PREVALENCE OF HYPERVIRULENT *Klebsiella pneumoniae* FROM PATIENT'S ADMITTED IN TERTIARY CENTERS AND THEIR CLINICAL PRESENTATION

DR AIMI BINTI KHAIRUDDIN

Dissertation Submitted in Partial Fulfilment of The Requirement for The Degree of Master of Pathology (Medical Microbiology)

SCHOOL OF MEDICAL SCIENCES
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ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim

In the Name of ALLAH , the Most Beneficent and the Most Merciful. I have completed my dissertation with the blessings granted by Him. I would like to give special thanks and appreciation to everyone who has been involved in this journey. My deepest gratitude goes to my supervisor, Associate Professor Dr. Siti Asma' Binti Hassan, and my co-supervisor, Professor Dr Chan Yean Yean, as well as my field supervisor, Dr Azura Binti Hussin, a clinical microbiologist from Hospital Raja Perempuan Zainab II (HRPZII), both co researchers, Dr Sharifah Aisyah Syed Hitam and Dr Rosnita Rashid from HRPZII and Hospital Tanah Merah(HTM) respectively. Thanks for their guidance, advice and encouragement throughout this dissertation period. A special thanks to Dr. Nik Zuraina binti Nik Mohd Noor and Nur Syafiqah binti Mohamad Nasir who had guided and assisted me in my laboratory work. I am also grateful that I received authorization from Director of Hospital Universiti Sains Malaysia to access the medical records for this research. Lastly, to my family, siblings and friends, thanks for supporting and helping in many ways. Special thanks to my dear husband, Mohd Adam Daniel b. Mat who have been my pillars of strengths, inspiration and motivation. Thank you to my children, Muhammad Fayyadh Mikael for being truly joyful. I am so grateful for all the help and support that has been given. May Allah rewards all the good that has been received with the best rewards and guide and bless all of us in this life and the hereafter.

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LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

Symbols/ Abbreviations	Meaning
-	Negative or subtraction
+	Positive or addition
±	Plus-minus
<	Less than
>	More than
≤	Less than or equal to
≥	More than or equal to
=	Equal to
×	Times or multiplication
/	Division or ‘or’
%	Percentage
°	Degree
µ	Micro
bp	Base pair
cKP	Classical *K. pneumoniae*
C	Celsius
CKD	Chronic Kidney Disease
COPD	Chronic obstructive pulmonary disease
DM	Diabetes mellitus
DNA	Deoxyribonuclease acid
et al.	

ESBL	Et alia (and others)
F	Extended spectrum β-lactamase
G	Forward
gapA	Gram
GNB	Housekeeping gene
HMKP	Gram negative bacilli
HvKp	Hypermucoviscous *K. pneumoniae*
HRPZ	Hypervirulent *K. pneumoniae*
HSIP	Hospital Raja Perempuan Zainab II
HTM	Hospital Sultan Ismail Petra
HUSM	Hospital Tanah Merah
iucA	Hospital Universiti Sains Malaysia
ICU	aerobactin
ID	Intensive care unit
IDSA	Identification
i.e.	Infectious Diseases Society of America
IPC	Id est (In other words)
JEPeM	Infection prevention and control
K1,K2	Jawatankuasa Etika Penyelidikan Manusia
L	Capsular serotyping K antigen
LIS	Liter
M	Laboratory information system
magA	Mili
mm	Mucoviscosity associated gene

M	Milimeter
MALDI-TOF-MS	Molar
MDR	Matrix assisted laser desorption ionization time-of-flight mass spectrometry
MDRO	Multidrug-resistant
n	Multidrug-resistant organism
NC	Number of samples
NMRR	Negative control
Peg-344	National Medical Research Register
PC	Putative transporter
PCR	Positive control
R	Polymerase chain reaction
RN	Resistant or reverse
rmpA,rmpA2	Registration number
rpm	Regulator of mucoid phenotype
S	Revolutions per minute
SPSS	Standard deviation
TBE	Statistical Package for Social Sciences
TSI	Tris/Borate/EDTA
U	Triple sugar iron
USM	Unit
UV	Universiti Sains Malaysia
XDR	Ultraviolet
	Extensive drug resistant

ABSTRACT

***Background*:** Hypervirulent variant of *Klebsiella pneumoniae* (hvKp) shows main characteristics of hypermucoviscosity and tendency to cause severe community-acquired infection. Patient infected with this strain may present with more severe illness can lead to fatality. Our study aimed to investigate its prevalence and patient clinical presentation associated with this infection.

***Methods*:** A cross-sectional study involving retrospective record review was done with a total of 180 isolates from various clinical specimens were collected from June 2020 till June 2021 in four major hospitals in Kelantan. All isolates were examined by string test for presumptive of hypermucoviscosity (HMKP) and the detection of their virulence genes (*rmpA, rmpA2, aerobactin, magA, peg-344, K1, K2*) were done by conventional PCR using published primers. HvKp strain is defined as *Klebsiella pneumonia* strain with presence of hypermucoviscocity and molecular detection of capsular serotyping K1 or K2. Patient's clinical informations were assessed from medical records and collected data was interpretated using SPSS software version 26.

***Results*:** One hundred and eighty *K. pneumoniae* isolates were obtained from various clinical specimens. String test were positive in 23.8% (n=43) and were identified as hypermucoviscous *K. pneumoniae* (HMKP). Capsular serotype K1 and K2 were detected in 11.1%(n=20) and 6.1%(n=11) respectively. Therefore, based on definition, prevalence

for hvKP was 9.4% (n=17). All virulence-associated genes were statistically significant associated with K1 (p<0.001). There was also significant association between *rmpA, rmpA2, aerobactin, peg-344* with K2 (p<0.05) except for *magA*. Majority of patient presented with respiratory infections.

***Conclusion*:** The prevalence of hvKp was found to be 9.4%, lower percentage as compared to others. There were significant association of K1 and K2 with various virulence genes. Respiratory infections were the most common clinical presentation among the hvKp group.

***Keywords*:** HvKP, String test, virulence genes K1,K2

ABSTRAK

Latar belakang: Varian hipervirulen *K. pneumoniae* (hvKP) menunjukkan ciri-ciri utama hipermukoviskositi dan kecenderungan untuk menyebabkan jangkitan teruk dalam komuniti. Pengenalpastian dan pembezaan strain ini daripada *K. pneumoniae* klasik boleh membantu doktor untuk menjangka tahap keterukan jangkitan dan merancang pengurusan. Data epidemiologi hvKp dan faktor virulensnya masih terhad di Malaysia, terutamanya di negeri Kelantan. Oleh itu, tujuan kajian ini adalah untuk menyiasat prevalens dan ciri-ciri klinikal pesakit yang berkaitan dengan jangkitan ini.

Kaedah kajian: Kajian keratan rentas yang melibatkan semakan rekod retrospektif telah dilakukan dengan sejumlah 180 isolasi, dikumpul dari Jun 2020 hingga Jun 2021 di empat hospital utama di Kelantan. Semua isolasi tersebut adalah daripada pelbagai sampel klinikal dan diperiksa dengan ujian "string test" dan pengesanan gen virulens (*rmpA, rmpA2, aerobactin, magA, peg-344,* K1, K2) dilakukan dengan PCR konvensional menggunakan primer yang sedia ada. Strain HvKp ditakrifkan sebagai strain *K. pneumoniae* dengan hipermukoviskositi dan pengesanan gen virulens K1 atau K2. Maklumat klinikal pesakit dinilai daripada rekod perubatan dan data yang dikumpul ditafsir menggunakan perisian SPSS versi 26.

Keputusan: Seratus lapan puluh (n=180) isolasi *K. pneumoniae* diperoleh daripada pelbagai spesimen klinikal. Antaranya, 43 (23.8%) positif untuk ujian 'string test" dan dikenal pasti sebagai *K. pneumoniae* hipermukoviskositi. Prevalens yang diperolehi untuk

HvKp ialah 9.4% (n=17). Semua gen yang berkaitan dengan virulens adalah signifikan secara statistik apabila dikaitkan dengan K1 ($p<0.001$). Terdapat juga perkaitan yang signifikan antara rmpA, rmpA2, aerobaktin, peg-344 dengan K2 ($p<0.05$) kecuali dengan magA. Majoriti pesakit datang dengan jangkitan pernafasan.

Kesimpulan: Prevalens HvKp didapati 9.4% iaitu lebih rendah daripada kajian lain. Terdapat perkaitan signifikan K1 dan K2 dengan pelbagai gen virulens. Kebanyakan jangkitan pernafasan diperhatikan dalam kumpulan hvKp sebagai ciri-ciri klinikal mereka.

Keywords**:** HvKp, String test, gen virulen K1,K2

CHAPTER 1 INTRODUCTION

1.1 Background of the study

A hypervirulent variant of *K. pneumoniae* (hvKP) shows main characteristics of hypermucoviscosity and tendency to cause severe community-acquired infection. This new variant often harbors the K1 or K2 capsular polysaccharide and several genes reported as severity determinants, including *rmpA* and *magA* (Ikeda *et al.*, 2018). This study will allow us to identify hypervirulent from classical *K. pneumoniae* with respect to patient background and microbiological characteristics. This study can contribute to gain understanding of the epidemiology of hvKp and its virulence factors. HvKp is difficult to treat despite the health of the host or its antimicrobial sensitivity. Challenges in the treatment of hvKp are to curtail its spread by the rapid initiation of therapy, detect occult metastases that may require source control, and to utilize appropriate site-specific antimicrobials. When hvKp strains were first recognized, they were largely antimicrobial susceptible. Unfortunately, multidrug resistant hypervirulent strains have emerged, creating a new challenge in combating this already dangerous pathogen (Choby *et al.*, 2020; Choby and Weiss, 2019). An increasing number of reports describe hvKp isolates that have acquired extended spectrum beta lactamase (ESBL) or carbapenemases or, conversely, extensive drug resistant-classic *K. pneumoniae* (XDR-cKP) strains that have acquired hvKp virulence determinants. For such isolates, the combination of virulence and antimicrobial resistance is highly problematic (Hvkpig, 2018).

1.2 Literature Review

K.pneumoniae is a gram-negative, lactose-fermenting, non-motile, aerobic rod-shaped bacterium, within the Enterobacterales family which commonly cause infections in hospital setting. Urinary tract infection, pneumonia, and bacteremia are the most common infections encountered, followed by liver abscess and other intraabdominal infections, wound infections, infections of intravascular and other invasive devices, meningitis and postneurosurgical infections. There are mainly two pathotypes that pose a threat to our health : hvKP and classical *K. pneumoniae* (Liu and Guo, 2019). Features that are highly suggestive of hvKP infection are its ability to infect healthy individuals of any age and the propensity of infected patients to present with multiple sites of infection and or develop subsequent metastatic spread. The hallmark clinical syndrome is a hepatic abscess in the absent of biliary tract disease (Russo and Marr, 2019). In one of study, healthy humans from community of Asian countries including Malaysia, the prevalence of *K. pneumoniae* colonic colonization ranged from 18.8% to 87.7%, while in Western countries are ranged from 5% to 35% (Marr and Russo, 2019). Despite the fact that HvKp colonization is requisite, it does not necessarily lead to subsequent infection. In microbiology laboratory, these strains grow in sticky colonies on agar plates and are identified by string test. A positive string test is indicated by an equal or more than 5mm viscous string from the colony on an agar plate when stretched by a standard bacteriological loop. The clinically invasive nature of some *K. pneumoniae* strains correlates with these microbiologic characteristics. Due to the characteristic hypermucoviscous aspect of the colonies produced by these hvKp, it was proposed that the polysaccharide capsule was the main virulence factor responsible for the hypervirulent phenotype (Catalán-Nájera *et al.*, 2017). A critical feature that contribute

to the hvKp phenotype is the ability to produce increased amount of capsular polysaccharide. This is mediated by *rmpA* and or *rmpA2* which are hvKp-specific factors located on the plasmid (Russo and Marr, 2019). *RmpA* was first described in1989 as a regulator of mucoid phenotype which encoded by virulence plasmid (Choby and Weiss, 2019). The *magA* gene, which encodes a polymerase involved in capsule synthesis, first emerged as one of the candidate virulence genes, but is now recognized as a K1 surrogate marker (Ikeda *et al.*, 2018). HvKp strains variably have the capability to produce four different siderophores that are secreted, bind to iron and reenter the bacterial cell through specific receptors. Molecular epidemiologic studies have shown that aerobactin, salmochelin, and yersiniabactin are more commonly present in hvKp strains than classical. Aerobactin accounts for increased siderophore production and is a major virulence determinant and new defining trait for hvKp based on genetic background (Zhang *et al.*, 2016).

1.3 History of hypervirulent *K. pneumoniae*

The first clinical report in 1986 published by Liu et al was recognized in Taiwan, reporting seven cases of invasive *K. pneumoniae* infection in community members who presented with hepatic abscess in the absence of biliary tract illness and septic endophthalmitis. Meningitis, pneumonia, and a prostatic abscess were among the symptoms experienced by several of the participants. *K. pneumoniae* strains that causing hepatic abscesses in Taiwanese patients were more likely to have a hypermucoviscous phenotype than noninvasive strains, reported by Fang et al in 2004. Hypermucoviscosity was characterised as the development of viscous strings equal to or greater than 5mm in length when a loop was used to stretch the colony on an agar plate, commonly known as

a positive string test. Hypervirulent strains were occasionally referred to as hypermucoviscous in the literature. However, due the variety of virulence factors and nonmucoid *K. pneumoniae* also observed make the term "hypervirulent *K. pneumoniae*" more appropriate.

1.4 Epidemiology of hypervirulent *K. pneumoniae*

Over the past three decades, the incidence of hvKp infection has increase in Asia including South Korea, Japan and China (Russo and Marr, 2019). Infection of hvKp initially begin with acquisition of and colonization with the organism (Marr and Russo, 2019). A study of healthy adults from Asian countries reported that putative hvKp strains comprised approximately 9.8% which isolated from intestinal *K. pneumoniae* (Marr and Russo, 2019). Majority of cases are reported to be acquired from the community, however infections developed in the healthcare settings are also increasing (Russo and Marr, 2019).

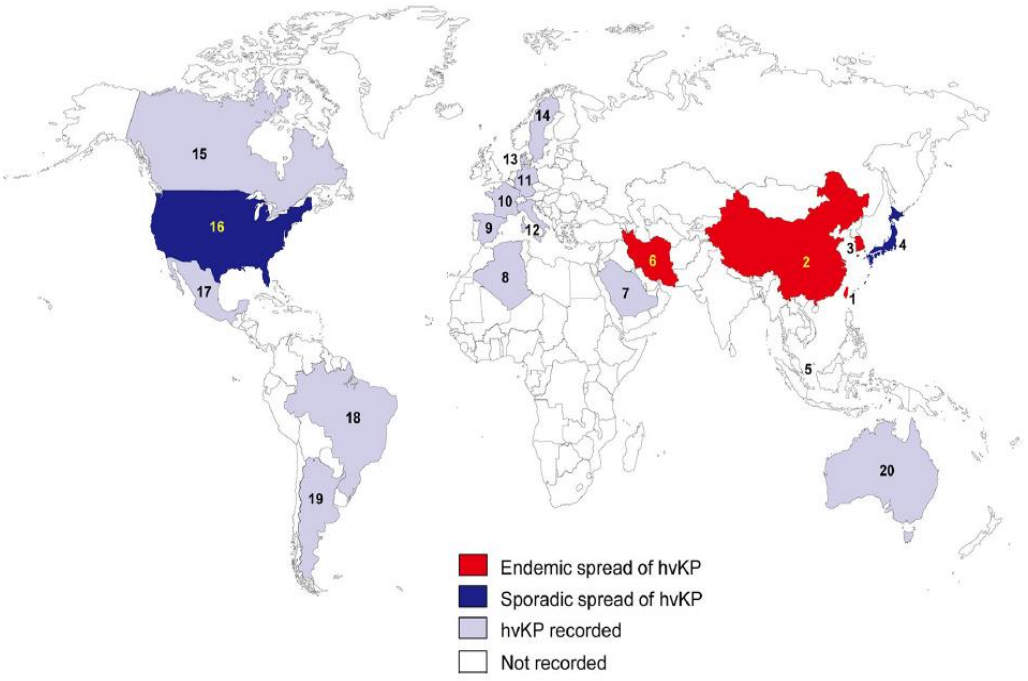


Figure 1.1 Prevalence assessment of *magA* gene and antimicrobial susceptibility of *K. pneumoniae* isolated from clinical specimens in Shahrekord, Iran. Iranian Journal of Micro. Source: Amraie, H., Shakib, P., Rouhi, S., Bakhshandeh, N., & Zamanzad, B. (2014).

1.5 Pathogenesis of hypervirulent *K. pneumoniae*

HvKp strains has increased virulence in a variety of infections. The hvKp-specific factor that mediate this phenotype include the regulators of the mucoid phenotype via increased capsule production (*rmpA* and *rmpA2*), capsular polysaccharides termed K antigens (K1 and K2, up through K78), *iucA* (aerobactin siderophore biosynthesis), *peg-344* (putative transporter), *magA* (mucoviscosity-associated gene) (Hvkpig, 2018). HvKp has emerged as an important pathogen capable of causing community-acquired in healthy individuals (Choby and Weiss, 2019). In most cases, the initial site of entry is undefined. This infection occurs in healthy individual for whom there is no overt disruption of these host barriers. Acquisition leading to colonization is probably prerequisite for subsequent

endogenous hvKp infection (Shon *et al.*, 2013). However, the time from acquisition to develop infection is unknown due to limited data from hvKp strain. The predominat colonization site appears to be the gastrointestinal tract followed by less frequent sites are oropharyngeal and skin (Shon *et al.*, 2013). Analysis from a study of Chinese adults who were residents of Taiwan, Hong Kong and China or living abroad in Japan, Thailand, Malaysia, Singapore and Vietnam had reported 9.8% were colonized with K1 or K2 serotype of *K. pneumoniae* respectively (Y. T. Lin *et al.*, 2012). In cases which patients presented with pneumoniae, there is possibility of prior oropharyngeal colonization followed by aspiration resulting in pneumoniae. HvKp reported to commonly associated with multiple sites of infection and/or metastatic spread. A "Trojan horse" mechanism has been postulated with neutrophils implicated as a possible vehicle in which the hvKp strains are able to survive within the neutrophils and resulted in disseminated infections (Russo and Marr, 2019). Most common disseminated infections include endophthalmitis or uveitis, pulmonary infections such as intraparenchymal disease or empyema and central nervous system including brain abscess, meningitis or epidural abscess (Choby and Weiss, 2019).

1.6 Risk factor for hvKp

HvKp infection is more common in the Asian Pacific Rim, although it can affect any ethnic groups. Patient with hvKp tend to be younger, otherwise healthy individuals but presented with severe disease compared to classical *K. pneumoniae* infection. Some studies discovered diabetes as a significant risk factor for acquiring a hvKp infection however there is no strong connection between this association and hvKp could vary by region or clinical presentation (Choby and Weiss, 2019). Uncontrolled diabetes mellitus

(DM) has been associated with an increase in disseminated infections. Men were more likely to be infected than women however Siu et al reviewed that there were demographic differences (Russo and Marr, 2019).

1.7 Basic laboratory identification methods

Basic laboratory identification of *K. pneumoniae* is based on the morphology, gram stain and biochemical tests. *K. pneumoniae* can be cultured on many types of media such as nutrient agar, Mac Conkey agar and blood agar and must be incubated at 35°C for 20 to 24 hours. This organism usually appears as mucoid colony with size ranging from 3-4mm in diameter on blood agar. On Mac Conkey agar, they will appear as lactose fermenter mucoid colonies. Biochemical tests such as triple iron sugar, motility, indole, citrate, urease and methyl red will further differentiate *K. pneumoniae* from other members of Enterobacterales (Farmer et al., 1985). Automated system such as Vitek2 Gram negative (GN) identification card (BioMerieux,Marcy Ietoile,France) and or MALDI-TOF (Bruker Daltonics,Bremen,Germany) may be required in difficult species identification.

1.8 Microbiology of hypervirulent *K. pneumoniae*

1.8.1 Phenotype-based method

A simple method known as the string test was used to detect hypermucoviscosity, which is defined as the formation of viscous strings of >5mm in length when a loop is used to stretch the colony on agar plate. Hypermucoviscosity phenotype is a known virulent

factor of *K. pneumoniae*. This method was proposed as possible clinical utility as routine microbiological surveillance for the hvKp (Hagiya *et al.*, 2014). However, the performance of string test only achieved 0.90 accuracy (Hvkpig, 2018).

Figure 1. 2. Lactose fermenter mucoid colonies on Mac Conkey agar and mucoid colony on blood agar.

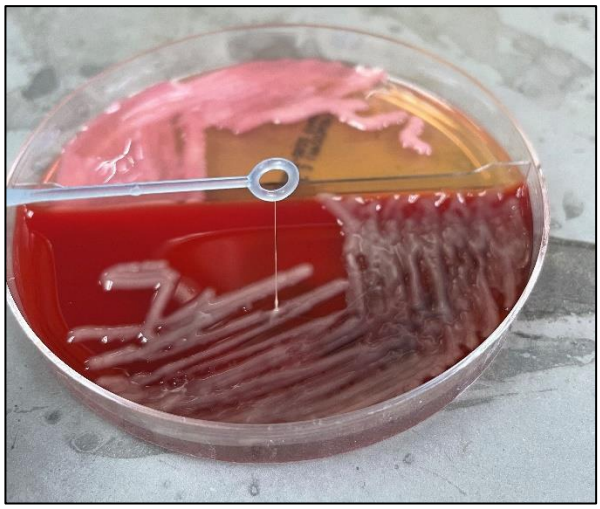


Figure 1. 3.Positive string test for the *K. pneumoniae* isolate. A positive string test was defined as a >5-mm viscous string from the colony, indicating the hypermucoviscous phenotype.

1.8.2 Molecular methods

Molecular methods are such as PCR assays for biomarker of virulence genes are widely used in studies and also surveillance. HvKp can be distinguished from classical *K. pneumoniae* strain by the detection of capsular serotyping and virulence genes. Capsule is a polysaccharide synthesized by all *K.pneumoniae* that acts as a protective layer which inhibit phagocytosis and also inhibit host inflammatory response induction. The most common capsule locus associated with hvKp is K1, followed by K2, K5 and K57 (Choby and Weiss, 2019). Virulence genes produced by hvKp include *iucA* (aerobactin siderophore biosynthesis), the plasmid-borne *rmpA* gene (*rmpA*; *rmpA2)*, and the chromosomal gene *rmpA* (*crmpA*) (regulators of the mucoid phenotype via increased capsule production), and *peg-344* (putative transporter)(Hvkpig, 2018). Molecular epidemiologic studies reported that common siderophores present in hvKp strains include aerobactin, salmochelin and yersiniabactin. Non-PCR based method, loop-mediated isothermal amplification (LAMP) is a rapid and easy to perform test was able to

distinguish hvKp from classical *K.pneumoniae* (cKp) at 65°C by detecting peg-344, was more sensitive than PCR assay.

Table 1. 1 Virulence factors of hvKp

Virulence factor	Genes	Molecular or cellular action	Proposed contribution for to direct pathogenesis
Regulator of mucoid phenotype	*rmpA, rmpA2*	Regulates capsule polysaccharide biosynthesis.	Inhibit phagocytosis and human defensin-mediated bactericidal activity.
Siderophore biosynthesis	*iucA, Iro, Ybt*	Aerobactin siderophore biosynthesis.	Siderophores bind to iron in the environment with extremely high affinity and are transported back into the cell for growth of bacterium.
Putative transporter	peg-344	Transporter of metabolites	It was hypothesized that it could contribute to selective nutrient transport within the pulmonary compartment that could enhance the local growth and/or enhance the extrapulmonary dissemination of hvKp (Bulger, 2017).
Mucoviscosity-associated gene	*magA*	Chromosomal gene encodes for a structural outer membrane protein involving biosynthesis, transfer and glycosylation of lipopolysaccharide (Amraie *et al.*, 2014)	Increased resistance to phagocytosis
Capsular polysaccharides (K antigen)	K1, K2, K5, K16, K20, K54, K57, KN1(Shon *et al.*, 2013)	K1 and K2 capsule types lack of mannose and rhamnose sugars which usually these sugars are recognized by macrophages (Choby and Weiss, 2019)	Prevent phagocytosis, increase survival and dissemination of hvKp

1.9 Disease caused by hvKp

The first recognition of hvKp in 1980 is from the pyogenic liver abscess which was reported from Taiwan. The increasing trend was observed which approximately 80% of all pyogenic liver abscess in Taiwan were caused by *K.pneumoniae*. Unlike other liver abscess, hvKp pyogenic liver abscess commonly occur in persons with normal biliary and hepatic function. HvKp pyogenic liver abscess often reported to be monomicrobial but non-hvKp pyogenic liver abscess commonly polymicrobial (Russo and Marr, 2019).

1.9.1 Respiratory infection

Lung infection related to hvKp was reported as the second most common after liver abscess (Wu *et al.*, 2017). A study from Taiwan analysed that hvKp has superseded *S. pneumoniae* as the most common pathogen for community-acquired pneumonia. Significantly patient with bacteremi community-acquired pneumonia caused by hvKp had higher respiratory failure, septic shock, bilateral lobar involvement and mortality (Y. T. Lin *et al.*, 2010). Although hvKp is more frequently associated with community-acquired disease, there have also been increasing reports of hypervirulent isolates causing healthcare-associated disease particularly pulmonary and ventilator-associated infections have been identified (Russo and Marr, 2019).

1.9.2 Endophthalmitis

Endogenous endophthalmitis occur as a complication in patients with hvKp bacteremia approximately 5%. It can occur later, up until 30 days after hvKp's initial presentation

and results in poor outcome despite intravitreal antibiotic treatment. Redness, painful ocular swelling and sudden blurred vision are typical presentation of endophthalmitis caused by hvKp. (Russo and Marr, 2019). Metastatic endophthalmitis was known to be associated with *K.pneumoniae* liver abscess, reported from Taiwan (Dehghani *et al.*, 2011).

1.9.3 Central Nervous System Infection

HvKp meningitis may be the primary infection or secondary to disseminated infection. There are no pathognomonic imaging findings for hvKp meningitis. However, one case from Japan reported that cord-like structures in the subarachnoid space was observed from the magnetic resonance images in a meningitis patient caused by hvKp (Takahashi *et al.*, 2015). Central nervous system is one of the common metastatic site which occur about one-third of patients who have disseminated infection (Choby and Weiss, 2019).

1.9.4 Musculoskeletal and soft tissue infection

HvKp can cause severe skin, soft tissue and bone infections including necrotizing fasciitis, neck and psoas abscesses and osteomyelitis (Choby et al., 2020). Less common site that has been associated to this strain including prostate, kidney and spleen (Choby and Weiss, 2019). Instead of being the result of a direct injury, osteomyelitis appears to be subsequent to haematogenous spread. However, no research has specifically linked the hypermucoviscous phenotype to osteomyelitis (Prokesch et al., 2016).

1.9.5 Genitourinary infection

Primary mechanism of urinary tract infection usually occurs via the ascending route, in which the fecal flora ascends through the urethra to infect the bladder and then reaching

the kidneys and systemic circulation. Study reported most of the genitourinary infections caused by hvKp resulting from hematogenous seeding from preceding bacteremia (Russo and Marr, 2019).

1.9.6 Bacteremia

HvKp bacteremia commonly occur as a complication from pyogenic liver abscess, although many other primary sites are possible. Patient infected with hvKp are likely to have positive blood culture even before the primary site of infection is identified (Russo and Marr, 2019). A study from Beijing reported that among 70 *K. pneumoniae* strains isolated from blood, about 31.4% were hvKP (J. Li and Ren, 2018).

1.10 The Rationale of the Study

A hypervirulent variant of *K. pneumoniae* shows main characteristics of hypermucoviscosity and tendency to cause severe community-acquired infection. This study can contribute to gain understanding of the epidemiology of hypervirulent *K.pneumoniae* and its virulence factors. It will also guide in the identification of hypervirulent from classical *K. pneumoniae* with respect to patient background and microbiological characteristics. Hypervirulent *K. pneumoniae* is difficult to treat despite the health of the host or its antimicrobial sensitivity. Challenges in the treatment of hvKp are to curtail its spread by the rapid initiation of therapy, detect occult metastases that may require source control, and to utilize appropriate site-specific antimicrobials (Choby *et al.*, 2020)

1.11 Objective of the Study

1.11.1 General Objective

The aim of this research is to determine the prevalence of hypervirulent *K. pneumoniae* strains among *K. pneumoniae* isolates from patients admitted in tertiary centers and patient's clinical presentation in Kelantan.

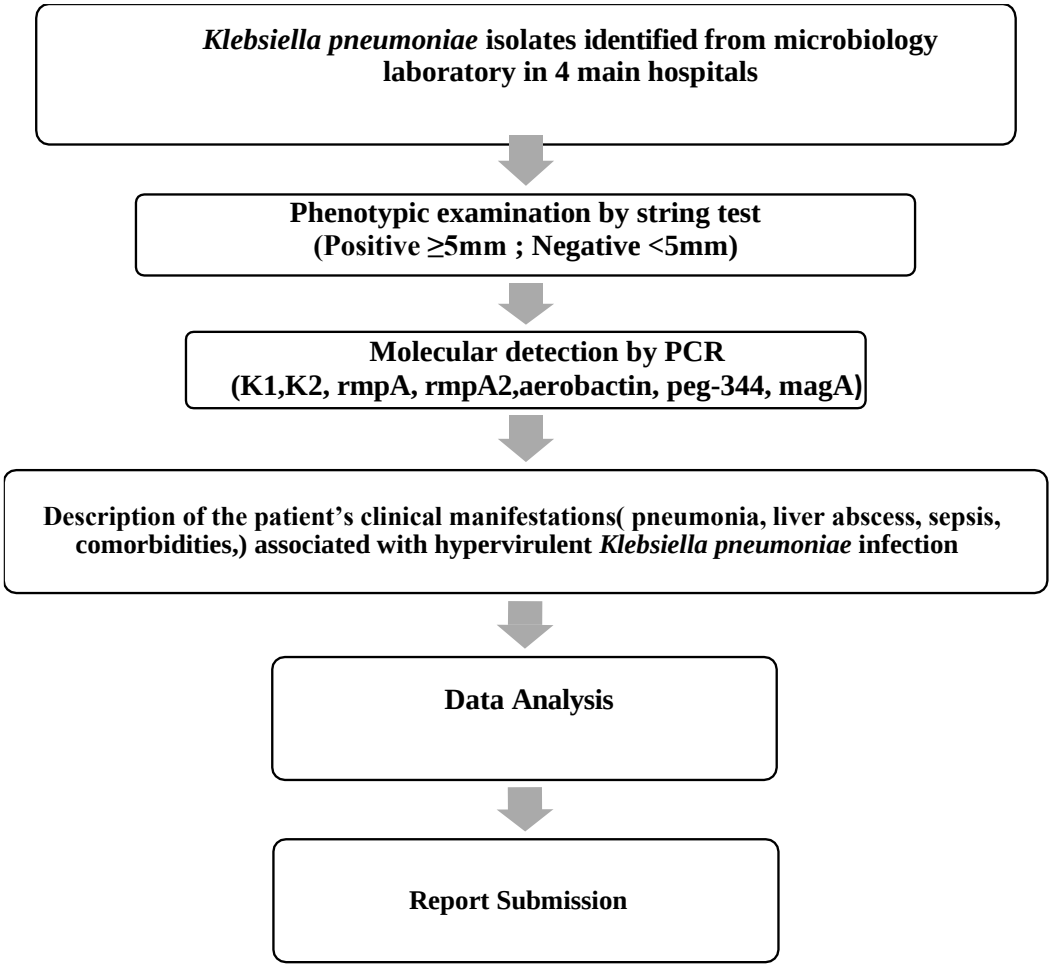
1.11.2 Specific Objective

a) To determine the prevalence of hvKp strains among *K. pneumoniae* isolates from patients admitted in tertiary care in Kelantan (HUSM, HRPZ II, HSIP and HTM) from June 2020 until June 2021.
    - Hospital University Sains Malaysia (HUSM)
    - Hospital Raja Perempuan Zainab II (HRPZ II)
    - Hospital Sultan Ismail Petra (HSIP)
    - Hospital Tanah Merah (HTM)

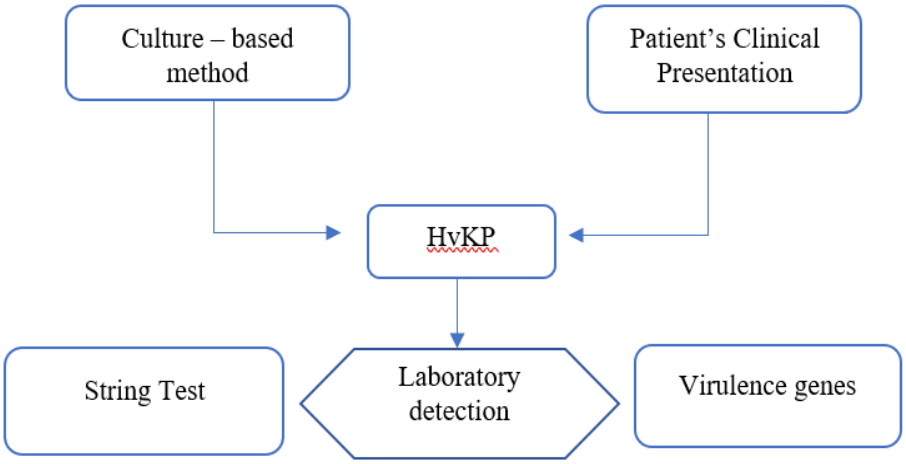
b) To describe the phenotypic and genotypic characteristics of hvKp among *K. pneumoniae* isolates.

c) To describe patient's clinical manifestations associated with hvKp infection.

1.12 Flow Chart of the study



1.13 Conceptual framework



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CHAPTER 2 STUDY PROTOCOL

2.1 Title

Prevalence of Hypervirulent *K. pneumoniae* Strains From Patients Admitted In Tertiary Centers and Their Clinical Presentation.

2.2 Objective

2.2.1 General objective

The aim of this research is to determine the prevalence of hypervirulent *K. pneumoniae* strains among *K. pneumoniae* isolates from patients admitted in tertiary centers and patient's clinical presentation in Kelantan.

2.2.2 Specific objectives

1)To determine the prevalence of hvKp strains among *K. pneumoniae* isolates from patients admitted in HUSM, HRPZ II, HSIP and HTM from June 2020 until June 2021.

2)To describe the phenotypic and genotypic characteristics of hvKp among *K. pneumoniae* isolates.

3)To describe patient's clinical manifestations associated with hvKp infection.

2.3 Methodology

2.3.1 Study design

This was a cross-sectional study conducted in four major hospitals in Kelantan from June 2020 until June 2021.

2.3.2 Study location

Samples of positive *K. pneumoniae* isolates and patient's data were collected from HUSM, HRPZ II, HSIP and HTM.

2.3.3 Reference population

All clinical isolates of *K. pneumoniae* in HUSM, HRPZ II, HSIP and HTM.

2.3.4 Source population

All clinical isolates of *K. pneumoniae* in HUSM, HRPZ II, HSIP and HTM from June 2020 until June 2021.

2.3.5 Inclusion criteria

1**)**All *K. pneumoniae* isolates confirmed with housekeeping gene (*gapA*).

2)*K. pneumoniae* isolates from the same patient with different episodes of infection.

2.3.6 Exclusion criteria

1)Repeated isolates from same patient with same episode of infection.

2)All *K. pneumoniae* isolates with negative detection of housekeeping gene (*gapA*).

2.3.7 Sampling size estimation

a. Objective 1

The aim was to determine the prevalence of hypervirulent *K. pneumoniae* strains among *K. pneumoniae* isolates from patients admitted in HUSM, HRPZ II, HSIP and HTM. The single proportion formula by Sample Size Calculator (Arifin, W.N.(2020) was used to calculate the sample size.

Variable	Proportion	Precision	Significance level	n	Dropout Rate, 10%	Total sample size,n	Literature Review
HvKp	0.38	0.07	0.05	185	206	206	Zhang *et al*, 2016

b. Objective 2

To determine the phenotypic characteristics of hypervirulent *K. pneumoniae* among *K. pneumoniae* isolates.

Phenotype	Proportion	Precision	Significance level	n	Dropout Rate, 10%	Total sample size,n	Literature Review
Hyper-mucovis cosity	0.75	0.06	0.05	147	164	164	Zhang *et al*, 2016

c. Objective 3

To determine the proportion of virulence-associated gene (*rmpA, rmpA2 , aerobactin, magA, peg344*) and capsular serotype-specific genes (K1 , K2).

Strains	Proportion	Precision	Significance level	n	Dropout Rate, 10%	Total sample size,n	Literature Review
K1	0.33	0.07	0.05	174	194	194	Zhang *et al*, 2016
K2	0.17	0.06	0.05	151	168	168	Zhang *et al*, 2016
rmpA	0.98	0.03	0.05	84	94	94	Zhang *et al*, 2016

d. Objective 4

To describe the patient's clinical manifestations associated with hypervirulent *K. pneumoniae* infection.

Factors	Proportion	Precision	Significance level	Power	n	Dropout Rate, 10%	Total sample size,n	Literature Review
Pneumonia	0.37	0.60	0.05	0.8	73	82	164	Zhang *et al*, 2016
Liver abscess	0.035	0.2	0.05	0.8	59	66	132	Zhang *et al*, 2016
Sepsis	0.35	0.55	0.05	0.8	96	107	214	Zhang *et al*, 2016
Diabetes mellitus	0.147	0.35	0.05	0.8	70	78	156	Zhang *et al*, 2016

Therefore, estimation of 214 *K. pneumoniae* acquisitions in 12 months was adequate.

2.3.8 Sampling method

Simple random sampling was applied in this study. All *K. pneumoniae* isolates from June 2020 until June 2021 were collected from four major hospitals in Kelantan state.

2.3.9 Operational definition

1) *K. pneumoniae* isolates : the organism was identified as *K. pneumoniae* based on colony morphology, gram staining, biochemical tests, Vitek2 and or MALDI-TOF, with presence of houskeeping gene (*gapA*).

2) Hypermucoviscosity: The isolated organism was identified by the string test, in which the formation of viscous string of equal or more than 5 mm is considered positive (Shon *et al.*, 2013).

3) Hypervirulent *K. pneumoniae* (hvKp) : In view of there is no consensus about the definition of hypervirulent, we used 2 microbiological characteristics that have been associated to it: A positive string test and positive PCR amplification of K1 or K2 genes. Those strain with these 2 indicators is considered as hypervirulent. A study in 2018 from Ikeda *et al* mentioned hypervirulent *K. pneumoniae* strains often showed hypermucoviscosity with K1 or K2 serotype.

4) Clinical presentation: primary diagnosis of the patient during hospitalization.

2.4 Research methodologies

2.4.1 Collection of bacterial isolates

All *K. pneumoniae* isolates used in this study were from the isolates which primarily identified and confirmed as *K. pneumoniae* organisms using the Standard Operating Procedure (SOP) from hospitals. Two-hundred and fourteen isolates were randomly selected from various clinical samples in four hospitals. The samples were collected from June 2020 until June 2021. However, only one-hundred and eighty isolates which fulfilled

the inclusion criteria were included in this study. The subsequent data analysis was based on these one-hundred and eighty isolates. Hypermucoviscosity were identified by the string test performance, in which the formation of a viscous string of more than 5 mm is considered positive. Then, all of the *K. pneumoniae* isolates were tested for virulence genes using PCR method. After all of the *K. pneumoniae* isolates have been analyzed, the isolates were destroyed after 12 months of storage.

2.4.2 DNA extraction

Genomic DNA extraction of all 180 isolates of *K. pneumoniae* was performed using DNA lysate technique. Overnight colonies of *K. pneumoniae* were prepared for cell lysis preparation. The cell lysate was used to break the cell wall of *K. pneumoniae* and expose its intracellular components. *K. pneumoniae* colonies were mixed with 100 µl sterile distilled water until a cloudy homogenous solution is obtained. The mixture was boiled for 10 minutes at 100°C thermostat. The sample was centrifuged at 12,000 rpm for 5 minutes to remove the cell debris. After centrifugation, the supernatant was transferred into the new tube, and the sediment was discarded. This lysate then was stored at -4°C before proceeded with specific hypervirulent *K. pneumoniae* virulence gene PCR.

2.4.3 Preparation of PCR master mix and PCR reaction

This study was carried out using multiplex PCR to target the housekeeping gene of *K. pneumoniae* (*gapA*) as a standard internal control, virulence-associated gene (*rmpA, rmpA2, iucA, magA, and peg344*), and two common hvKp capsule serotypes (K1 and