (45.9)	13 (21.3)	Yu et al., 2007
India	1 (0.27%)	8 (2.16%)	Remya et al., 2018

In addition to serotype-specific genes, multiplex PCR is widely used to detect β-lactamase genes. The wide range of β-lactamase genes include bla^{TEM}, bla^{CTX-M}, bla^{SHV}, bla^{PER}, bla^{VEB}, bla^{GES} (Class A), bla^{IMP}, bla^{VIM}, bla^{KPC} (Class B) and bla^{OXA} (Class D) (Branger et al., 2005 & Ramazanzadeh et al., 2015). Among these, bla^{TEM}, bla^{CTX-M} and bla^{SHV} are often considered the most clinically significant β-lactamase genes variants (Bush and Fisher, 2011). Each of these genes expresses different levels of resistance against different antibiotics. Isolates detected with bla^{SHV} genes were found to express a high level of resistance against ceftazidime but not to cefotaxime or cefazolin. At the same time, those with bla^{CTX-M} showed higher effectiveness against cefotaxime (Liakopoulos et al., 2016). On the other hand, bla^{TEM} and a variant of bla^{SHV}, bla^{SHV-1}, confer resistance to oxyimino cephalosporins, such as cefotaxime,

ceftazidime and aztreonam (Delmas et al., 2006). Detecting these β-lactamase genes that are usually associated with multidrug resistance can provide crucial data regarding infection epidemiology (Tumbarello et al., 2006).

In conclusion, multiplex PCR genotyping offers a sensitive, specific, reproducible and rapid detection of K1 and K2 virulence genes as well as β-lactamase genes carried by *K. pneumoniae* isolates (Turton et al., 2018; Compain et al., 2014). It will also be essential to compare the virulence profile between ESBL and non-ESBL isolates of *K. pneumoniae* (Compain et al., 2014). Early and rapid detection of infections caused by the resistant variant of *K. pneumoniae* might result in better infection control management, lower treatment complications and lower mortality rate.

2.14 Multilocus sequence typing (MLST) of *Klebsiella pneumoniae*

Genotyping is crucial for identifying cases or outbreaks of *K. pneumoniae* as well as tracking the source and spread of infections. The most commonly known genotyping methods are multilocus sequence typing (MLST), multiple-locus variable number tandem repeat analysis (MLVA) and pulsed-field gel electrophoresis (PFGE) (Diancourt et al., 2005 & Turton et al., 2010). Multilocus Sequence Typing (MLST) is a method that is widely utilised to characterise the genetic relationship between bacterial isolates (Enright & Spratt, 1999; Feil et al., 2004). It is a nucleotide sequence-based method that allows multi-user international databases to be implemented through the delivery of transferable data (Jolley et al., 2004). It carries the advantage of being easier to manipulate and compare than the traditional PFGE (Aanensen & Spratt, 2005 & Diancourt et al., 2005). The unambiguous data provided through MLST might be helpful for molecular epidemiology and the clinical characteristics of *K. pneumoniae* isolates involved in the current study.

The MLST scheme for *K. pneumoniae* isolates was developed in 2005 by Diancourt et al. and has since been used globally to characterise the diversity and epidemiology of *K. pneumoniae*. The MLST scheme consists of seven housekeeping genes; *pgi* (phosphoglucose isomerase), *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *rpoB* (beta-subunit of RNA polymerase), *mdh* (malate dehydrogenase, *phoE* (phosphorine E), *tonB* (periplasmic energy transducer) and *infB* (translation initiation factor 2) (Diancourt et al., 2005). Through this scheme (Diancourt et al., 2005), 40 different sequence type (STs) were identified, which consists of ST1, ST2, ST3, ST4, ST5, ST6, ST8, ST9, ST10, ST12, ST13, ST14, ST15, ST16, ST17, ST18, ST19, ST20, ST21, ST22, ST23, ST24, ST25, ST26, ST29, ST31, ST32, ST33, ST34, ST35, ST36, ST37, ST38, ST39, ST40, ST41, ST42, ST43, ST44 and ST45. Strains with similar ST were revealed to belong to suspected epidemiological clusters. The discriminatory index (Simpson index) when only isolates with no documented epidemiological link were considered was 96%, indicating the ability of MLST to discriminate most epidemiologically unrelated strains (Diancourt et al., 2005)

MLST study involving *K. pneumoniae* clinical isolates conducted across Malaysia revealed high diversity of STs among them. In the study carried out by Mobasseri et al. (2020), 24 different STs were yielded from 97 *K. pneumoniae* clinical isolates. ST23 were identified as the predominant STs, making up almost 21% of the total isolates. The other isolates belong to several distinct type of STs, indicating high genetic diversity of *K. pneumoniae* in their sample collection. Meanwhile, in the study conducted by Gan at al. (2020), no new ST was uncovered from eight carbapenem-resistant strains of *K. pneumoniae* despite the scarcity of the representation of this strain in the MLST database. The result from their MLST analysis also showed high diversity as shown by the distinct STs recovered from their sample collection.

CHAPTER 3

METHODOLOGY

3.1 Ethics approval

Ethical approval for this cross-sectional study was obtained from the Medical Research and Ethics Committee of the Ministry of Health Malaysia, the National Medical Research Register (approval no. NMRR-20-3087-57426) and the Universiti Putra Malaysia (UPM) Medical Research Ethics Committee (JKEUPM-2021-057). The study used de-identified *Klebsiella pneumoniae* isolates collected at the microbiology laboratories of the selected hospitals. Since this study did not meet the definition of research involving human subjects, informed consent was not required; only data related to the source of the isolates and the patients' age, gender, and ethnicity were derived from the medical records. These were not traceable to the sampled patients.

3.2 Isolates collections

Simple/single proportion sampling formula were used in calculating the sample size (Lemeshow et al., 1990). From the calculation, a total of 174 samples, comprising 87 ESBL and 87 non-ESBL *K. pneumoniae* clinical isolates were aimed to be collected. However, at the end of the study, 192 clinical isolates of *K. pneumoniae*, consisting of 87 ESBL strains and 105 non-ESBL strains were collected. All the isolates were collected between March 2021 to April 2022 from the Microbiology Laboratories of Hospital Sultanah Aminah, Johor Bahru (HSAJB) and Hospital Pengajar Universiti Putra Malaysia (HPUPM), Selangor from March 2021 to April 2022. Hospital Sultanah Aminah Johor Bahru, which was founded in 1882, is a government-funded multi-speciality hospital located in Johor Bahru, Johor, Malaysia. Since it is the state's main referral and tertiary health centre, this hospital has an exceptionally high patient load daily. On the other hand, HPUPM is a private and government-funded multi-speciality hospital located in the Serdang area. This hospital is located near Putrajaya and the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM). It is a teaching hospital for the Faculty of Medicine and Health Sciences of Universiti Putra Malaysia and provides comprehensive medical care to the surrounding communities. The hospital was built to serve the roughly 570,000 population of Serdang, Putrajaya, Kajang and Bangi districts.

HSAJB was chosen to be included in this study since it serves a large population, being the largest and the main referral centre for Johor. Therefore, samples collected from this hospital may represent all ethnicity in Malaysia. Meanwhile, HPUPM were included in this study as it serves the southern Selangor. Data

from this study would help to provide information about the disease trend in the respective hospitals which will benefit them in the long run.

Any non-duplicated clinical isolates identified with *K. pneumoniae* were the criteria used for selecting the sample population. Non- *K. pneumoniae* samples and/or duplication samples were excluded from this study.

$$n = \frac{z^2 p(1-p)}{d^2} \tag{3.1}$$

Where:
n= number of samples.
p= prevalence of *K. pneumoniae* in Malaysia in 2019 = 0.13 (NSAR, 2019)
z= statistical for a level of confidence = 1.96
d= margin of error = 0.05

$$n = \frac{(1.96)^2 0.13(1-0.13)}{(0.05)^2}$$

$$n = \frac{0.43448496}{0.0025}$$

$$n = 174 samples$$

3.3 Identification of isolates

Klebsiella pneumoniae isolate identification was carried out at the hospital laboratories as part of the routine laboratory operation. Following temporary storage and transportation from the hospital laboratory, re-identification based on phenotypic colony morphology using MacConkey agar medium was further carried out to ensure the viability and purity of the isolates. Following overnight incubation, colonies were examined for colour and the presence of lactose fermentation. The presence of a pinkish colony, indicating lactose fermentation, would suggest a positive result.

These isolates were also biochemically identified through Triple Sugar Iron (TSI), citrate utilization, urease and motility test according to the Manual of Clinical Microbiology (2020). A positive TSI test will be indicated by the presence of a yellow slant and yellow butt, indicating the presence of acidity in both regions. No gas bubbles were observed at the butt of the agar as *K. pneumoniae* did not produce hydrogen sulphide. As for the citrate utilization test, the colour change of the medium from green to blue will indicate a positive result as citrate was utilized as a carbon source. Next, a positive urease test will be indicated by the

colour change from yellow to pink due to the increment of the pH of the medium as urea was converted to ammonia. Lastly, the motility test would observe no lateral movement or diffusion for *K. pneumoniae* isolates.

3.4 Preparation of stock cultures and subculturing

Brain Heart Infusion (BHI) broth + 10% glycerol was used as suspension broth to preserve *K. pneumoniae* isolates. A duplicate of each sample was preserved; one for working stock and the other for long-term preservation (cryo state -80^{o}C). All samples were then cultured on routine culture media by semi-quantitative method (WHO). Using a calibrated loop, each sample was inoculated on MacConkey agar and incubated aerobically for 24 hrs at 37^{o}C.

3.5 Antibiotic Susceptibility Test (AST)

The susceptibility of *K. pneumoniae* isolates against aztreonam (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg) from Oxoid Ltd, Basigstokes UK was tested using Kirby Bauer's disc diffusion method as recommended by Clinical and Laboratory Standard Institute (CLSI, 2020). The antibiotic-containing discs were first taken out from the chiller and allowed to reach room temperature. Subsequently, colonies grown on nutrient agar overnight were suspended in 10 ml of normal saline, and the turbidity was adjusted to 0.50 McFarland. A sterile cotton swab was then dipped into the bacterial suspension and pressed against the tube wall to remove excess fluid. The surface of a Mueller-Hinton agar was then evenly swabbed. 30 µg aztreonam, 30 µg ceftazidime, 30 µg ceftriaxone, and 30 µg cefotaxime discs were placed firmly onto the surface of inoculated agar plates. The plates were then incubated at 37^{o}C overnight. Following overnight incubation, the diameters of the inhibition zone (IZD) were measured.

3.6 ESBL screening

Interpretation of the results from the antibiotic susceptibility test above was carried out to screen for ESBL phenotypes as outlined in the Clinical and Laboratory Standards Institute guidelines (CLSI, 2020). Isolates with a diameter of inhibition zone of ≤ 17 mm against ceftazidime, ≤ 22 mm against aztreonam, ≤ 27 mm against cefotaxime and ≤ 27 mm against ceftriaxone were suspected to be ESBL producers. *Klebsiella pneumoniae* ATCC 700603 was used as the quality control strain.

3.7 ESBL confirmatory test

All isolates were then phenotypically screened to confirm ESBL production using ceftazidime (30 µg), ceftazidime-clavulanate (30/10 µg), cefotaxime (30 µg), cefotaxime-clavulanate (30/10 µg) from Oxoid Ltd, Basigstokes UK and Becton Dickinson, USA on Mueller-Hinton agar plates already inoculated with the test bacteria (adjusted to 0.5 McFarland turbidity standard). After incubation for 16-18 hrs at 37^{o}C, the diameter of inhibition zones was measured. A ≥5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanate versus when the agent is tested alone were regarded as ESBL producers according to the CLSI breakpoints 2020.

3.8 Extraction of DNA from bacteria

One millilitre of fresh bacterial cultures grown in Luria-Bertani broth was used to prepare template DNA (Green and Sambrook, 2012), followed by DNA extraction using a DNA extraction kit (GeneAll Biotechnology, Korea). Following the manufacturer's protocols, cells were harvested by centrifugation for 1 min at 15 000 rpm. The supernatant was discarded, and the cell pellets were resuspended completely in 200 µl of Buffer CL. Subsequently, 20 µl of Proteinase K solution (20 mg/ml) was added and vortexed vigorously to mix before incubation at 56°C for 15 min. Following incubation, 200 µl of Buffer BL was added to the tube, vortexed and further incubated at 70°C for 10 min. The suspension was allowed to cool at room temperature before proceeding. Next, 200 µl of absolute ethanol was added to the sample and pulse-vortexed to mix thoroughly. The mixture was then transferred to the SV column and centrifuged for 1 min at 15 000 rpm.

Following centrifugation, the collection tube was replaced with a new one, and 600 µl of Buffer BW was added. The mixture was then again centrifuged for 1 min at 15 000 rpm, and the collection tube was replaced with a new one. Next, 700 µl of Buffer TW was applied and centrifuged for 1 min at 15 000 rpm. The pass-through was discarded, and the SV column was reinserted into the collection tube. The mixture was then centrifuged again at 15 000 rpm for 1 min to remove residual wash buffer. The SV column was placed into a fresh centrifuge tube. Finally, 200 µl of Buffer AE was added into the tube and incubated for 1 min at room temperature before being centrifuged at full speed for 1 min. Nanodrop spectrophotometers were then used to measure DNA concentration. A sample absorbance ratio of between 1.8 and 2.0 at 260/280 nm wavelengths respectively was considered "pure DNA" (Green and Sambrook, 2012). Storage of the extracted DNA was at -20^{o}C.

3.9 Multiplex PCR for the detection of K1 and K2 serotypes

The detection of K1 and K2 serotypes of *K. pneumoniae* isolates was determined using the multiplex PCR method. The primers employed in this study were chosen from previous studies as listed in Table 3.1, and they were synthesized by Apical Scientific (Malaysia). This includes a primer pair targeting a conserved region of *K. pneumoniae* which served as an internal positive control. The extracted DNA and fragments from *magA*, *K2A* and 16s rRNA genes were amplified using a BioRadMyCycler™ Thermal Cycler (BioRad, USA). PCR amplification was performed by thoroughly mixing the components listed in Table 3.2 in a reaction mixture with a final volume of 15 uL.

The PCR conditions for amplification were adapted from Al-Jailawi (2014) with a slight modifications for optimization according to Elnifro et al., (2000), as described in Table 3.3. PCR gradient was set at +/-5 from the annealing temperature of 60°C while keeping the denaturation and elongation constant. The temperature that gave the best band was retained as the annealing temperature. Subsequently, the denaturation temperature was optimized by keeping the annealing, elongation and number of cycles constant. Next, variable extension time were applied to the protocol to find the fastest and the best band observed. Finally, the number of cycles were optimized using the best annealing, denaturation and extension temperature obtained earlier.

Table 3.1: Primers used for the identification of K1 and K2 serotypes

Gene		Primer	Product size (bp)	Reference
magA	F	5'- GGT GCT CTT TAC ATC ATT GC- 3'	1283	Fang et al. (2004)
	R	5'- GCA ATG GCC ATT TGC GTT AG- 3'		
K2A	F	5'- CAA CCA TGG TGG TCG ATT AG- 3'	543	Turton et al. (2008)
	R	5'- TGG TAG CCA TAT CCC TTT GG- 3'		
16s rRNA	F	5'- ATT TGA AGA GGT TGC AAA CGA T- 3'	130	Turton et al. (2010)
	R	5'- TTC ACT CTG AAG TTT TCT TGT GTT C- 3'		

Table 3.2: Multiplex PCR components for detecting K1 and K2 serotypes among *K. pneumoniae* (Adapted from Al-Jailawi, 2014 with slight modification)

Component	Volume
HS Bioline Mastermix	7.5 uL
magA – Forward primer	0.5 uL
magA – Reverse primer	0.5 uL
K2A – Forward primer	0.5 uL
K2A – Reverse primer	0.5 uL
16s rRNA – Forward primer	0.5 uL
16s rRNA – Reverse primer	0.5 uL
Nucleus-free water	3 uL
DNA sample	1.5 uL
Final volume	15 uL

Table 3.3: Multiplex PCR conditions for detecting K1 and K2 serotypes among *K. pneumoniae*, (Adapted from Al-Jailawi, 2014 with slight modification)

Stage	Cycling parameter
Initial denaturation	95°C for 60 seconds
Denaturation	95°C for 15 seconds
Annealing	58°C for 15 seconds
Elongation	72°C for 70 seconds
Repeat cycle (2 to 4)	30x
Final extension	72°C for 300 seconds

3.10 Electrophoresis and identification

Following amplification, 1.7% agarose gel was used to analyse amplified products. Five microliters of the amplified PCR products were electrophoresed in 80V for 60 minutes using GelRedTM Nucleic Acid Gel Stain. 100 bp DNA ladder was used as a marker to compare the molecular weight of the amplified PCR products. The amplicon of the PCR product was then visualized using the UV transilluminator GelDoc (1000 System. Bio-Rad, USA). PCR products with a size of 1283 bp (*magA*) and 543 bp (*K2A*) were regarded as K1 and K2 capsular serotypes respectively.

3.11 Multilocus Sequence Typing (MLST) and sequencing

Eleven isolates were included for MLST. This set of isolates with diverse origins was intended to assess the discrimination of MLST among isolates. Isolates that were included for MLST were those that were positive for ESBL and K1 or K2 capsular serotypes. MLST was performed based on an existing scheme of *K. pneumoniae* targeting seven loci, including *gapA, infB, mdh, pgi, phoE, rpoB* and *tonB,* as described in Table 3.4 (Diancourt et al., 2005). PCR amplification was performed using a BioRadMyCycler™ Thermal Cycler (BioRad, USA) in a 50-uL reaction mixture consisting of the components listed in Table 3.5. Every sample was individually tested for the seven housekeeping genes in separate vials (one vial for only one primer pair from one gene). Cycling conditions were as described in Table 3.6. From the 50 uL PCR products, 5 uL was used for gel electrophoresis and 45 uL for DNA sequencing.

Sequences of the seven housekeeping genes were obtained from our isolates through 1st Base Sequencing at a commercial facility (Apical Scientific, Malaysia). Subsequently, DNA alignment was carried out using Molecular Evolutionary Genetic Analysis version 11 (MEGA11) for all the seven gene loci sequences according to 10 reference sequences available on GenBank, National Center for Biotechnology Information (NCBI). The allele number for the specific aligned sequences was obtained from the Institute Pasteur MLST Databases (https://bigsdb.pasteur.fr/). Isolates were assigned according to their sequence types (STs) from the allele combination in all seven loci.

Table 3.4: Housekeeping genes, their primers and conditions included for *K. pneumoniae* MLST analysis

Locus	Primer sequence	Size (bp)	Temperature (°C)
rpoB	F: GGC GAA ATG GCW GAG AAC CA R: GAG TCT TCG AAG TTG TAA CC	501	50
mdh	F: CCC AAC TCG CTT CAG GTT CAG R: CCG TTT TTC CCC AGC AGC AG	477	50
gapA	F: TGA AAT ATG ACT CCA CTC ACG G R: CTT CAG AAG CGG CTT TGA TGG CTT	450	60
pgi	F: GAG AAA AAC CTG CCT GTA CTG CTG GC R:CGC GCC ACG CTT TAT AGC GGT TAA T	432	50
tonB	F: CTT TAT ACC TCG GTA CAT CAG GTT R: ATT CGC CGG CTG RGC RGA GAG	414	45
infB	F: CTC GCT GCT GGA CTA TAT TCG R: CGC TTT CAG CTC AAG AAC TTC	318	50
phoE	F: ACC TAC CGC AAC ACC GAC TTC TTC GG R: TGA TCA GAA CTG GTA GGT GAT	420	50

Table 3.5: MLST components for *K. pneumoniae*

Component	Volume
TopTaq MasterMix	25 uL
CL stain	5 uL
Forward primer	1.6 uL
Reverse primer	1.6 uL
Nucleus-free water	10.8 uL
DNA sample	6 uL
Final volume	50 uL

(Diancourt et al., 2005)

Table 3.6: PCR conditions for MLST of *K. pneumoniae*

Stage	Cycling parameter
Initial denaturation	94°C for 120 seconds
Denaturation	94°C for 20 seconds
Annealing	50°C (45°C for *tonB* and 60°C for *gapA*) for 30 seconds
Elongation	72°C for 30 seconds
Repeat cycle (2 to 4)	34x
Final extension	72°C for 300 seconds

(Diancourt et al., 2005)

3.12 Phylogenetic analysis

Using the MEGA11 software, the sequences and STs obtained from MLST analysis were used to conduct phylogenetic analysis using the maximum likelihood method based on the Tamura-Nei model (1000 bootstrap replicates). Relevant reference sequences retrieved from MLST Global Database consisting of ID:723, ID:62, ID:847, ID: 968, ID: 3017, ID: 942, ID: 2998, ID:3900, ID:2731, ID: 14156 and ID:4799 were included to increase the validity of our findings.

3.13 Statistical analysis

Statistical analysis, including descriptive statistics of demographic data, antimicrobial susceptibility patterns, and the association between serotypes and ESBL production and other variables, was done using Statistical Package for

Social Sciences (SPSS) software (version 25.0). The tabulation of variables was analyzed using Pearson Chi-square test and independent t-test. Proportions were also compared using Chi-square test. Differences of $P< 0.05$ were considered statistically significant.

CHAPTER 4

RESULTS

4.1 *Klebsiella pneumoniae* general characteristics and identification

A total of 192 *Klebsiella pneumoniae* clinical isolates were included in this study. They are collected from Hospital Sultanah Aminah Johor Bahru (HSAJB) and Hospital Pengajar Universiti Putra Malaysia (HPUPM) for 13 months, from March 2021 to April 2022. One hundred fifty-six isolates were obtained from HSAJB, while 36 isolates were from HPUPM, accumulating to a total of 192 *K. pneumoniae* isolates, as mentioned in Table 4.1.

Table 4.1: Summary of *K. pneumoniae* isolates from HSAJB and HPUPM collected between March 2021 to April 2022

Hospital	Total isolates collected
Hospital Sultanah Aminah Johor Bahru (HSAJB)	156
Hospital Pengajar Universiti Putra Malaysia (HPUPM)	36
Total	**192**

4.1.1 Growth on MacConkey agar

All of our isolates displayed typical characteristics for *K. pneumoniae* when grown on MacConkey agar, where pink colour colonies were observed following overnight incubation at 37°C, which indicates *K. pneumoniae* growth (Figure 4.1). This is due to their ability to ferment lactose.

Figure 4.1: A representative isolate depicting the morphology of *K. pneumoniae* following overnight incubation on MacConkey agar

4.1.2 Biochemical identification of *Klebsiella pneumoniae*

Biochemical tests were carried out by utilizing the triple sugar iron (TSI), urease, oxidase, citrate utilization and motility tests. All of the isolates were reidentified as *K. pneumoniae*. In the triple sugar iron test, *K. pneumoniae* isolates presented with a yellow slant and yellow butt, which indicates acidity in both regions. As for the urease test, *K. pneumoniae* isolates showed changes in the colour of agar from yellow to pink, indicating a pH increment resulting from the conversion of urea to ammonia. In the oxidase test, negative test results were displayed by *K. pneumoniae* isolates. In contrast, isolates showed positive test results for the citrate utilization test, which proves their ability to utilize citrate as a carbon source. Finally, *K. pneumoniae* isolates showed no lateral diffusion or movement in the motility test since this pathogen is non-motile.

4.2 Demographic data

The demographic data of the whole collection included in this study are shown in Table 4.2. Among these, 109 (56.8%) were isolated from males and 83 (43.2%) from females. Our *K. pneumoniae* isolates were also isolated from various ethnicity; which majority of them were Malays (n = 132, 68.8%), followed by Chinese (n = 32, 16.7%), Indian (n = 19, 9.9%) and others (n = 9, 4.7%). On the other hand, the age group 50 – 59 years old (19.3%) contributed to the majority of the clinical isolates, followed by 60 – 69 years old (18.2%), 30 – 39 years old (14.6%), 40 – 49 years old (13.5%), 70 – 79 years old (13.0%), 20 – 29 years old (8.3%), 0 – 9 years old (7.3%), 10 – 19 years old (3.1%) and 80 – 89 years old (2.6%).

Table 4.2: Demographic characteristics of *K. pneumoniae* clinical isolates from HSAJB and HPUPM between March 2021 to April 2022

Demographic characteristics	Categories	Number of isolates	Percentage (%)
Gender	Male	109	56.8
	Female	83	43.2
Ethnicity	Malay	132	68.8
	Chinese	32	16.7
	Indian	19	9.9
	Others	9	4.7
Age group	0 – 9 years old	14	7.3
	10 – 19 years old	6	3.1
	20 – 29 years old	16	8.3
	30 – 39 years old	28	14.6
	40 – 49 years old	26	13.5
	50 – 59 years old	37	19.3
	60 – 69 years old	35	18.2
	70 – 79 years old	25	13.0
	80 – 89 years old	5	2.6

4.3 Prevalence of ESBL strains among *K. pneumoniae* clinical isolates

From the 156 isolates collected from HSAJB, 69 were ESBL producers, while the remaining 87 were non-ESBL producers. Meanwhile, from HPUPM, 18 isolates were ESBL producers and 18 were non-ESBL producers (Table 4.3). Of the 192 isolates, the majority of them were isolated from blood = 106 (55.2 %),

followed by urine = 29 (15.1%), sputum = 9 (4.7%), tissue = 15 (7.8%), BBA = 10 (5.2%), pus = 10 (5.2%) and others 13 = (6.8%) as shown in Table 4.4.

Table 4.3: Prevalence of ESBL strains among *K. pneumoniae* strains collected from HSAJB and HPUPM

Hospital	ESBL isolates	Non-ESBL isolates	Total
Hospital Sultanah Aminah Johor Bahru (HSAJB)	69	87	156
Hospital Pengajar Universiti Putra Malaysia (HPUPM)	18	18	36
Total	**87**	**105**	**192**

Table 4.4: Distribution of ESBL strains among varying clinical specimens

Type of specimens	Frequency (n = 192)	Percentage
Blood	106	55.2
Urine	29	15.1
Sputum	9	4.7
Tissue	15	7.8
BBA	10	5.2
Pus	10	5.2
Others*	13	6.8

**Others: bone, bile, wound swab, peritoneal fluid, tracheal aspirate, BAL from lung, nasopharyngeal aspirate, vaginal swab, body fluid*

4.4 Association between ESBL status and demography data of the patients

When the isolate collection was divided into ESBL and non-ESBL types, the demographic data were as described in Table 4.5. Chi-square analysis showed that sex was not significantly associated with ESBL expression (P= .684). Similarly, no significant association was observed between ethnicity and ESBL status (Fisher-Freeman-Halton Exact Test) (P= .550). On the other hand, statistically significant associations were reported between ESBL status and age group (P= .023) as well as sample type (P= .045) (Fisher-Freeman-Halton Exact Test).

Table 4.5: Baseline characteristics of *K. pneumoniae* isolates involved in this study

	ESBL n = 87	Non – ESBL n = 105	Percentage ESBL (%)	P-value
Sex				
Female	39	44	47.0	.684
Male	48	61	44.0	
Ethnicity				
Malay	60	72	45.5	.550
Chinese	16	16	50.0	
Indian	9	10	47.4	
Others	2	7	22.2	
Age group				
0 – 9 years old	12	2	85.7	.016
10 – 19 years old	4	2	66.7	
20 – 29 years old	7	9	43.8	
30 – 39 years old	11	17	39.3	
40 – 49 years old	6	20	23.1	
50 – 59 years old	19	18	51.4	
60 – 69 years old	17	18	48.6	
70 – 79 years old	10	15	40.0	
80 – 89 years old	1	4	20.0	
Sample type				
Blood	43	63	40.6	.045
Urine	13	16	44.8	
Sputum	6	3	66.7	
Tissue	10	5	66.7	
BBA	8	2	80.0	
Pus	4	6	40.0	
Others*	3	10	23.1	

4.5 Antimicrobial Susceptibility Test

Antibiotic susceptibility testing was performed using the disk diffusion method to classify *K. pneumoniae* isolates into ESBL and non-ESBL. For ESBL screening, four antibiotics were employed which include aztreonam (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg) and cefotaxime (30 µg). The results were as tabulated in Table 4.6. The results were interpreted based on the guideline set by CLSI,

2020. Isolates that showed resistance to any of these four antimicrobials were suspected to be ESBL producers and were therefore subjected to the confirmatory combination disc test (CDT) using ceftazidime/clavulanate (30 µg /10 µg) and cefotaxime/clavulanate (30 µg /10 µg). They were confirmed to be ESBL producers if the diameter of zone inhibition is ≥ 5 mm bigger with clavulanate than without (CLSI, 2020).

Table 4.6: Distribution of antibiotic susceptibility test among *K. pneumoniae* isolates against aztreonam, ceftriaxone, ceftazidime and cefotaxime

Antibiotics	Susceptible	Intermediate	Resistant
Aztreonam (30 µg)	100	0	92
Ceftriaxone (30 µg)	95	3	94
Ceftazidime (30 µg)	81	12	99
Cefotaxime (30 µg)	88	5	99

4.5.1 The association between ESBL status and antibiotic susceptibility pattern

Table 4.7 shows that there was a higher percentage of ESBL-producing *K. pneumoniae* isolates that displayed resistance against aztreonam, ceftriaxone, ceftazidime and cefotaxime compared to non-ESBL-producing *K. pneumoniae* ($P < 0.001$).

Table 4.7: Difference in antibiotic susceptibility pattern among ESBL and non-ESBL *K. pneumoniae* isolates

Antimicrobial	ESBL status	Susceptible	Intermediate	Resistance	Percentage resistant (%)	P-value
Aztreonam	ESBL	5 (2.6%)	0 (0%)	82 (42.7%)	94.25	<.001
	Non-ESBL	95 (49.5%)	0 (0%)	10 (5.2%)		
Ceftriaxone	ESBL	4 (2.1%)	0 (0%)	83 (43.2%)	95.40	<.001
	Non-ESBL	91 (47.4%)	3 (1.6%)	11 (5.7%)		
Ceftazidime	ESBL	1 (0.5%)	4 (2.1%)	82 (42.7%)	94.25	<.001
	Non-ESBL	80 (41.7%)	8 (4.2%)	17 (8.9%)		
Cefotaxime	ESBL	4 (2.1%)	0 (0%)	83 (43.2%)	95.40	<.001
	Non-ESBL	84 (43.8%)	5 (2.6%)	16 (8.3%)		

4.5.2 Mean zone of inhibition of *K. pneumoniae* isolates against ß-lactam antibiotics

Table 4.8 shows that the mean zone of inhibition of all *K. pneumoniae* isolates was lowest for ceftriaxone (17.63 ± 13.06 mm) and highest for cefotaxime-clavulanate (26.33 ± 7.39 mm).

Table 4.8: Mean zone of inhibition (± SD) of *K. pneumoniae* isolates

Antibiotics	Mean
Aztreonam	19.89 (11.93)
Ceftriaxone	17.63 (13.06)
Ceftazidime	17.91 (9.36)
Cefotaxime	18.76 (13.12)
Ceftazidime-Clavulanate	23.42 (6.86)
Cefotaxime-Clavulanate	26.33 (7.39)

This study also found that ESBL-producing isolates had statistically significantly lower mean zones of inhibition towards aztreonam (9.66 ± 7.41), ceftriaxone (6.00 ± 7.41), ceftazidime (10.46 ± 5.42), cefotaxime (7.01 ± 7.30), ceftazidime/clavulanate (22.74 ± 6.27) and cefotaxime/clavulanate (23.68 ± 6.82) compared to non-ESBL producing isolates with mean zones of inhibition towards aztreonam (28.37 ± 7.48), ceftriaxone (27.26 ± 7.83), ceftazidime (24.09 ± 7.20), cefotaxime (28.49 ± 7.83), ceftazidime/clavulanate (23.98 ± 7.30) and cefotaxime/clavulanate (28.53 ± 7.15) (Table 4.9). The mean zones of inhibition of aztreonam, ceftriaxone, ceftazidime and cefotaxime were all statistically significantly associated with ESBL-producing isolates (Independent samples t-test).

Table 4.9: The association between mean zones of clearance of aztreonam, ceftriaxone, ceftazidime and cefotaxime and ESBL status among *K. pneumoniae* clinical isolates isolated from HSAJB and HPUPM from 2021-2022

Antibiotics	Mean zones of clearance (SD)		P-value
	ESBL	Non-ESBL	
Aztreonam	9.66 (7.41)	28.37 (7.48)	<0.001
Ceftriaxone	6.00 (7.41)	27.26 (7.83)	<0.001
Ceftazidime	10.46 (5.42)	24.09 (7.20)	<0.001
Cefotaxime	7.01 (7.30)	28.49 (7.83)	<0.001
Ceftazidime-Clavulanate	22.74 (6.27)	23.98 (7.30)	0.212
Cefotaxime-Clavulanate	23.68 (6.82)	28.53 (7.15)	<0.001

4.6 Detection of K1 and K2 serotypes by multiplex PCR

Multiplex PCR was performed on all isolates to detect K1 and K2 serotypes through the presence of *magA* and *K2A* genes respectively (Figure 4.2). *magA* genes (1283 bp) were detected in 16/192 (8.3%) of the *K. pneumoniae* clinical isolates (Table 4.10). The prevalence of *magA* gene among ESBL and non-ESBL *K. pneumoniae* isolates were 4.6% (4/87) and 11.4% (12/105) respectively. On the other hand, 21/192 (10.9%) isolates contained the *K2A* gene (543 bp). The prevalence of *K2A* gene was 9.2% (8/87) and 12.4% (13/105) among ESBL and non-ESBL strains, respectively.

Table 4.10: Prevalence of K1 and K2 serotype-associated genes among *K. pneumoniae* isolates isolated from HSAJB and HPUPM from 2021-2022

Isolates strain	Number of isolates	Isolates with *magA* gene (K1 serotype) (%)	Isolates with *K2A* gene (K2 serotype) (%)
ESBL	87 (45.3%)	4 (2.1%)	8 (4.2%)
Non-ESBL	105 (54.7%)	12 (6.3%)	13 (6.8%)
Total	192 (100%)	16 (8.3%)	21 (10.9%)

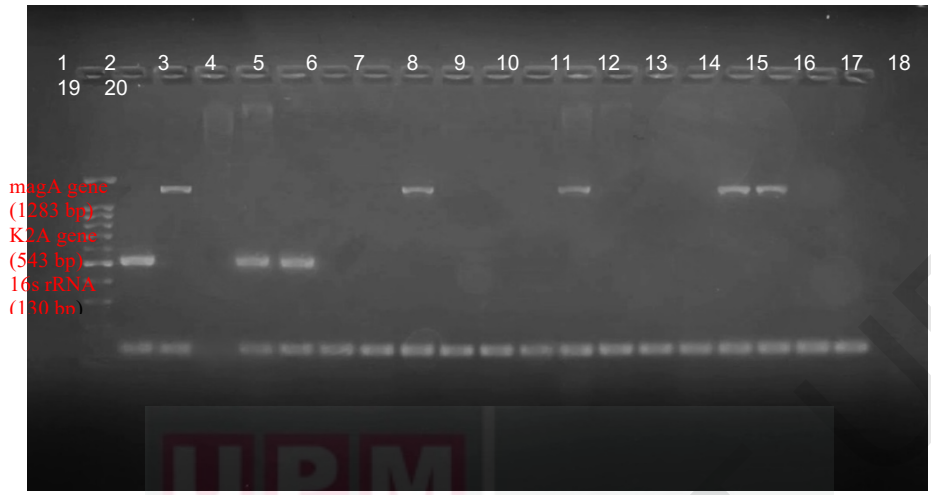

Figure 4.2: Agarose gel electrophoresis of multiplex PCR products of K1- and K2-positive *K. pneumoniae* isolates. Lane 1: Molecular size marker (100bp DNA ladder), 2: *K. pneumoniae* K2 positive control, 3: I K1 positive control, 4: *K. pneumoniae* negative control (nucleus-free water), 5: HSA114 (K2 positive), 6: HSA115 (K2 positive), 7: HSA116, 8: HSA117, 9: HSA118 (K1 positive), 10: HSA119, 11: HSA120, 12: HSA122, 13: HSA123 (K1 positive), 14: HSA124, 15: HSA125, 16: HSA126, 17: HSA127 (K1 positive), 18: HSA128 (K1 positive), 19: HSA129, 20: HSA130

4.7 String test

As tabulated in Table 4.11, string test revealed that 54 out of 192 isolates (28.1%) tested positive and showed hypermucoviscosity phenotype. Among these, 50% (n = 27) of the isolates were K1 (n = 13) and K2 (n = 14) capsular serotypes, respectively. The remaining 50% of the isolates that tested positive for the string test were non-K1 or K2 serotypes. Meanwhile, among our 87 ESBL-producing isolates, only 19.5% (n = 17) displayed positive string test. A higher prevalence which is 35.2% positive string test, was observed among the non-ESBL isolates. A Chi-square test was performed, and it revealed a significant association between capsular serotypes and hypermucoviscosity ($P < .001$) as well as between ESBL status and hypermucoviscosity ($P = .023$).

Table 4.11: String test result for *K. pneumoniae* isolates

Characteristic	Categories	Total	String Test		P-value
			Positive	Negative	
Serotypes	K1	16	13 (81.3%)	3 (18.8%)	<.001
	K2	21	14 (66.7%)	7 (33.3%)	
	Non-typable	155	27 (17.4%)	128 (82.6%)	
ESBL status	ESBL	87	17 (19.5%)	70 (80.5%)	.023
	Non-ESBL	105	37 (35.2%)	68 (64.8%)	
		Total	**54 (28.1%)**	**138 (71.9%)**	

Figure 4.3: A representative isolate showing a positive result for string test

4.8 Multilocus Sequence Typing

A total of 12 isolates were confirmed through PCR and ESBL tests to possess ESBL and K1 or K2 serotypes. All of them were subjected to MLST analysis using seven housekeeping genes (*rpoB, tonB, phoE, pgi, mdh, gapA and infB*) and the results showed that they were all genetically diverse. Based on genetic variation in the seven housekeeping genes, seven different sequence types were yielded from the MLST analysis. ST23 (*n* = 4) was the common ST among these strains. Other strains belonged to ST86 (n = 2), ST14 (*n* = 1), ST65 (*n* = 2), ST628 (*n* = 1), ST657 (*n* = 1), ST792 (*n* = 1), as tabulated in Table 4.12.

Table 4.12: Allele number assigned at each gene loci of selected *K. pneumoniae* isolates and their resulting STs

Samples	Serotypes	*gapA*	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*	ST
HSA4	K1	2	1	1	1	9	4	12	23
HSA80	K1	2	1	1	1	9	4	12	23
HSA118	K1	2	1	1	1	9	4	12	23
HSA146	K1	2	1	1	1	9	4	12	23
HSA41	K2	2	7	1	26	1	4	4	792
HSA111	K2	2	1	2	1	10	4	4	657
HSA114	K2	9	4	2	1	1	1	27	86
HSA132	K2	1	6	1	1	1	1	1	14
UPM2	K2	2	60	11	1	4	8	24	628
UPM21	K2	2	1	2	1	10	4	13	65
UPM37	K2	2	1	2	1	10	4	13	65
UPM39	K2	9	4	2	1	1	1	27	86

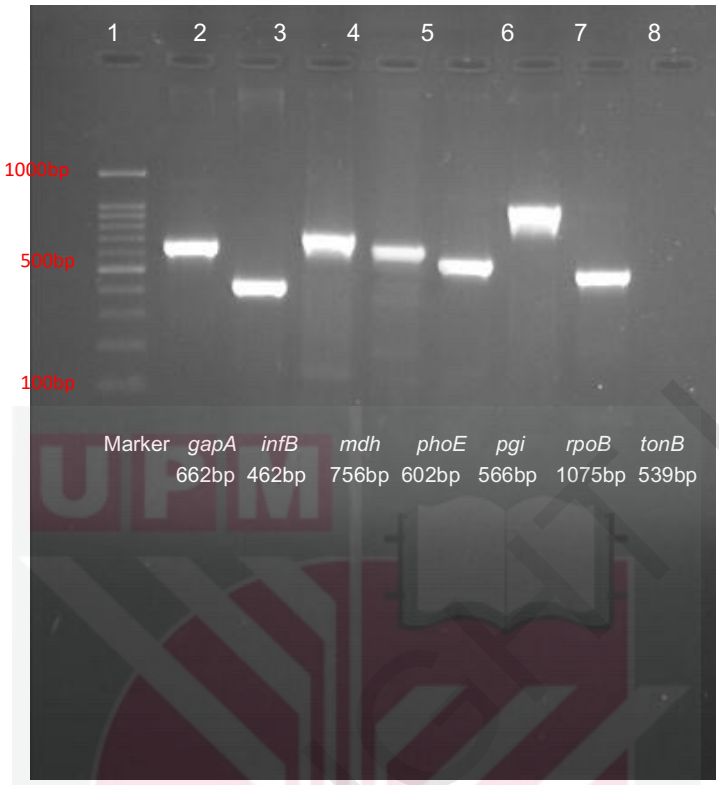

Figure 4.4: Agarose gel electrophoresis of PCR products of the seven housekeeping genes in selected isolate (HSA80). Lane 1: Molecular size marker (1000bp DNA ladder), 2: gapA gene (662 bp), 3: infB gene (462 bp), 4: mdh gene (756 bp), 5: phoE gene (602 bp), 6: pgi gene (566bp), 7: rpoB gene (1075bp), 8: tonB gene (539bp)

4.9 Phylogenetic analysis

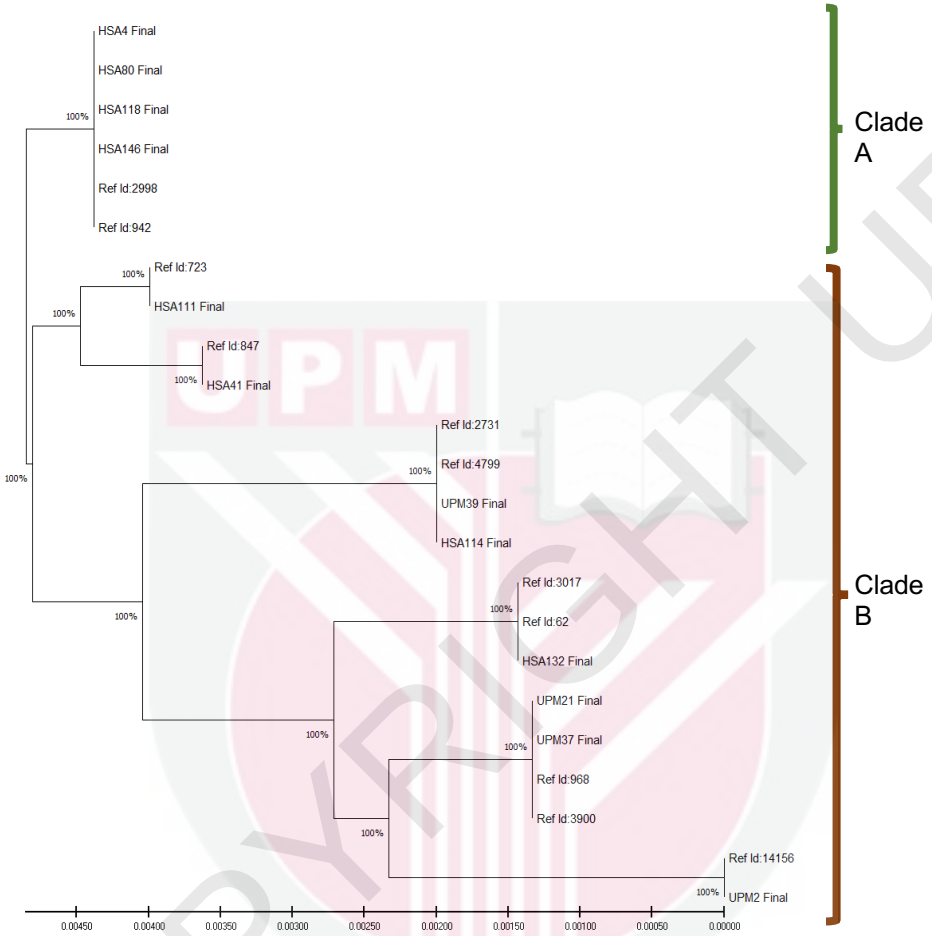

Figure 4.5: Phylogenetic tree generated based on the aligned nucleotide sequence from the MLST analysis of 12 *Klebsiella pneumoniae* clinical isolates

From the 12 selected *K. pneumoniae* isolates included for MLST, the nucleotide sequences of the seven housekeeping genes from the MLST were aligned in a specific manner and were subjected to phylogenetic analysis. Eleven reference sequences obtained from the MLST database were also included in the run (labelled with Ref followed by identity number).

The phylogenetic tree was constructed using MEGA11 software, and the result revealed two distinct clusters, Cluster A and Cluster B, with 100% bootstrap values as all branching, as shown in Figure 4.5. Cluster A consisted of four isolates from our study (HSA4, HSA80, HSA118 and HSA146) with the addition of two reference isolates retrieved from the MLST global database (ID: 942 and ID: 2998). Interestingly, this cluster only comprises ST23 isolates. Meanwhile, cluster B was made up of the remaining eight isolates (HSA41, HSA111, HSA114, HSA132, UPM2, UPM21, UPM37, UPM39) with the addition of nine reference isolates retrieved from the MLST global database (ID62, ID723, ID847, ID968, ID2731, ID3017, ID3900, ID14156). The Bootstrap sampling was set to 1000 replicates to ensure high reliability of the result. Information including the specimen source, capsular serotype, ESBL status and sequence type of the twelve isolates in Cluster A and B, were tabulated in Table 4.13 and Table 4.14 respectively.

Table 4.13: Characteristics of isolates belonging to Cluster A in this study

Samples	Source	Serotype	ESBL status	ST
HSA4	Sputum	K1	ESBL	23
HSA80	Blood	K1	ESBL	23
HSA118	Tissue	K1	ESBL	23
HSA146	Blood	K1	ESBL	23
Ref942	Blood	K1	ND	23
Ref2998	Tissue	K1	ESBL	23

Table 4.14: Characteristics of isolates belonging to Cluster B in this study

Samples	Source	Serotype	ESBL status	ST
HSA111	BBA	K2	ESBL	657
Ref723	ND	ND	ND	657
HSA41	Urine	K2	ESBL	792
Ref847	Urine	ND	ND	792
HSA114	Pus	K2	ESBL	86
UPM39	Urine	K2	ESBL	86
Ref2731	CSF	K2	ESBL	86
Ref4799	Bronchial	K2	Non-ESBL	86
HSA132	Blood	K2	ESBL	14
Ref62	Tissue	K2	ESBL	14
Ref3017	Anal swab	K2	ESBL	14
UPM21	Sputum	K2	ESBL	65
UPM37	Others	K2	ESBL	65
Ref968	Pus	K2	ND	65
Ref3900	Tissue	K2	ESBL	65
UPM2	Sputum	K2	ESBL	628
Ref14156	Blood	ND	ND	628

CHAPTER 5

DISCUSSION

5.1 Sociodemographic characteristics of *Klebsiella pneumoniae* isolates

This study found that ESBL-producing strains have higher prevalence rates in males compared with females (56.8% vs 43.2%). This finding is similar to a study by Nirwati et al. (2019), who collected 167 *K. pneumoniae* samples from a hospital in Indonesia and reported that *K. pneumoniae* infection among males was higher compared to females (64.07% vs 35.93%). Another study conducted by Akter et al. (2013) also revealed that male patients were at greater risk of contracting *Klebsiella* infection than females. The association between gender and the incidence of *K. pneumoniae* infection might be attributed to poor lifestyle, which includes smoking and consuming alcohol (Osagie et al., 2017). Even so, chi-square test showed that sex was not significantly associated with ESBL production among isolates in the current study (P= 0.684).

On the other hand, there is a statistically significant association between age group and ESBL producer among our study isolates (P= 0.016). The prevalence of ESBL producers was the highest in the 50 - 59 age group, accounting for 19.3% of the total isolates, followed by 60 – 69 years old (18.2%). The high prevalence of colonization incidence in older people (50 and above) might be attributed to various factors, including malnutrition, underlying chronic diseases, prolonged hospital stays, and environmental factors (Chen et al., 2022). Identifying the prevalence of ESBL is crucial for countries to monitor antibiotic resistance changes. These findings may provide important data on the epidemiology of ESBL and may help physicians to select the most appropriate regimen to treat infections caused by ESBL-producing *K. pneumoniae*.

5.2 Distribution of *Klebsiella pneumoniae* among varying clinical specimens

The highest number of *K. pneumoniae* isolates were isolated from blood samples (55.2%) which means these patients were diagnosed with bacteraemia. This is not new as the report on the 20-year SENTRY program published by Diekema et al., (2019) stated that *K. pneumoniae* is among the most common pathogen (6.6% – 9.9%) that causes bloodstream infections (BSIs). The findings from this study contrast with the report published by the National Surveillance of Antimicrobial Resistance, Malaysia, which reported that most of the *K. pneumoniae* isolated in 2020 and 2019 were from sputum (23% and 26% respectively). Another publication by Zhang et al., 2022 in Sichuan, China also reported that sputum and broncho-alveolar lavage make up the majority of *K. pneumoniae* isolated from 2017 to 2020 (48.7%, 56.4%, 49.2% and 43.7%).

Similar results were reported by a study conducted in a tertiary hospital in Hangzhou, China from 2006-2020 (Lin et al., 2022), where 53.77% of their *K. pneumoniae* strains were mostly isolated from sputum.

Whereas among the 87 ESBL producers in this study, majority of them were isolated from blood (n = 43, 49.43%), followed by urine (n = 13, 14.94%), tissue (n = 10, 11.49%), BBA (n = 8, 9.20%), sputum (n = 6, 6.90%), pus (n = 4, 4.60%) and others (n = 3, 3.45%). Further Fisher-Freeman-Halton exact test revealed that ESBL phenotype in *K. pneumoniae* was significantly associated with sample type (p-value = 0.0.45). The difference between prevalence rates among specimens depends on a range of factors such as patient group, geographic locality and many other factors.

5.3 Prevalence and characteristics of ESBL strain

In the current study, ESBL test revealed that 87 of the isolates (45.3%) were ESBL producers. The prevalence of ESBL strain among *K. pneumoniae* clinical isolates involved in this study was considerably high compared to a similar study by Mobasseri et al., (2020) (27.8%) involving *K. pneumoniae* isolates isolated from a tertiary hospital in Malaysia in 2014 (45.3% vs 27.8%). This finding should be a major concern as this strain is often associated with multidrug-resistant, therefore limiting the options of antibiotics. The variation in ESBL production might be attributed to different geographical locations, types of samples and antibiotics used to treat infections caused by these pathogens.

Susceptibility patterns may serve as a guideline for clinicians to determine the appropriate antibiotic for therapy. Antibiotic susceptibility test observed in this study confirms that the ESBL-producing *K. pneumoniae* study isolates showed significantly higher resistance to aztreonam, ceftriaxone, ceftazidime and cefotaxime compared to non-ESBL-producing isolates ($P< 0.001$). Based on the current findings, ESBL-producing study isolates also have a resistance of more than 90% towards all antibiotics tested in this study (ceftriaxone = 95.40%, cefotaxime = 95.40%, aztreonam = 94.25%, and ceftazidime = 94.25%). Chi-square test revealed that there was a significant association between ESBL status and resistance against the antibiotics employed in this study ($P< 0.001$). The interpretative criteria used were based on CLSI M100-ED31 (2021), where for aztreonam (Resistant ≤17 mm; Intermediate 18-20 mm; Susceptible ≥ 21 mm) and ceftriaxone (Resistant ≤19 mm; Intermediate 20-22 mm; Susceptible ≥ 23 mm). These results are comparable to those reported by Gharrah et al., 2017 and Shin and Ko, 2014 where ESBL-producing isolates exhibited significantly greater resistance ($P< 0.0001$ and $P< 0.05$, respectively) to most β-lactams compared to non-ESBL-producing isolates.

5.4 Prevalence of K1 and K2 serotypes among *Klebsiella pneumoniae* isolates

Other virulence factors commonly associated with *K. pneumoniae* include capsules, fimbriae, siderophores and lipopolysaccharides (Paczosa & Mecsas, 2016). Of these, capsules have long been viewed as the main and most important virulence factors (Huang et al., 2022). Although 79 capsular serotypes have been identified thus far, K1 and K2 recorded the highest prevalence in many countries, especially Southeast Asia. They were also commonly associated with liver abscesses, bacteraemia and endophthalmitis (Fung, 2002, Tsay et al., 2002, Lin et al., 2004; Wang et al., 2019).

Screening for the K1- and K2-serotype associated genes to assess the prevalence of K1 and K2 capsular serotypes of *K. pneumoniae* isolates was carried out using multiplex PCR. The amplification products were subjected to identification according to their sizes through electrophoresis in nucleic acid-stained agarose gels. Analysis of multiplex PCR confirmed that all isolates were *K. pneumoniae* by the presence of 16s rRNA band. The amplification of 16S rRNA was a control for bacterial identification as they are highly species-specific (Song et al., 2003). These findings were also confirmed by a study by Turton et al. (2010). All *K. pneumoniae* that has been previously identified biochemically gave a clear band of 130 bp in molecular size (Turton et al., 2010).

In this study, *K. pneumoniae* possessing capsular serotype K1 was confirmed through the amplification of *magA* gene using a primer pair *magA*-F and *magA*-R. Table 4.10 revealed that among our study isolates, 16 (8.3%) were positive for *magA* gene. Therefore, they were diagnosed with K1 serotype. Studies by Struve et al. (2005) and Yeh et al. (2006) involving 495 and 134 isolates respectively show that *magA* was located in the gene cluster of capsular serotype K1 of *K. pneumoniae*. More importantly, all non-K1 strains in both studies were *magA* negative.

On the other hand, *K. pneumoniae* possessing capsular serotype K2 was confirmed through the amplification of *K2A* gene using a primer pair *K2A*-F and *K2A*-R. 21 (10.9%) isolates were detected with *K2A* fragment gene, referring them to K2 serotype. Similar to the *magA* in the gene cluster of *K. pneumoniae* with capsular serotype K1, *K2A* is restricted to the capsular serotype K2' *cps* gene cluster.

Of the two serotypes sought, K2 (n= 21) serotype recorded a higher prevalence compared to K1 (n= 16) serotype. This is in concordance with the finding reported in a study from south India by Remya et al. in 2018, where 2.16% of the total isolates possessed K2 serotypes while only 0.27% possessed K1 serotypes. However, our findings disagreed with the findings of studies conducted in several Asian countries, where K1 is said to be the main serotype,

followed by K2 (Bilal et al., 2014). In a study from East China by Qu et al., K1 and K2 serotypes were reported in 68.9% and 20% of the total isolates. Similarly, in Taiwan, K1 is the most predominant serotype (63.4%), followed by K2 serotype (14.2%) (Fung, 2002). However, this might be attributed to the inclusion criteria of the study, which includes only liver abscess that is commonly known to be predominantly caused by strains with K1 capsular serotype.

5.5 Hypermucoviscosity as hypervirulent determinant

Strains possessing K1 or K2 capsular serotypes and ESBL phenotypes were commonly associated with hypervirulent phenotypes due to the extent of clinical manifestations of infection. Traditionally, string test was used as an indicative test to identify hypervirulent (hypermucoviscous) strains of *K. pneumoniae*. The result of string test is interpreted based on the length of the viscous string extended from the colony, where if the string formed is at least 5mm, then the isolate is said to be positive for hypermucoviscosity (Fang et al., 2004). However, present study and several other studies have suggested that this might not be the case. Furthermore, this test was not performed routinely in clinical laboratories as it remains unclear if all hvKP strains express hypermucoviscosity (Shon et al., 2013).

Of the 192 isolates included in our study, 54 (28.1%) tested positive for string test despite their capsular serotypes and ESBL status. A high prevalence of 81.3% (n = 13/16) and 66.7% (n = 14/21) of isolates belonging to the K1 and K2 serotypes respectively, tested positive for string test. However, 17.4% of isolates with non-K1 or K2 serotypes also tested positive. This finding suggests that hypermucoviscosity should not be solely restricted to K1 or K2 serotypes, although they are most commonly associated with hypervirulent infection.

A similar finding was reported by Luo et al. (2014), where 93.8% and 70% of isolates belonging to the K1 and K2 capsular serotype respectively, showed positive results for string test. Interestingly, a considerable proportion of non-K1 or K2 clinical isolates also tested positive for the test (46.7%). Therefore these findings suggest that hypermucoviscosity solely does not determine hypervirulence among isolates. The reliability of string test has been found increasingly disappointing due to its impreciseness in hypervirulent *K. pneumoniae* determination (Sanikhani et al., 2021). Genotype and clinical characteristics of the infections must also be taken into huge consideration when rendering an isolate hypervirulence (Luo et al., 2014).

5.6 MLST and phylogenetic analysis of hypervirulent *Klebsiella pneumoniae*

In the present study, four isolates with K1 serotype and eight isolates with K2 serotype were confirmed to be ESBL producers. Since K1 and K2 capsular serotypes were widely known to be among the hypervirulent serotypes, their co-existence with ESBL production raises major concerns. The simultaneous presence of two virulence factors together might result in hypervirulent strain with greater resistance that can cause serious infection and difficulty in infection management. Therefore, isolates positive for ESBL production and K1 or K2 capsular serotype were subjected to MLST to evaluate the diversity of genetic backgrounds.

Multilocus sequence typing (MLST) revealed that the predominant sequence type (ST) was ST23 (n= 4; 33.33%). All isolates belonging to this ST share the same capsular serotype, K1. They were clustered into a single clade that was obviously isolated from other isolates, indicating a high degree of clonality of the ST23 group (Cluster A). ST23 was revealed through a previous study to be predominant in the hypervirulent *K. pneumoniae* (hvKP) and K1 serotypes of *K. pneumoniae* (Siu et al., 2011; Qu et al., 2015; Guo et al., 2017). This is further supported by a more recent study conducted by Lin et al. (2020) which found that ST23 genotype was more commonly found among hvKP than classical *K. pnuemoniae* (cKP), indicating the correlation between hypervirulence and ST23. Serotype K1 *K. pneumoniae* has also been associated with ST23, which can cause a wide range of invasive infection cases (Struve et al., 2015).

In contrast, the second cluster (Cluster B) contains isolates with K2 capsular serotypes that were found to belong to several distinct, unrelated sequence types, consisting of ST14, ST65, ST86, ST628, ST657 and ST729. This is in accordance with the findings of previous studies which reported that K2 strains of *K. pneumoniae* appear to belong to a wider range of ST groups (Lin et al., 2014; Struve et al., 2015). Our findings revealed that ST65 (n=2; 16.7%) and ST86 (n=2; 16.7%) were the joint-highest prevalent ST found among K2 serotype. This is similar to the findings reported by several studies which identified ST14, ST65 and ST86 as the most prevalent sequence types found in K2 serotypes (Lin et al., 2014:;Harada et al., 2018).

Based on the analysis of seven housekeeping genes, our study showed that there was no genetic relatedness between ESBL producers of *K. pneumoniae* with K1 and K2 serotypes as they were placed in two different clusters. Our findings are in accordance with that reported in a study by Struve et al. (2015), which found that all the K1 serotypes belonged to a cluster that is totally separated from another cluster containing K2 serotypes of *K. pneumoniae* isolates. The study also emphasized the absence of genetic relatedness between the two serotypes. Interestingly, it was discovered that both K1 and K2 serotypes carry large plasmids expressing similar virulence-associated genes

such as *iro* (gene specific for salmochelin siderophore regulation), *rmpA* (gene specific for mucoid phenotype regulation) and *iuc* (gene specific for aerobactin siderophore regulation). Therefore, further analysis of these virulence-associated genes might unveil the genetic relevance between these two hypervirulent serotypes.

CHAPTER 6

CONCLUSION AND FUTURE RECOMMENDATIONS

The spread of hypervirulent *K. pneumoniae* is a huge concern and should be closely monitored. Infections caused by these strains are very difficult to treat and become even more challenging when inappropriate treatments are given. Since K1 and K2 capsular serotypes are most commonly associated with hypervirulent infections and complications, early detection may alert physicians and help them to confirm appropriate therapy for patients infected with this strain. Multiplex PCR has been shown to be a reliable and rapid method for screening K1 and K2 capsular serotypes *among K. pneumoniae* isolates. This study generally aimed to characterize a collection of clinically isolated *K. pneumoniae* isolates with respect to their serotypes, hypermucoviscosity, ESBL-producing ability and antibiotic susceptibility. In addition, sequence types were also included for isolates characterization, which was applied only to a collection of isolates that met the criteria for MLST in this study.

In this study, a total of 192 *K. pneumoniae* isolates were collected and analyzed. However, this study suffers from limitations where only two hospitals were included in this study which is HSAJB and HPUPM. Furthermore, there was inequal distribution of isolates collected between the two hospitals, in which only 36 isolates were collected from HPUPM compared to 156 from HSAJB. The huge difference in the number of samples collected from these two hospitals were due to the different number of patient populations that they serve. The present study showed that blood was the predominant site for *K. pneumoniae* infection. Furthermore, almost half of the isolates displayed susceptibility towards aztreonam (48.5%), ceftriaxone (49.5%), ceftazidime (51.5%), and cefotaxime (52%). Accordingly, the remaining half of the isolates showed resistance against the antibiotics mentioned. Since they are considered commonly used antibiotics, their use in the clinical setting as the drugs of choice for treating *K. pneumoniae* infections should be re-evaluated. To ensure effective management, resistance must be monitored when deciding on empirical treatments for *K. pneumoniae* infection.

Multiplex PCR was employed in this study to detect K1 and K2 serotypes, and it was successfully applied to our *K. pneumoniae* isolates collection. Based on the PCR amplification, all our isolates were detected to carry 16s rRNA gene, which serves as the control for *K. pneumoniae* identification. Only a small proportion of our isolates were categorized as K1 (n = 16, 8.2%) and K2 (n = 21, 10.8%) capsular serotypes respectively. The remaining 157 isolates were identified as non-typeable (NT) since no molecular target, either *magA* and *K2A* were amplified. K1 and K2 capsular serotypes are globally known to contribute to invasive infections.

In regard to ESBL-producing ability, 87/192 (45.3%) of our isolates were revealed to be ESBL producers. These isolates displayed a much higher percentage of resistance against antibiotics employed in this study compared to non-ESBL isolates. As a result of which, these common antibiotics might no longer be effective against infections caused by this strain and therefore, require the development and use of novel antibiotics. Even so, continuous monitoring and surveillance programs are still needed as bacteria are known to evolve constantly in response to changes.

The predominant ST among our selected isolates for MLST analysis was ST23 (n= 4). Interestingly, all of the ST23 detected belong to K1 capsular serotype. Meanwhile, among K2 serotype, the ST detected was ST14, ST65, ST86, ST628, ST657 and ST729. No novel STs were identified. Based on these findings, it can be concluded that our isolates that were selected for MLST analysis are well-established global clones, which confirms their widespread distribution. It was also revealed through this study that K1 and K2 serotypes of ESBL-producing *K. pneumoniae* carry no genetic relatedness since they were separated into two distinct clusters. However, these two serotypes might express similar virulence-associated genes, which requires further analysis to unveil the genetic association contributing to hypervirulence in these two serotypes.

That said, further in-depth investigation of MLST analysis involving a large sample size of *K. pneumoniae* clinical isolates might provide a greater understanding of the evolutionary origin and dissemination of hypervirulent strain. Coupled with genetic analysis on the common virulence-associated gene, it would help in the molecular typing of hypervirulent strains. It might also help determine the clonal relationships of strains and provides insight into their epidemiology.

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APPENDICES

Appendix A

Letters of Ethic Approval for Research Conduct

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(MEDICAL RESEARCH & ETHICS COMMITTEE)
KEMENTERIAN KESIHATAN MALAYSIA
MINISTRY OF HEALTH MALAYSIA
Kompleks Institut Kesihatan Negara (NIH)
No.1, Jalan Setia Murni U13/52,
Seksyen U13 Bandar Setia Alam,
40170 Shah Alam, Selangor. Tel.: +(6)03-33628888/ 33628205

Ref. : KKM/NIHSEC/P21-385(12)
Date : **29-March-2021**

DR. NURSHAHIRA BINTI SULAIMAN
UNIVERSITY PUTRA MALAYSIA (UPM)

Dear Dato'/ Dr/ Sir/ Madam,

LETTER OF ETHICAL APPROVAL:

NMRR-20-3087-57426 (IIR)
OCCURRENCE OF ESBL AND NON-ESBL-PRODUCING KLEBSIELLA PNEUMONIAE HARBOURING MAGA AND K2A GENES IN CLINICAL SAMPLES ISOLATED FROM HOSPITALS IN MALAYSIA

This letter is made in reference to the matter above.

2. The Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (MOH) has provided ethical approval for this study. Please take note that all records and data are to be kept strictly **CONFIDENTIAL** and can only be used for the purpose of this study. All precautions are be taken to maintain data confidentiality. Permission from the District Health Officer / Hospital Administrator/ Hospital Director and all relevant heads of departments /units where the study will be carried out must be obtained prior to the study. You are required to follow and comply with their decision and all other relevant regulations including the Access to the Biological and Benefit Sharing Act 2017.

3. The investigators and sites involved in this study are:

University Putra Malaysia (UPM)
Dr. Nurshahira Binti Sulaiman (Principal / Coordinating Investigator)
Dr. Mohd Nasir Mohd Desa
Dr. Siti Norbaya Binti Masri
Nurul Syazrah Binti Anuar

Hospital Pengajar Universiti Putra Malaysia
Dr. Siti Norbaya Binti Masri

Hospital Sultanah Aminah
Dr. Nur Afiza Binti Aziz

4. The following study documents have been received and reviewed with reference to the above study:

Documents received and reviewed with reference to the above study:

1. Cover letter to MREC (Version 2, dated 23-03-2021)
2. Declaration of Conflict of Interest (COI) (Version 1, dated 19-01-2021)
3. Protocol (Version 2, dated 24-03-2021)
4. English: Patient Information Sheet/ Informed Consent Form (Version 2, dated 23-03-2021)

…/2-

ETHICS COMMITTEE FOR RESEARCH INVOLVING HUMAN SUBJECTS
(JKEUPM)
UNIVERSITI PUTRA MALAYSIA

Research title	:	**Prevalence and Serotype Comparison of ESBL and Non-ESBL *Klebsiella pneumoniae* from Hospital Pengajar Universiti Putra Malaysia, Serdang and Hospital Sultanah Aminah, Johor Bahru.**
Study Site	:	**Hospital Pengajar Universiti Putra Malaysia, Serdang and Hospital Sultanah Aminah, Johor Bahru.**
JKEUPM Ref No.	:	**JKEUPM-2021-057**
Researcher	:	**Nurul Syazrah Binti Anuar**
Supervisor	:	**Dr. Nurshahira Binti Sulaiman**

Documents received and reviewed with reference to the above study:

1. Ethics Application Form, Version 2 dated 31/3/2021
2. Proposal (English), Version 2 dated 31/3/2021
3. Curriculum Vitae of:
    a. Dr. Nurshahira Binti Sulaiman
    b. Assoc. Prof. Dr. Mohd Nasir Bin Mohd Desa
    c. Assoc. Prof. Dr.Siti Norbaya Binti Masri
    d. Nurul Syazrah Binti Anuar

The University Research Ethics Committee, Universiti Putra Malaysia (JKEUPM) operates in accordance to the ICH-GCP Guidelines.
Decision by JKEUPM:

[X] Approved

[X] **Permission MUST BE OBTAINED from the respective hospitals/ institutions before conducting the research**

[ ] Disapproved

Please note that the approval is **VALID UNTIL 14 APRIL 2022**

Researchers should comply with the following:

I. Complete a Study Final Report upon study completion (Form 3.2).

II. Ethical approval is required in the case of amendments/ changes to the study documents/ study sites/ study team.

III. Applicable for Clinical Trial Studies and Clinical interventional Studies only: Progress Report has to be submitted to JKEUPM at every 6 months from the date of approval (Form 3.1). Report occurrences of all Serious Adverse Events (SAEs), Suspected Unexpected Serious Adverse Reaction (SUSARs) and Protocol Deviation/ Violation at all JKEUPM approved sites to JKEUPM. SAEs are to be reported within 15 calendar days from awareness of event by investigator. Initial report of SUSARs are to be reported as soon as possible but not later than

Approved at JKEUPM Meeting on 24 February 2021, attended by:

NAME	DESIGNATION	GENDER	TICK IF PRESENT
Prof. Dr. Zamberi Sekawi	Professor of Medical Microbiology, Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences.	Male	√
Prof. Dr. Johnson Stanslas	Professor of Pharmacology, Department of Medicine, Faculty of Medicine and Health Sciences.	Male	√
Prof. Dr. Rukman Awang Hamat	Professor of Clinical Microbiology, Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences.	Male	
Prof. Dr. Normala Ibrahim	Associate Professor of Psychiatry, Department of Psychiatry, Faculty of Medicine and Health Sciences.	Female	√
Assoc. Prof. Dr. Wan Aliaa Wan Sulaiman	Associate Professor of Neurology, Department of Medicine, Faculty of Medicine and Health Sciences.	Female	√
Assoc. Prof Dr. Shamala Paramasivam	Associate Professor of English Language, Department of English, Faculty of Modern Languages and Communication.	Female	√
Prof. Dr. Muhammad Najib Bin Mohamad Alwi (Independent member)	Associate Professor of Psychiatry and Psychiatric Consultant, Cyberjaya University College of Medical Sciences (CUCMS)	Male	√
Assoc. Prof. Dr. Nor Afiah Mohd. Zulkefli	Senior Lecturer of Public Health Medicine Specialist, Department of Community Health, Faculty of Medicine and Health Sciences	Female	√
Assoc. Prof. Dr. Hayati Kadir@Shahar	Senior Lecturer of Public Health Medicine Specialist, Department of Community Health, Faculty of Medicine and Health Sciences	Female	√
Assoc Prof. Dr. Rosliza Abdul Manaf	Associate Professor of Public Health Medicine Specialist, Department of Community Health, Faculty of Medicine and Health Sciences.	Female	√
Assoc. Prof. Dr. Nur Surayyah Madhubala Abdullah	Senior Lecturer of Moral and Citizenship Education, Department of Language and Humanities Education, Faculty of Educational Studies	Female	√
Assoc. Prof. Dr. Syamsiah Mashohor	Associate Professor of Computer Engineering, Department of Computer and Communication Systems, Faculty of Engineering	Female	√
Assoc. Prof. Dr Chew Boon How	Senior Lecture of Family Medicine Specialist, Department of Family Medicine, Faculty of Medicine and Health Sciences.	Male	√
Pn. Mimi Nora Binti Mansor (Independent Member/Layperson)	Retired Government Staff	Female	√

Appendix B

Gel Image of Serotyping and MLST of *K. pneumoniae*

Multiplex PCR

Image of Agarose Gel Electrophoresis of K1 and K2-capsular serotype associated gene together with the internal control for *K. pneumoniae,* 16s rRNA

MLST

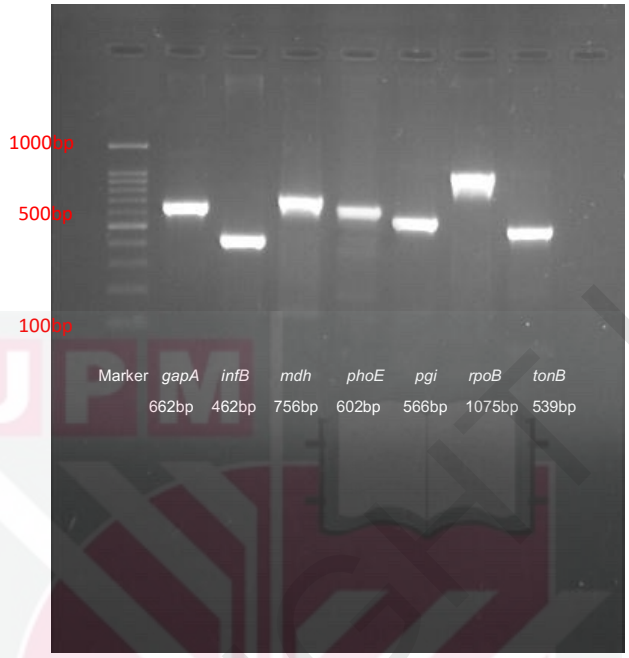

Image of Agarose Gel Electrophoresis of Seven Housekeeping Genes (*rpoB, tonB, phoE, pgi, mdh, gapA and inf)* employed in the MLST scheme for *K. pneumoniae*.

LIST OF PUBLICATION

Under Review:

Anuar, N. S., Sulaiman, N., Hazman, H., Desa, M. N. M., Masri, S. N., Saidi, H. I., & Aziz, N. A. (2022). Occurrence of K1 and K2 serotypes and genotypic characteristics of extended-spectrum beta-lactamases (ESBL)-producing *Klebsiella pneumoniae* isolated from selected hospitals in Malaysia. *Asian Pacific Journal of Tropical Medicine).*

Conference

Nurul Syazrah Anuar, Siti Norbaya Masri, Mohd Nasir Mohd Desa, Nur Afiza Aziz, Hazmin Hazman, Nurshahira Sulaiman. Occurrence of *Klebsiella pneumoniae* K1/K2 serotypes in ESBL and non-ESBL clinical isolates. 3rd International Anatomical and Biomedical Scientific Conferences (IABS) 2022.