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MOLECULAR DETECTION OF CAPSULAR SEROTYPES K1 AND K2 Klebsiella pneumoniae ESBL CLINICAL ISOLATES

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A PROJECT PAPER SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE (BIOMEDICAL SCIENCES)

DEPARTMENT OF BIOMEDICAL SCIENCES
FACULTY OF MEDICINE AND HEALTH SCIENCES
UNIVERSITI PUTRA MALAYSIA

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ABSTRACT

MOLECULAR DETECTION OF CAPSULAR SEROTYPES K1 AND K2 *Klebsiella pneumoniae* ESBL CLINICAL ISOLATES

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Introduction: *Klebsiella pneumoniae* is a gram-negative, non-motile encapsulated bacteria which is commonly associated with hospital acquired infections (HAIs). Studies have shown that infections caused by hypervirulent *Klebsiella pneumoniae* (hvKp) isolates occurred in patients with chronic diseases including asthma, sepsis, pneumonia and liver abscesses. *Klebsiella pneumoniae* exhibits the highest virulent activity with the presence of K1 and K2 capsule-associated genes, *magA* and *K2A*, respectively. Importantly, the treatment of *K. pneumoniae* infection becomes greatly limited with its ability to produce extended spectrum β - lactamase (ESBL) which causes resistant against almost all β - lactam antibiotics. **Objective:** The study aims to determine the occurrence of serotypes K1 and K2 in *K. pneumoniae* ESBL isolates. **Methodology:** A total of 40 *K. pneumoniae* ESBL clinical isolates collected from Hospital Pakar Sultanah Fatimah Muar, Johor from 2009 to 2012 were analysed using Multiplex PCR for the presence of *magA* and *K2A* genes. Hypermucoviscosity test (String test) was performed to observe the formation of viscous string of *K. pneumoniae* isolates with more than 5 mm in length. Extraction of DNA was carried out using a commercial extraction kit. Identification of *magA* and *K2A* genes were performed using Multiplex PCR followed by DNA sequencing for phylogenetic tree analysis. **Result:** Five percent (5%) of the isolates were detected as K1 (n = 2) and K2 (n = 2), respectively. High number of the isolates (72.5%) showed positive for the hypermuscoviscosity test. However, hypermuscoviscity test showed no significance associations between K1/K2 and non-K1/K2 capsular serotypes (p = 0.194). The results of phylogenetic analysis of *K. pneumoniae* K1 and K2 capsular serotypes showed two distinct clusters. Cluster A is represented by K1 while Cluster B is represented by K2 capsular serotypes. **Conclusion:** High occurrence of non-K1/K2 were observed in this study (90%). Further surveillance of *K. pneumoniae* isolates from larger number of clinical isolates is important to provide a better understanding and evaluation of the genotypic characteristics and resistance mechanisms of this organism.
Keyword: K. pneumoniae, ESBL, capsular serotype, multiplex PCR

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ABSTRAK

Pengesanan Molekular Serotaip Kapsul K1 dan K2 *Klebsiella pneumoniae* STBL Isolat Klinikal

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Pengenalan: *Klebsiella pneumoniae* ialah bakteria yang merangkumi gram negatif, nyahgerak dan mengandungi lapisan selaput kapsul; serta, ia amat biasa dijumpai dalam jangkitan persekitaran hospital, "*hospital acquired infection, (HAIs)"*. Kajian ini menunjukkan bahawa jangkitan ini menyebabkan oleh hipervirulen *Klebsiella pneumoniae* (hvKp) isolat terjadi dalam kalangan pesakit mengalami penyakit kronik termasuk asma, sepsi, radang paru-paru dan kebenjolan nanah pada hati. *Klebsiella pneumoniae* muncul dengan peringkat virulen yang tinggi bersama kehadiran kapsul gabungan gen K1 dan K2, iaitu *magA* dan *K2A* mengikut masing-masing. Pentingnya, rawatan untuk jangkitan *Klebsiella pneumoniae* menjadi sangat terhad apabila dengan adanya ia menghasilkan spektrum terluas β-laktamase (STBL), "*extended spectrum β-lactamas, (ESBL)*" di mana ia berlakunya kerintangan terhadap hampir semua β – laktam antibiotik. **Tujuan:** Penyelidikan ini bertujuan mengenalpastian sekumpulan serotaip K1 dan K2 dalam *K. pneumoniae* STBL isolat klinikal. **Metodologi:** 40 *K. pneumoniae* STBL isolat klinikal kesemuanya telah dikumpul dari Hospital Pakar Sultanah Fatimah, Muar, Johor pada tahun 2009 hingga 2012 untuk mengenal pasti kehadiran gen *magA* dan *K2A* melalui Multipleks reaksi rantai polimerase (RRP), "*polymerase chain reaction (PCR)*" analisis. Ujian hipermukoviskositi dijalankan untuk mengesan kebolehan satu formasi likatan benang dihasil oleh *K. pneumoniae* isolat dengan melebihi 5 mm panjang. Pengestrekan DNA dilakukan dengan menggunakan kit komersial pengestrekan. Pengenalpastian gen *magA* dan *K2A* dilakukan dengan Multipleks RRP; lalu, penjujukan DNA dilaksanakan untuk penghasilan rangkaian filogenetik. **Keputusan:** Lima peratus (5.0%) isolat telah didapati gen K1 (n = 2) dan gen K2 (n = 2), mengikuti masing-masing. Angka yang tinggi pada isolat-isolat (72.5%) menunjukkan positif pada ujian hipermukoviskositi. Walau bagaimanapun, ujian hipermukoviskositi telah membuktikan bahawa tiada signifikan penggabungan antara kapsul serotaip K1/K2 dengan bukan K1/K2 (p = 0.194). Hasilan rangkaian filogenetik analisis *K. pneumoniae* kapsul serotaip K1 dan K2 menunjukkan mengandungi dua (2) kluster yang asing. Kluster A terdiri daripada kapsul serotaip K1 malah Kluster B terdiri daripada kapsul serotaip K2. **Kesimpulan:** Kajian ini menunjukkan majoriti isolate klinikal tidak mengadungi gen K1/K2 (90.0%). Pada masa akan datang, pengawasan *K. pneumoniae* isolat dari kumpulan nombor yang bsear ia menzahirkan satu kepentingan dan ia amat membantu menyediakan kefahaman dan penilaian secara dipermudahkan dalam menerokai ciri-ciri genotaip dan mekanisme rintangan organisma ini.

Kata kunci: Klebsiella pneumoniae, hipervirulen, spektrum terluas β-laktamase (STBL), Multipleks PCR, rangkaian filogenetik

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LIST OF ABBREVIATIONS

β	Beta
γ	Gamma
°C	Degree Celsius
%	Percentage
µL	Microliter
bp	Base pair
g	Gram
kb	Kilobase
L	Litre
M	Molar
AMR	Antimicrobial resistance
AST	Antimicrobial Susceptibility Testing
CDC	Centre of Diseases Control and Prevention
cKp	Classical *Klebsiella pneumoniae*
CLSI	Clinical & Laboratory Standard Institute
CR	Carbapenem resistance
DNA	Deoxyribonucleic acid
ESBL	Extended Spectrum β lactamase
hvKP	Hypervirulent *Klebsiella pneumoniae*
LB	Luria Bertani
LPS	Lipopolysaccharides
MDR	Multidrug resistance
PCR	Polymerase chain reaction

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CHAPTER 1

INTRODUCTION

1.1 Background

Klebsiella pneumoniae is a rod-shaped gram-negative bacterium presented with capsule. It appears with mucoidal characteristic once it has been cultured on MacConkey agar. *Klebsiella pneumoniae* is a pathogen of the human respiratory system which can be transmitted through person-to-person contact. These bacteria can be found in soil, human skin and intestine. Interestingly, studies have shown that 78 capsular serotypes were reported in *K. pneumoniae* isolates (Pan et al., 2008). Over the past decades, *K. pneumoniae* have been highly observed among severe infection cases in hospitals. Importantly, *K. pneumoniae* ranks among the leading causes of nosocomial infections including pneumoniae, liver abscesses, septicaemia, bacterial meningitis, urinary tract infection and catheter-related infections.

K. pneumoniae isolates have strong interaction between hosts, especially in humans. Indeed, *K. pneumoniae* have become higher risk when the patient presented with underlying diseases such as diabetes mellitus, hepatobiliary diseases, cancer, community-acquired infection, alcoholism, chronic renal failure and immunosuppression as reported in previous studies (C. R. Lee et al., 2017; Siu et al., 2012).

The commonly reported virulence factors which enables *K. pneumoniae* to evade the host defence mechanism is the polysaccharide capsule. The capsule plays an important role in protecting the bacteria form opsonophagocytosis, antibiotics permeability and adverse environmental conditions. Several important virulence

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factors were reported in *K. pneumoniae* include the capsule, lipopolysaccharide (LPS), siderophores and fimbriae. This study focused on the occurrence and genetic relatedness of capsular serotypes K1 and K2 hypervirulent *K. pneumoniae* isolates recovered from the clinical isolates.

Additionally, antimicrobial resistance was increasingly reported globally in the Enterobacteriaceae family, especially *Klebsiella pneumoniae*, which mainly affected infection cases in the hospital sites. In this study, Extended Spectrum Beta (β)-Lactamase (ESBL) was investigated where it functions as a major beta-lactam resistant mechanism; the bacterial defense system produced a beta-lactamase enzyme. In Malaysia, the prevalence of *K. pneumoniae* ESBL-producing isolates were reported between 27.8% to 47%. Clinical isolates showed relatively great resistance to cephalosporins and ampicillin were highly reported, implying the likelihood of effective treatment for infections caused by these strains when treated with these agents (Mohd Helmi et al., (2016); Shah et al., (2017)). This is in concordance with the report released by Malaysian National Surveillance on Antibiotic Resistance Report in 2008 which stated high resistance rate were observed in ampicillin (95%) as compared to imipenem (0.5%).

1.2 Problem Statement

Klebsiella pneumoniae is increasingly reported to cause infections in patients diagnosed with liver abscesses, pneumoniae, meningitis and nosocomial infection. The increasing trend observed in EBSL-producing *K. pneumoniae* serotypes K1 and K2 clinical isolates calls for the urgent need to understand the virulence factors associated with this condition.

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1.3 Objective

1.3.1 General Objective

The study aims to determine the occurrence of capsular serotypes K1 and K2 in *Klebsiella pneumoniae* ESBL clinical isolates.

1.3.2 Specific Objective

1. To determine the hypermucoviscosity phenotype of *Klebsiella pneumoniae* using String Test.
2. To observe the association between hypermucoviscosity phenotype and capsular serotypes K1 and K2.
3. To identify the distribution of capsular serotypes K1 and K2 using Multiplex PCR.
4. To observe the genetic relatedness between K1 and K2 by phylogenetics tress analysis.

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CHAPTER 2

LITERATURE REVIEW

2.1 Morphology *Klebsiella pneumoniae*

Klebsiella pneumoniae is gram – negative, non – motile, encapsulated, rod – shaped bacteria. It grows in facultative anaerobic condition and appears as a mucoid lactose fermenter on MacConkey agar. It has about 1 – 2 µm sized and is arranged singly in pair or in short chains formed in cluster. According to taxonomic presented, it was followed by Bacteria, Proteobacteria, Enterobacteriaceae, *Klebsiella* and *K. pneumoniae* on their domain, phylum, class, order, family, genus and species respectively. *K. pneumoniae* was found commonly in soil, animal, and human especially on skin, mouth, and intestines (Ryan et. al., 2004) (Postgate, 1998). Also, biochemical reaction and media culture showed on Table 2.1.1 and Table 2.1.2 respectively as the identification of *K. pneumoniae.*

The genus is named for German physician and bacteriologist Edwin Klebs. *Klebsiella pneumoniae*, also called Friedländer's bacillus, was first described in 1882 by Carl Friedländer, German microbiologist and pathologist. *K. pneumoniae* is best known as a pathogen of the human respiratory system that causes pneumoniae. Nowadays, *K. pneumoniae* was evolulated and resistance to antibiotic provided in hospital especially community-acquired infections (CAI) (Siu et al., 2012)(Piperaki et al., 2017).

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Table 2.1.1: Biochemical reaction on *Klebsiella pneumoniae*. "+" as positive test; present, "- "as negative test; absent.

Biochemical Test	Result
Gas from Glucose	+
Lactose (acid)	+
Sucrose (acid)	+
Dulcitol (acid)	+
ONPG	+
Methyl red	+
Voges-Proskauser	-
Citrate	+
Urease	+
Malonate	+
Lysine decarboxylase	+

Table 2.1.2 Media culture and *Klebsiella pneumoniae* colony characteristics

Culture media	**Colony characteristics**
MacConkey agar (MA)	Lactose fermenting, mucoid, pink colonies; exception is *Klebsiella pneumoniae* subsp, rhinoscleromatis, which gives lactose non-fermenting colonies
Blood agar (BA)	Greyish white, large, mucoid, non-hemolytic colonies
Cysteine Lactose Electrolyte Deficient (CLED) agar	Yellow to bluish mucoid colonies
Xylose Lysine Deoxycholate (XLD) agar	Yellow coloured colonies
Hektoen enteric (HE) agar	Yellow coloured colonies

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2.2 Epidemiological of *Klebsiella pneumoniae* isolates in the clinical area

An epidemiological study helps to understand the prevalence and distribution of *K. pneumoniae* isolates. Therefore, this study is important to observe the occurrence of *K. pneumoniae* in clinical settings. Importantly, studies reported that *Klebsiella spp*. was placed in the third rank after *Staphylococcus aureus* and *Pseudomonas aeruginosa,* where occurs in North America (9.0%), Europe (11.0%) and Asia-Pacific Region (14.2%) (Sader et al., 2019). The study was based on SENTRY Antimicrobial Surveillance Program which was carried out from 1997 to 2016, and the samples were from clinical isolates.

Based on the number of *K. pneumoniae* isolates in clinical sites and as stated by Navon-Venezia et al. (2017) that *K. pneumoniae* contributes significantly to the global antibiotic resistance burden. Most important, there related to antimicrobial susceptibility profiles of pathogens such as extended-spectrum β-lactamase (ESBL), multidrug resistance (MDR), and carbapenem resistance (CR). According to CDC (2019), the Enterobacteriaceae family included *K. pneumoniae,* was issued on the serious treat listed as ESBL-producing isolates. Moreover, Jean et al. (2016) expressed that the rate of ESBL-producing *K. pneumoniae* isolates was represented tabulation in nine countries in 2013; including Philippines (61.3%), South Korea (55.6%), China (54.5%), Thailand (50.0%), New Zealand (45.5%), Malaysia (42.3%), Singapore (29.2%), Australia (20.7%), Taiwan (16.3%), and Hong Kong (12.5%). As a result of previous studies based on Jean et. al. (2016), Malaysia is 5th rank of all countries stated and among together issues on antimicrobial resistance (AMR). Latest, Ministry of Health Malaysia (2019) insisted that *K. pneumoniae* clinical isolates showed a resistance rate on cefotaxime with increases by 13.8% on MyAp-AMR (2017-2021) program.

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2.3 Method of screening Hypervirulent *Klebsiella pneumoniae*

Hypervirulent *K. pneumoniae* (hvKp) is an evolving phenotype that is more virulent than classical *K. pneumoniae* (cKp). hvKp primarily infected individuals from community acquired infection (CAI) where happen in the health care setting also risk patients. The infections are more common in the Asian Pacific Rim but are occurring globally (Shon et al., 2013; Siu et al., 2012; Russo & Marr, 2019). Although that, there are some detection methods was discovered by several scientists on hvKp followed by:

2.3.1 Antimicrobial Susceptibility Testing (AST)

AST also called Disk Diffusion Test which means as an antibiotic susceptibility test the determine measure of *in vitro* bacteria' susceptibility to it. Following the interpretation data, the diameter of the inhibition zone for each antibiotic will be measured and interpreted as susceptible, intermediate and resistant after incubation period. This method was mostly used all researchers for detection outbreak of hvKp isolates on the sample (C. R. Lee et al., 2017)(Bengoechea & Sa Pessoa, 2019). Furthermore, AST was used by several scientist for standardized by Clinical and Laboratory Standards Institute (CLSI).

Meanwhile, AST also can detection on ESBL producing *K. pneumoniae* more specific by disk diffusion clavulanate inhibition (CSLI, 2018). Furthermore, phenotypic confirmatory test for ESBL production was detected by double-disc synergy test with disks of amoxicillin–clavulanic acid surrounded at a radius of 30 mm by cefotaxime, ceftazidime, aztreonam and cefepime on a Muller–Hinton agar plate (Abdel-Hady et al., 2008).

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2.3.2 String test

String test or another name as hypermucoviscosity test to determine the general characteristics of hvKp (Shon et al., 2013). Because of the hypermuviscous appearance of colonies grown on an agar plate, it can be defined by a positive “string test”. To show that string test is positive, the bacteriology inoculation loop or needle is able to generate a viscous string > 5 mm in length by stretching bacterial colonies on an agar plate as show in Figure 2.3.1. And some researcher stated that the hypervirulent phenotypes was influenced by hypermuviscous characteristics as well (Zheng et al., 2018)(Lin et al., 2020) and to differences between hvKp and cKp isolates (Shah et al., 2017).

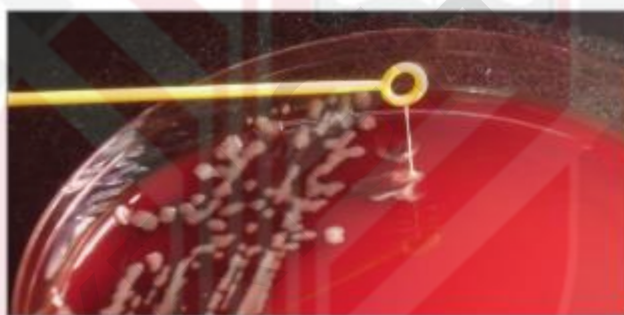

Figure 2.3.1 show that the positive “string test” result on a hypervirulent strain of *K. pneumoniae* (was adapted from (Taylor et al., 2015))

2.3.3 Commercial Kit Detection *Klebsiella pneumoniae* isolates

Commercial kit detection *K. pneumoniae* isolates one of method believe will provide result at a short time, reducing used technology laboratory, reduce manpower and high sensitivity. As well based on several doctors, microbiologist, medical laboratory technologist and health teams in flavour to use this method. At early stage, this method was commercialised on Real – Time Polymerase Chain Reaction (PCR) (Poh et al., 2004) which studied on the result of *K. pneumoniae* identified from LightCycler real-time PCR hybridization probe-based assay and time saved compared to conversional

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method at that time. Several previous research, this commercial kit was supported by researchers on their study; detection *K. pneumoniae* serotype K1 and K2 by colloidal gold-based immunochromatographic strip (ICS) as showed in Figure 2.3.2 (Huang et al., 2019) (Wang et al., 2019).

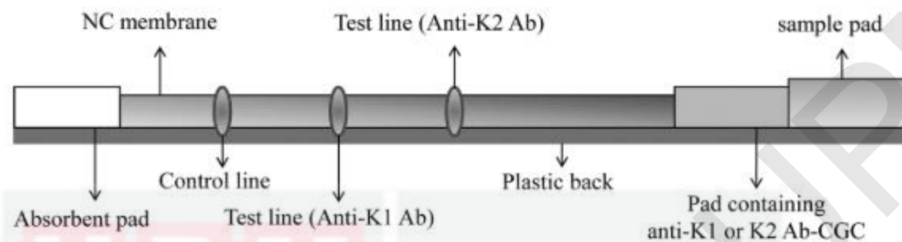

Figure 2.3.2 shows the Schematic illustration of ICS with labelled materials. Ab; antibody, NC; nitrocellulose. (was adapted from (Huang et al., 2019)).

2.3.4 Serotyping of Antisera detection

Serotyping of antisera as well can be detection for *Klebsiella pneumoniae* by capsular other surface polysaccharides stand up with K – Antigen and O – Antigen respectively. Also, this method has been rationally used *K. pneumoniae* serotype. Starting that introduced serotyping method on 1926 (Julianelle, 1926). Then, there previous study was detected that 77 capsular serotypes (K – antigen) from K1 to K82 and eight other surface polysaccharides (O – antigen) form O1 to O12; but O types was less diverse and it was going on further observation (Fang et al., 2016; Ørskov & Ørskov, 1984). In addition, Fung et al. (2000) used antisera detection in their study and recognized serotyping of capsular types *K. pneumoniae* isolates. Not only the isolates were serotypes by the counter current immunoelectrophoresis (CIE) method but also its were observed by the Quelling (capsular swelling) reaction with antisera if cross-reactions happened or if equivocal results were resulted by CIE. However, this method needed high cost and anti – sera provided was limited and experienced manpower is needed to carry out the procedure (Pan et al., 2013). Therefore, Wang et al., 2019 stated

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other the alternative methods maybe can replaced uses anti – sera detection serotyping such as are polymerase chain reactions (PCRs) to identify K. pneumoniae capsular serotypes.

2.3.5 Complement C3

Complement C3 is a blood test that measures the activity of a certain protein. The proteins work with immune system and play a role to protect the body from infections, and to remove dead cells and foreign material. People may inherit deficiency of some complement proteins which prone to certain infections or autoimmune disorders.

2.4 Factor virulence of *Klebsiella pneumoniae* to invade into deep organs

2.4.1 Capsule

The capsule would be one of their organelles important for protection, communication, and defense mechanisms if environmental changes and adaptation appeared to help bacteria survive. It was made up of heteropolysaccharides (Tankeshwar, n.d.); whereas as known sugar polysaccharides combined polypeptides. This reasons it supporting the capsulated *K. pneumoniae to* protect themselves; thus, it developed more into antimicrobial resistance (AMR) and weak the host defense mechanisms on the human immune system (Fleeman et al., 2020). Mainly, this factor is caused by the presence of capsular serotype genes on hvKp isolates. In contrast, they are responsible formation of the capsule on *K. pneumoniae* species in the previously studied (Paczosa & Mecsas, 2016). Some studied showed that this capsule has a higher chance of protection compare to non-capsulated *K. pneumoniae* while in humans or some animals' populations (Struve & Krogfelt, 2003). Therefore, capsular serotypes in *K.*

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pneumoniae consist of 78 serotypes (named from K1 to K78) (Pan et al., 2008), but the essential K1 and K2 serotypes were classified as category hvKp.

2.4.2 Lipopolysaccharide (LPS)

Lipopolysaccharides can be LPS; similarly, endotoxin is an important and required element of all Gram-negative bacteria's external membrane of the cell surface. Therefore, it can be expressed by O antigens such as a core oligosaccharide and lipid A; whereas, it is composited on LPS. LPS can categorize by nine O antigen serotypes (O1 until O9 serotypes) on *K. pneumoniae* species (Hansen et al., 1999). The function of LPS and capsule is quite the same; thus, both can collaborate. However, LPS depends on a crucial virulence factor that protects against humoral defences and can also be a potent immune activator (Paczosa & Mecsas, 2016).

2.5 Virulence gene characteristic *Klebsiella pneumoniae*

Some virulence genes have obtained characteristic phenotypes on *K. pneumoniae* species. There is act as carries information on their specific phenotypes on this bacterium. Several genes were responsible for this virulence determinants may be utilized to study *K. pneumoniae* virulence factors. First, regulator mucoid phenotype A, *rmpA* gene acts as a transcriptional activator of capsular polysaccharide (CPS). It underwent gene transcription CPS synthesis, thus forming hypervirulent in K1 and K2 serotypes of *K. pneumoniae* (Shah et al., 2017). Second, *uge* gene encodes for uridine diphosphate (UDP) galacturonate 4 -epimerase; thus, for expression of on both smooth O antigen molecules and K antigen on the surface of cell membrane *K. pneumoniae* (Regué et al., 2004). Fourth, aerobactin was coding on formation siderophores, which helps and acts as a compactor between the host and bacteria to take iron molecules

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(Quinn et al., 1995). Next, *kfu* gene was carried information for an iron uptake system related to aerobactin's siderophores (Hsieh et al., 2008). Then, the *fim-H gene* can be *mrkD* gene responsible for forming type 1 and type 3 fimbriae for attachment to the host's cells (Podschun & Ullmann, 1998). Most important, capsular serotypes in this study were on *magA* gene for encoded on K1 serotypes while *K2A* gene for encoded on K2 serotypes stands of characteristics of capsule polypeptides molecules of *K. pneumoniae* (Paczosa & Mecsas, 2016). Previous studies found that these several genes on their samples have shown no significant difference in *fim-H* and *mrkD* genes (Shah et al., 2017).

2.5.1 Extended Spectrum Beta Lactamase (ESBL) producing *Klebsiella pneumoniae*

Extended Spectrum Beta (β) Lactamase, short-form ESBL is a bacterial defence mechanism on their bacteria life. This system had to create beta-lactamase enzymes as a major beta-lactam resistant mechanism. Hence, these mechanisms have to resist penicillin groups, cephalosporins group and monobactam group and do not affect carbapenems (Shah et al., 2017); that is why it is called extended-spectrum region to resistant some at antibiotics. First recorded have found ESBL producing *K. pneumoniae* isolate at early 1984 (Knothe et al., 1983); instead, this situation becomes numerous cases in hospital or community environmental was reported (Fils et al., 2021). Alternatively, Ogutu et al. (2015) and Surgers et al. (2019) explored that ESBL producing *K. pneumoniae* isolates can be detected on several genes' sequences; such as bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ and $bla_{OXA\text{-}1}$ genes where bla stand for β-lactamase genes and can be seen by multiplex polymerase chain reaction (MPCR). ESBL genes were detected in the range of 86% to below of *K. pneumoniae* isolates found in Malaysian district hospitals in previous studies (Mohd Helmi et al., 2016).

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2.6 Diseases of clinical manifestation *Klebsiella pneumoniae*

The disease of clinical manifestation *K. pneumoniae* isolates can be challenging to detect characteristics on MacConkey agar or Iodine test or identification system (such as API system). The site of infection is a point of information on how it spreads and grows on the specific organ. In particular, the entry transmission of this pathogen can be entered either by nosocomial entry or gasteriointernal tract (Li et al., 2016); afterward, this pathogen will be transmitted on the bloodstream with their advantaged structure help them. Following the first report on the hvKp of liver abscess in 1986 (Y.-C. Liu et al., 1986); these were investigated between septic endophthalmitis. In addition, liver abscesses increased the number of cases reported in Taiwan (Chang et al., 2000). Then, it was informed that numerous instances of clinical manifestation were related to this situation, such as community acquired pneumoniae, urinary tract infection, and bloodstream infection (Keynan & Rubinstein, 2007; Premathilake et al., 2018). Indeed, there was associated with hvKp, and ESBL producing *K. pneumoniae* isolates especially on it have formation hypercapsule association between either presence of hypermucosvicosity phenotypes or presence of capsular serotypes K1 and K2 genes(Chuang et al., 2006; Martin et al., 2016; Yu et al., 2006).

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2.7 Molecular detection using Multiplex PCR techniques

Multiplex Polymerase Chain Reaction can stand for M-PCR or MPCR. It is one modification of transitional PCR where two or more target primers sequences run together at one time or one vial PCR tube (Markoulatos et al., 2002). They also stated that MPCR was a suitable method for genes detection and at optimum specificity and sensitivity, especially in microbiology diagnostics; thus, this moment is supported by previous studies by Compain et al. (2014). This study detects the capsular serotypes K1 and K2 in *K. pneumoniae* clinical isolates. Some previous studies were discovered on how the MPCR sees on K1 and K2 serotypes isolates. According to Fangyou et al. (2018), it functional acts as rapid detection of the presence of wzyK1 and wzyK2 genes on K1 and K2 serotypes, respectively; using MPCR on carbapenem-resistant and hypervirulent of *K. pneumoniae* isolates. Table 2.7.1 shows the several target primer sequences involved in detecting capsular serotypes K1 and K2 in *K. pneumoniae* isolates with several previous studies.

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Table 2.7.1 Tabulation of primer DNA sequences based on their previous studies.

Primer	Primer DNA Sequences (5' to 3')	Size of Product (bp)	Reference
K1 (magA)	Forward: GGTGCTCTTTACATCATTGC Reverse: GCAATGGCCATTTGCGTTAG	1243	Wang et al. (2019)
K2 (K2A)	Forward: GACCCGATATTCATACTTGACAGAG Reverse: CCTGAAGTAAAATCGTAAATAGATGGC	643	
K1 (magA)	Forward: GGTGCTCTTTACATCATTGC Reverse: GCAATGGCCATTTGCGTTAG	1283	Compain et al. (2014)
K2 (K2A)	Forward: CAACCATGGTGGTCGATTAG Reverse: TGGTAGCCATATCCCTTTGG	531	
*K1 (magA)	Outer; Forward: GGTGCTCTTTACATCATTGC Reverse: GCAATGGCCATTTGCGTTAG Inner; Forward: CGCCGCAAATACGAGAAGTG Reverse: GCAATCGAAGTGAAGAGTGC	1200	Fang et al. (2004)
K2 (K2A)	No study		
K1 (magA)	Forward: GGTGCTCTTTACATCATTGC Reverse: GCAATGGCCATTTGCGTTAG	Not Stated	(Yu, Fung, Ko, & Chuang, 2007)
K2 (K2A)	Forward: CAACCATGGTGGTCGATTAG Reverse: TGGTAGCCATATCCCTTTGG	Not Stated	

Note: *The study utilized Nested PCR analysis

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CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Collection

In this study, 40 vials of clinicals samples were collected from Microbiology Department of Hospital Pakar Sultanah Tunku Fatimah, Muar, Johor. There samples were collected from 2009 to 2012. All samples were detected positive for the presence of Extended-Spectrum Beta-Lactamase (ESBL) genes by polymerase chain reaction carried out by Ujir et. al. (2014). These include bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$ and bla_{TEM} detected by simplex PCR. The ethical approval for this study was obtained by Mohd. Helmi Bin Ujir for the study entitled "Distribution of ESBL genes and activities in clinical isolates of *Klebsiella pneumoniae* at a district hospital Malaysia", from Medical Research & Ethics Committee with reference number NMRR-12-1111-13791.

3.2 Bacteria Identification

There are two methods used for the identification of *K. pneumoniae* bacteria;

3.2.1 Gram Staining

All *K. pneumoniae* isolates were identified through gram staining method. This staining technique classifies the bacteria as Gram - negative or Gram - positive bacteria. Gram staining stain the cell wall of bacteria by chemical reaction. Gram – negative bacteria will be stained pink in colour while Gram – positive bacteria will be stained violet or purple colour. Firstly, the *K. pneumoniae* isolates were cultured on Columbia Agar with 5 % of Sheep Blood (generally called 5% Blood Agar) or on LB

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agar and incubated overnight at 37ºC with anaerobic condition. Secondly, a single colony of *K. pneumoniae* was placed on the glass slide and mixed with a drop of distilled water. Then, the glass slides were fixed with flames at few seconds and let it dry on the table.

The glass slides were stained with crystal violet solution for 1 minute. Then, it was rinse thoroughly. This is followed by the addition of iodine stain for 1 minute to enhance the binding between crystal violet and cells. The glass slides were rinse thoroughly. This is followed by the addition a few of drops of 95% of ethanol for 4 seconds where decolourisation with ethanol was applied to interact with lipids of the cell membrane. The glass slides were rinsed thoroughly to completely remove the primary stain. Finally, safranin solution was added for 1 minute for counterstain the cells.

These glass slides were observed and examined under light microscope at 100X magnification with oil immersion. The observation appears on the bacterial cells will be stained by two colours; pink colour for gram – negative bacteria while violet colour for gram – positive bacteria presented.

3.2.2 Biochemical Tests for identification of *Klebsiella pneumoniae*

First and foremost, biochemical tests were used for one of the determinations, particularly in clinical isolates; it is relevant and provides key biochemical characteristics required for organism identification, information for susceptibility testing is indicated, and information on the appropriate antimicrobial agents to use. All the isolates of *K. pneumoniae* were going to the biochemical test for identification of bacteria. These were tested with some tests; citrate utilization tests, Sulphur, Indole, and Mortality (SIM) test, Triple Sugar Iron (TSI) Agar and Urease Hydrolysis test.

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Citrate Utilization test is used to determine the ability of an organism to utilize sodium citrate as its only carbon source and inorganic ammonium salts as its only nitrogen source. Bacteria that can grow on this medium turn the bromothymol blue indicator from green to blue. On based expected results positive result will appear growth on the medium, with or without a change in the colour of the indicator. Also, the colour change of the indicator is due to acid or alkali production by the test organism as it grows on the medium. Growth usually results in the bromothymol blue indicator, turning from green to blue. The observation was expected changes form blue to green colour and showed positive test.

Other biochemical tests such as the SIM test or Sulphur, Indole, and Mortality tests were carried to identify *K. pneumoniae*. Firstly, indole test is used to determine the ability of an organism to split tryptophan to form the compound indole. On expected result, positive result will be appearing pink to wine – coloured ring after addition of Kovac's reagent; while negative result will be no colour change after the Kovac's reagent. The observation on indole test will be appeared no colour change and it classified negative test on these isolates.

Secondly, motility test is used to determine if an organism is motile. An organism must possess flagella to be motile. So expected result, positive result will be the motile organisms will spread out into the medium from the site of inoculation; while negative result will be non-motile organisms remain at the site of inoculation. The observation was expected this test remain at the site of the inoculation and presented as negative test.

Thirdly, sulphur test or H_2S production test is carried out to determine the certain bacterial species liberate sulphur from sulphur-containing amino acids or other compounds in the form of H_2S. Bacterial species capable of producing H_2S release

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sulphide from cysteine or thiosulfate present in the medium by their enzymatic action. Bacteria that produce cysteine desulfhydrase are able to remove the sulfhydryl and amino groups from cysteine, yielding hydrogen sulphide, ammonia, and pyruvic acid. Hydrogen sulphide is also produced by the reduction of thiosulfate in anaerobic respiration by the enzyme thiosulfate reductase. Thus formed H_2S gas, which is colourless, combines with H_2S indicators (iron, bismuth or lead) present in the medium producing insoluble, heavy metal sulphides that appear as a black precipitate. As the results, no change colour will be observed on these isolates.

Triple Sugar Iron (TSI) Agar was used to determine whether a gram – negative rod utilizes glucose and lactose or sucrose fermentatively and forms hydrogen sulphide (H_2S). Therefore, expected result can be several observations; alkaline slant or no change in the butt (K/NC) = for glucose, lactose and sucrose non utilizer; also recorded as K/K (alkaline slant /Alkaline butt). It remains red colour agar. Alkaline slant/acid butt (K/A) = glucose fermentation. It will change form red to yellow at butt while red colour remains unchanged on slant. Acid slant /Acid butt (A/A) = glucose, sucrose and/or lactose fermenter. It will appear fully change from red to yellow colour agar. Bubbles or crack in the tube indicate the production of CO_2 or H_2. Drawing a circle around the A or just write 'G' stand for 'Gas' for the acid butt; usually indicates this means the organism ferments glucose and sucrose, glucose and lactose or glucose, sucrose, lactose with the production of gas. A black precipitate in the butt indicates production of ferrous sulfide and H_2S gas (H_2S^+). The expected results showed A/AG on this test.

Urease Hydrolysis test was used to determine the ability of an organism to produce the enzyme urease, which hydrolyzes urea to produce the ammonia and CO_2. Therefore, expected result will be appears positive result by changes in colour of slant

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from light orange to magenta; while, negative result will no colour change (agar slant and butt remain light orange). The result of urease test was expected change light orange to magenta colour thus positive test on these isolates. All tests were prepared by manufacture company and were cultured samples isolates. Then, it was incubated 18 – 24 hours at 37ºC. The results were observation and recorded.

3.3 Bacteria Cultivation

3.3.1 MacConkey Agar

The MacConkey agar was used as selective and differential media to differentiate between Gram – negative and Gram – positive bacteria. It contains protease, peptone, lactose monohydrate, bile salts and sodium chloride which inhibit the growth of Gram – positive bacteria. Bile salt helps to inhibit non-intestinal bacteria. Combination of lactose and neutral red are to distinguish the lactose fermenting from non-lactose fermenting Salmonella and Dysentery groups. After an overnight of 37°C of incubation period, the cultured *K. pneumoniae* showed mucoid and pink colonies.

3.3.2 5% of Blood Agar

The colonies then were sub cultured onto blood agar supplemented with 5% sheep blood agar thus it also formed with Colombia agar and incubated for overnight. Blood agar is used to detect fastidious organisms as well as to detect haemolytic activity. The *K. pneumoniae* showed greyish white, large, and mucoid colonies. It also showed gamma haemolysis which referring to the lack of haemolytic activity. In other words, the *K. pneumoniae* does not haemolyse the blood agar.

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3.3.3 Luria-Bertani Agar

LB agar is also known as nutrient agar as it provides nutritionally rich medium for growth of bacteria. 20 g of LB powder was weighed and mixed with 1000 ml of distilled water a bottle. The solution was mixed thoroughly to make sure all blended very well without any clumping pieces. Label the bottle and ensure the tip was not closely tightly to be prepared with pressure in autoclave machine. The LB solution was autoclaved for 21 minutes at 121°C. Autoclave is purposely for sterile and kill micro flora. Agar medium to be poured into plates is sterilized in a flask, and then poured afterward.

3.4 Preservation and Sub-culturing

Brain Heart Infusion (BHI) or LB broth with extra of 20% glycerol was used as suspension broth to preserve *K. pneumoniae* isolates. Each isolate was preserved in duplicate as one for working stock and another stored in cryo state for long term preservation at - 80°C. A loopful thawed working stock was inoculated onto MacConkey media and incubated at 37°C overnight. Purity of the colony was inspected the next day prior to further laboratory procedures.

3.5 Hypermucoviscosity Testing (String Test)

The string test was carried out to observe the formation of a mucoviscous string of more than (>) 5 mm in length. A positive string test with *K. pneumoniae* strains were designated as hypervirulence as showed by Fang et al. (2004). Firstly, colonies grown overnight on 5% Sheep Blood with Colombia agar were placed on a glass slide. Using a sterile loop, the bacterial colonies were mixed with 3% potassium hydroxide (KOH). When exposed to 3% KOH, *K. pneumoniae* underwent cell lysis thereby releasing its

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viscous nucleic acid, which form a string when drawn up. The measurement of viscous string more than 5 mm demonstrates *K. pneumoniae* having hypervirulent characteristics. In this study, the *K. pneumoniae* isolates positive for hypermucoviscosity string test were selected for DNA extraction followed by PCR analysis for the detection of K1 and K2 genes.

3.6 DNA extraction

DNA extraction of *K. pneumoniae* isolates was done with Commercial DNA extraction kit from GeneAll® Exgene™ (GeneAll Biotechnology Co. Ltd, Seoul, South Korea). This kit includes several ready to use buffers such as Buffer CL, BL, BW, TW and AE. Firstly, few colonies of *K. pneumoniae* isolates where grown on LB agar were suspended in 200 µl LB Broth in 1.5ml microcentrifuge tube by using a sterile wire loop. The bacterial suspension was then centrifuged for 1 minute at 15,000 rpm. The supernatant was discarded and the pelleted bacterial cells were left in the microcentrifuge tube. The cell pellets were resuspended completely with 200 µl of Buffer CL. Thus, 20 µl of Proteinase K solution (20 mg/ml) were pipetted and its vortex vigorously to mix completely. Then, it's were incubated 56°C for 15 minutes in water bath for make sure the sample complete lysis.

Continue with another set's protocol, 200 µl of Buffer BL were added to the tube and vortex the tube to mix thoroughly. Then, the samples were incubated at 70°C for 10 minutes in dry block heater machine and check the lid does not have the drops of samples. The samples were taken rest at 5 minutes at room temperature. 200 µl of 100% ethanol were added with to samples, pulse – vortex to mix thoroughly. Next, the samples were transferred all to the SV column carefully, centrifuged for 1 minute at 15,000 rpm and replaced the collection tube with a sterile collection tube. Secondly,

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600 µl of Buffer BW were added into the samples, centrifuged for 1 minute at 15,000 rpm and replaced the collection tube with another sterile tube. Suddenly, 700 µl of Buffer TW into the samples and centrifuged for 1 minute at 15,000 rpm. The pass – through solution was discarded and the SV column is placed back into the collection tube. The SV column centrifuged at full speed (15,000 rpm) for 1 minute to remove the residual wash buffer. Then, the SV columns were placed into a fresh 1.5 ml tube. 200 µl of Buffer AE was added and incubated for 1 minute at room temperature tprior to centrifugation at 15,000 rpm for 1 minute. Finally, the SV columns were removed and the sample tubes containing the bacterial DNA were stored in the refrigerator at – 20°C for 1 – 3 months.

3.7 Multiplex PCR Assay for K1 and K2 Genes Detection

Polymerase Chain Reaction (PCR) is the common method used to produce multiple copies of DNA template. One of the important components in PCR is the primer pairs. They are used to bind to certain nucleotide sequences complementary to the target sequence along the DNA strand. A set of primers was used, which were magA Forward (also stand *magA* – F) and magA Reverse (also stand *magA* – R) for K1 serotypes detection; K2A Forward (also stand *K2A* – F) and K2A Reverse (also stand *K2A* – R) for K2 serotypes detection shown on Table 3.7.1. Thus, the internal control was used on Multiplex PCR that is K16S primers for identification of *K. pneumoniae* isolates from the presence of other contamination. The total volume of PCR components utilized in this analysis was 15 µl. These include 7.5 µl of MyTaq HSTM DNA Polymerase Master Mix, 0.5 µl of K16S Forward, 0.5 µl of K16S Reverse, 0.5 µl of K1 Forward, 0.5 µl of K1 Reverse, 0.5 µl of K2 Forward, 0.5 µl of K2 Reverse, 1.5 µl of DNA extracted samples and 3.0 µl of nucleus-free water solution as shown in Table

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3.7.2. Initial duration was carried out at 95°C for 3 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, elongation at 72°C for 10 seconds. The final extension was carried out at 72°C for 10 minutes as shown Table 3.7.3.

Table 3.7.1 DNA Sequences of K1 and K2

Target gene	Sequences of primer (5'-3')	Size of amplified products (bp)
magA (K1)	Forward: GGTGCTCTTTACATCATTGC	1283
	Reverse: GCAATGGCCATTTGCGTTAG	
K2A (K2)	Forward: GGTGCTCTTTACATCATTGC	531
	Reverse: GCAATGGCCATTTGCGTTAG	

Table 3.7.2 Multiplex PCR components for K1 and K2 capsular serotype detection

Components		Volume per 15µl reaction (µl)
MyTaq HSTM DNA Polymerase Master Mix (Sydney University, Save Sight Institute, Sydney, Australia)		7.5
Internal Control K16S	K16S Forward	0.5
	K16S Reverse	0.5
magA (K1)	K1 Forward	0.5
	K1 Reverse	0.5
K2A (K2)	K2 Forward	0.5
	K2 Reverse	0.5
DNA Extracted samples		1.5
Nucleus-free water		3.0

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Table 3.7.3 Cycling condition for Multiplex PCR.

Target gene	Cycling conditions				
	Initial Denaturation	Denaturation	Annealing	Elongation	Final Extension
magA(K1) *K2A* (K2) K16S	95°C	95°C	58°C	72°C	72°C
	3 minutes	30 seconds	30 seconds	10 seconds	10 minutes
		Repeated for 30 cycles			

The PCR products were resolved through 1.7 % agarose gel in 1x TBE buffer at 80 V for 60 minutes with the addition of 5 µl of DNA staining solution. The product was run using gel electrophoresis to analyse and separate the PCR products according to their respective sizes and charges by using electric field. 5 µl of 100bp DNA ladder (HS Bioline) was loaded into the first well and 1000 bp DNA ladder was loaded into the last well of the agarose gel to compare the molecular weights of amplified PCR products. When the separation is completed, the agarose gel was then viewed under UV Transilluminator and Molecular Image Gel Doc XR system (Bio Rad, United States). The isolates are classified as *K. pneumoniae* K1 and K2 serotypes based on the presence of the PCR products appeared at 1283bp and 533bp, respectively (Wang C et al., 2019).

3.8 DNA Sequencing by Phylogenetics Analysis

Phylogenetics analysis based on the capsular serotypes of K1 and K2 genes were determined by using Molecular Evolutionary Genetics Analysis version 7 (MEGA7). Appropriate reference sequences ID EF221827.1 and MF417542.1 were utilized for K2 serotypes while AB355924.1 and AB085741.1 were used for K1 serotypes. All references gene sequences were retrieved from the GenBank, National Center for

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Biotechnology (NCBI) and included in the analysis as control. The phylogenetic tree was constructed using MEGA7 using the maximum likelihood method based on the Tamura-Nei model, while the reliability of the tree was estimated via bootstrap analysis with 1000 replicates.

3.9 Statistical analysis

The Chi – Square, X_2 test was used to test the significant association between the categorical variable with the significant value with less than 0.05 ($p < 0.05$). The data obtained were analysed by using Statistical Package for the Social Sciences (SPSS) Version 20. There were two parts of variable tested, which are the dependent variables and independent variables. The presence of capsular serotypes K1 and K2 would be the independent variables whereas the hypermucoviscosity test would be the dependent variables.

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CHAPTER 4

RESULTS

4.1 Morphological identification of *Klebsiella pneumoniae*

4.1.1 Luria-Bertani Agar

Luria Bertani (LB) agar was used to grow *K. pneumoniae* isolates and acted as a nutrient-rich media for growth. The colony was formed on a punctiform, convex elevated, entire margin and circular shape as shown in Figure 4.1.1. The size of the colony was less than 0.2 cm in diameter. No morphology changes were observed on the LB agars where white opaque and mucoid.

Figure 4.1.1 shows that the appearance of *K. pneumoniae* isolates on the LB agar. (Right side: form the top; left side: from the 45° close up shoot).

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4.1.2 5% Blood Agar

5 % blood agar was used to grow *K. pneumoniae* isolates. Blood agar act as enriched media and additional support for growing bacteria in nutritional media. Also, blood agars can be utilized for recognizing their type of haemolysis. The appearance of *K. pneumoniae* colonies formed on blood agar were gamma - haemolysis (γ – haemolysis) as shown in Figure 4.1.2. In other words, *K. pneumoniae* showed no haemolysis pattern surrounding the colonies on blood agar.

Figure 4.1.2 shows that the appearance of *K. pneumoniae* isolates on the 5% blood agar. (Right side: form the top; left side: from the 45° close up shoot).

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4.1.3 MacConkey Agar

MacConkey agar acts as differential media and play an important role in the isolation and differentiation of lactose fermenting and non – lactose fermenting enteric bacilli. In addition, this agar was used to inhibit the growth of gram-positive bacteria and allow the growth of gram-negative bacteria. Klebsiella pneumoniae grown on MacConkey showed the appearance of pink colonies were formed in the agar as shown in Figure 4.1.3. This indicate that *K. pneumoniae* are lactose fermenters; meanwhile, the colonies appeared as mucoid.

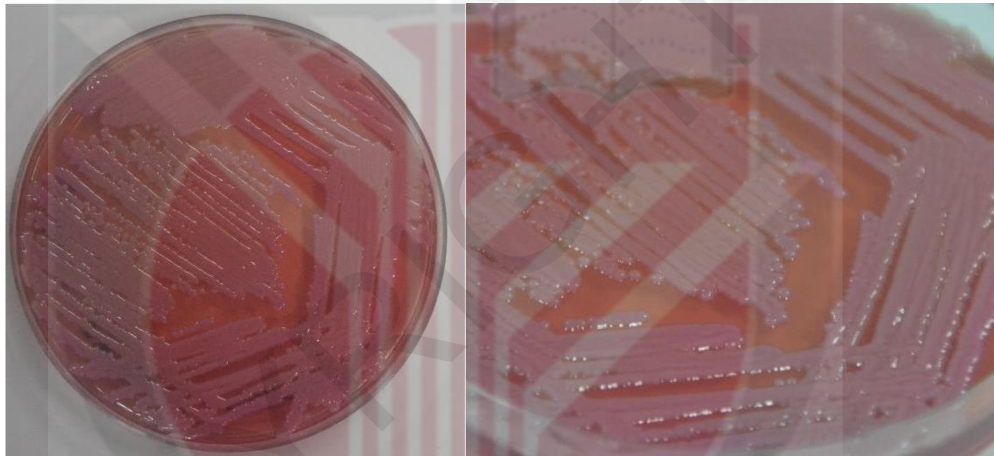

Figure 4.1.3 shows that the appearance of *K. pneumoniae* isolates on the MacConkey agar. (Right side: form the top; left side: from the 45° close-up shoot).

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4.1.4 Gram Staining on *Klebsiella pneumoniae*

Gram staining was used to classify the bacteria as Gram positive or negative based on the thickness of peptidoglycan layers in the bacterial cell wall structure. In Figure 4.1.4, *K. pneumoniae* isolates were classified as gram-negative bacteria wherein pink colour formed. Meanwhile, the microscopic structure isolates were in bacilli shaped and arranged in single or pair formed.

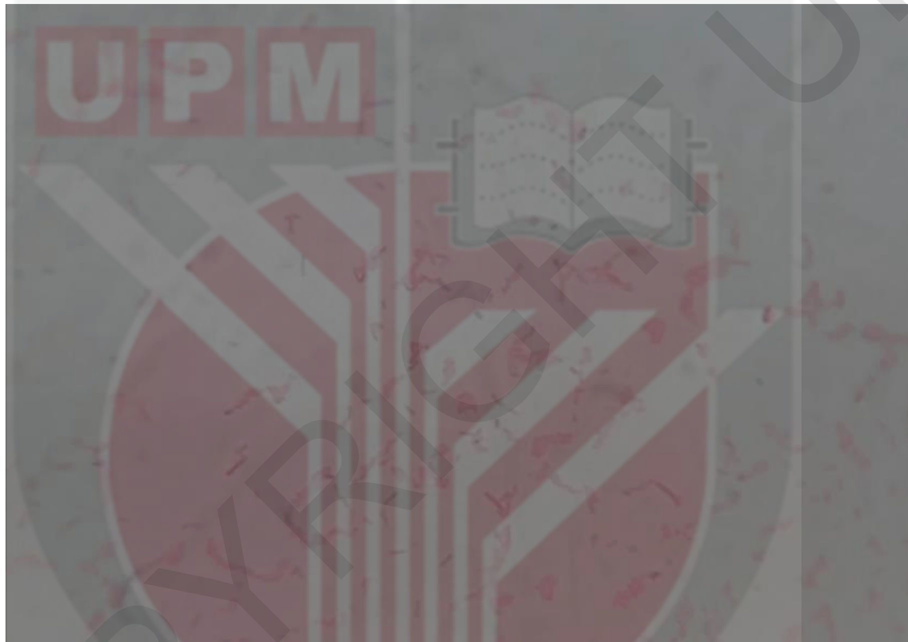

Figure 4.1.4 shown that the gram staining of *K. pneumoniae* isolates under a light microscope with oil immersion. (Gram Staining; 100X magnification).

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4.1.5 Biochemical test on *Klebsiella pneumoniae*

The biochemical tests were carried out in *K. pneumoniae* isolates to further identify characterize the bacteria based on the biochemical reactions. There are results of biochemical reactions as summary (on Table 4.1.1) and figure at below on based observation as shown in Figure 4.1.5 and Figure 4.1.6

Figure 4.1.5 shows several biochemical tests on *K. pneumoniae* isolates. Left to the right direction; SIM agar, Citrate agars, Urea agars, TSI agars

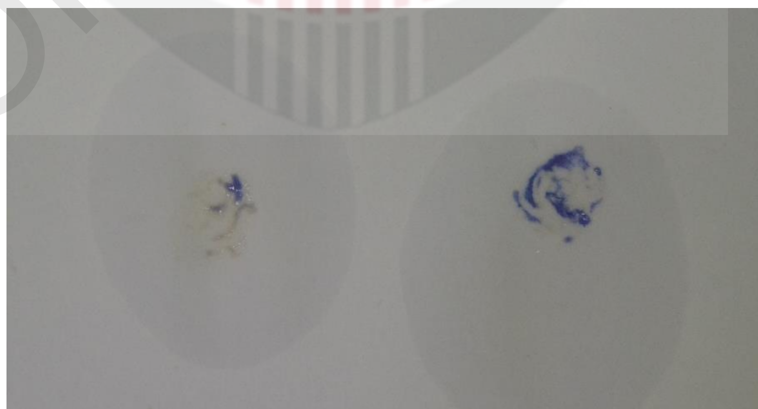

Figure 4.1.6 shows the oxidase test results. Right side: *Pseudomonas aeruginosa* (Positive); Left side: *K. pneumoniae* (Negative).

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Table 4.1.1 shows the tabulation of summary observation on the biochemical tests of *K. pneumoniae* isolates.

Biochemical Test	Observation
TSI Agars	1. A/A with the presence of gas 2. Acid Slant and Acid Bottom (change colour from red to bright yellow) 3. Absence of hydrogen sulfide, H_2S (absent of black precipitate formed between slant and bottom).
SIM Agars	1. Absence of hydrogen sulfide, H_2S (absent of black precipitate formed). 2. No change after added Kovac solution (Negative, -). 3. The bacteria remain at the site of inoculation (Negative, -).
Urea Agars	It changes in colour from light orange to magenta at slant and bottom (Positive, +).
Oxidase Test	It changes in colour from light orange to magenta at slant and bottom (Positive, +).
Citrate Agars	It was turning from green to blue (Positive, +).

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4.2 Demographic of *Klebsiella pneumoniae* isolates

Among 40 isolates were collected; it has several demographic tabulations as shown on Table 4.2.1. There have majority on male (n = 23; (57.5%)) and then female (n = 17; (42.5%)). Sputum was the most common (n = 26; (65%)) clinical specimens from *K. pneumoniae* was cultured, followed by urine (n = 3; (7.5%)), nasal swab (n = 3; (7.5%)), tissues (n = 2; (5.0%)), endotracheal aspirate (ETT) (n = 2; (5.0%)), body fluids (n = 1; (2.5%)), vaginal swab (n = 1; (2.5%)), blood (n = 1; (2.5%)), and tracheal aspirate (n = 1; (2.5%)). The patients were infected by *K. pneumoniae* ESBL isolates majority effected at 81- to 90-year-olds (n = 8; (20%)); meanwhile, no data presented at 0- to 10-year-olds and 21- to 30-year-olds range groups with mean (SD); 58.78 (21.528) as shown in Figure 4.2.1.

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Table 4.2.1 shows the demographic of *K. pneumoniae* isolates classified by gender, biological specimens, and age ranges (N = 40 samples).

Variable	Distribution	
	n	%
Gender		
Female	17	42.5
Male	23	57.5
Biological Specimen		
Tissue	2	5.0
Sputum	26	65.0
Urine	3	7.5
Body fluids	1	2.5
ETT	2	5.0
Vaginal swab	1	2.5
Blood	1	2.5
Nasal swab	3	7.5
Tracheal aspirate	1	2.5
Age Range		
0 - 10	0	0
11 - 20	4	10
21 - 30	0	0
31 - 40	3	7.5
41 - 50	5	12.5
51 - 60	7	17.5
61 - 70	5	12.5
71 - 80	6	15
81 - 90	8	20
91 - 100	2	5

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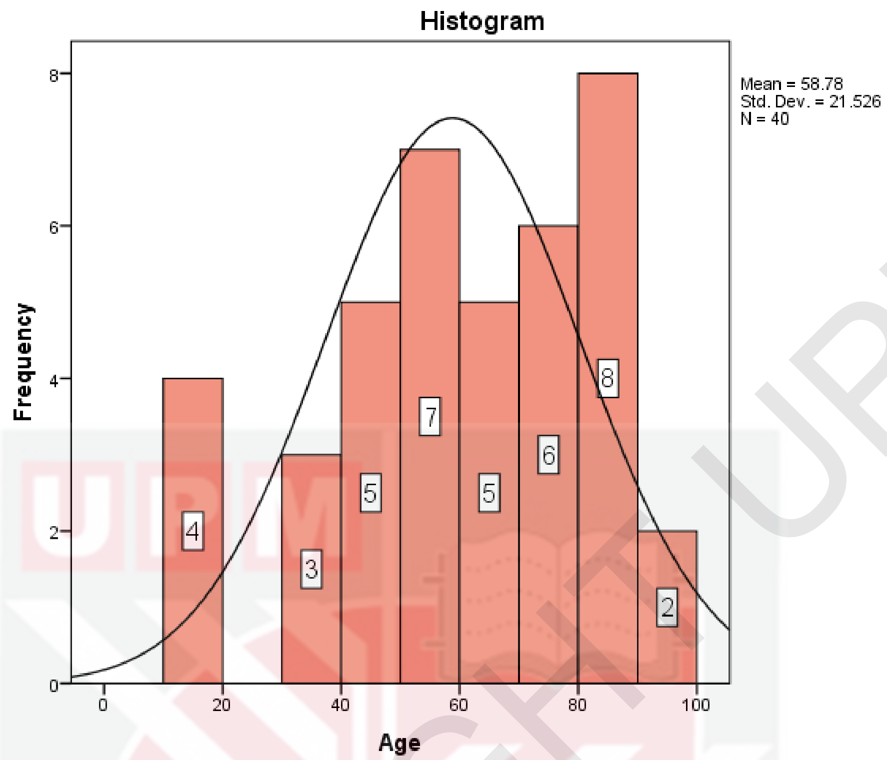

Figure 4.2.1 shows the histogram graph of frequency patients' samples against age range. Mean (SD): 58.78 (21.598).

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4.3 Hypermiscoviscosity Test

The hypermucoviscosity test or string test was used to observe the phenotype of hypermucoviscosity characteristics of *K. pneumoniae* isolates. It is defined by the formation of viscous strings more than (>) 5 mm in length when a loop is used to stretch the colony on an agar plate; as a result, it was a positive string test. In Table 4.3.1, the positive result (n = 29; (72.5%)) was more than negative result (n = 11; (27.5%)) on these isolates.

Table 4.3.1 shows the tabulation of hypermucoviscosity test results. N = 40 isolates.

Hypermucoviscosity Test	Distribution	
	n	%
Negative (-)	11	27.5
Positive (+)	29	72.5

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4.4 Detection of Multiplex PCR Capsular Serotype K1 and K2 in *Klebsiella pneumoniae* isolates

The Multiplex PCR was performed on the 40 *K. pneumoniae* isolates to identify the presence of *magA* (K1 serotype) and *K2A* (K2 serotype) genes. Based on Table 4.4.1, two isolates were detected positive for K1 gene (n = 2; (5.0%)). The result also showed two isolates were positive for K2 gene (n = 2; (5.0%)); meanwhile, a total of 36 isolates showed absence of K1 and K2 genes (n = 36; (90.0%)). In Table 4.4.1, the appearance of K1 and K2 genes were observed at 1283 bp and 583 bp, respectively as shown in Figure 4.4.1. However, the band of K1 genes was slightly below 1000 bp.

Table 4.4.1 shows the presence of capsular serotypes data on *K. pneumoniae* isolates. N = 40 isolates

Capsular Serotype	Distribution	
	n	%
Not Detected (Non – K1/K2)	36	90.0
K1	2	5.0
K2	2	5.0

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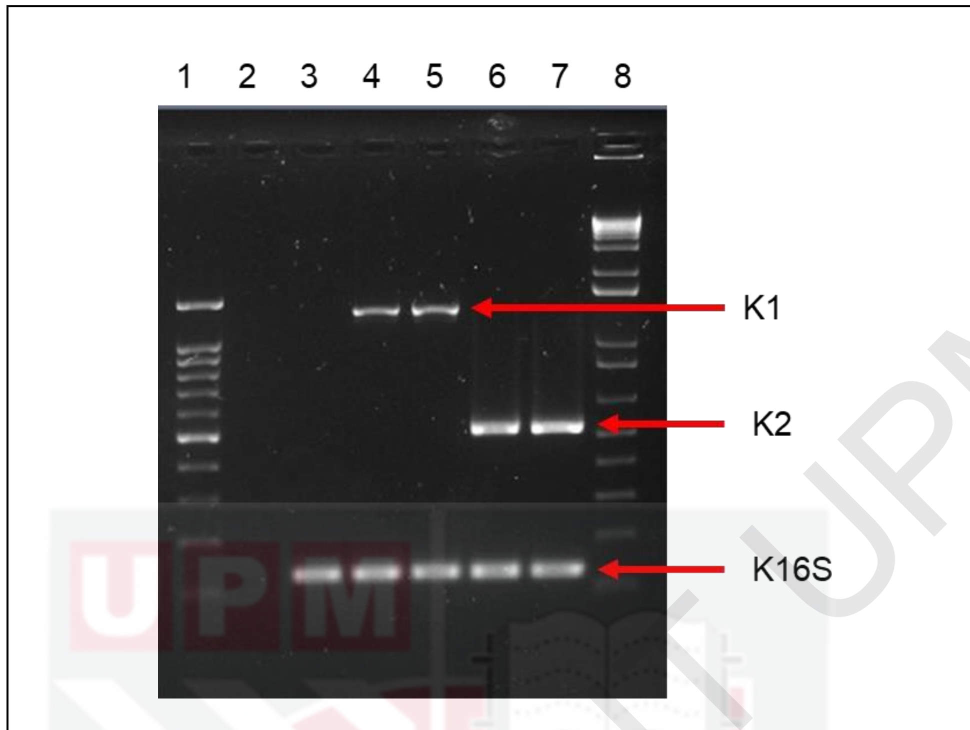

Figure 4.4.1 shows the gel electrophoresis of Multiplex PCR on the presence of K1 and K2 genes in *K. pneumoniae* isolates. Line 1: 100 bp DNA ladder; Lane 2: dH_2O solution (as negative control); Lane 3: No. 42375 (no capsular serotype carried); Lane 4 and 5: representative of K1 serotypes isolates (No. 45554 and No. 45945 respectively) that showed the band at 1283 bp; Lane 6 and 7: representative of K2 serotypes isolates (No. 8996 and No. 6643 respectively) that showed the band at 531 bp; Lane 8: 1000 bp DNA ladder. Note internal control; K16S gene for identification of *K. pneumoniae* isolates were observed for all *K. pneumoniae* isolates.

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4.5 Phylogenetics Tree Analysis

A phylogenetic tree was constructed using MEGA 7.0 software. In Figure 4.5.1, the results of the phylogenetic placement of four *K. pneumoniae* ESBL isolates showed two distinct clusters. Cluster A is represented by capsular serotype K1, while Cluster B is represented by capsular serotype K2. All the results showed 100% data coverage, and bootstrap sampling at 1000 replicates. The reference sequences were retrieved from the NCBI database comprising identity (ID) numbers; EF221827.1 (Taiwan), MF4175542.1 (Brazil), AB355924.1 (Taiwan) and AB085741.1 (Taiwan) for respective their capsular serotype.

Both clusters were completely separated by two different capsular serotypes *K. pneumoniae* isolates. The distribution of sources, presence of ESBL, capsular serotype, string test and reference for respective isolates are shown in the right columns.

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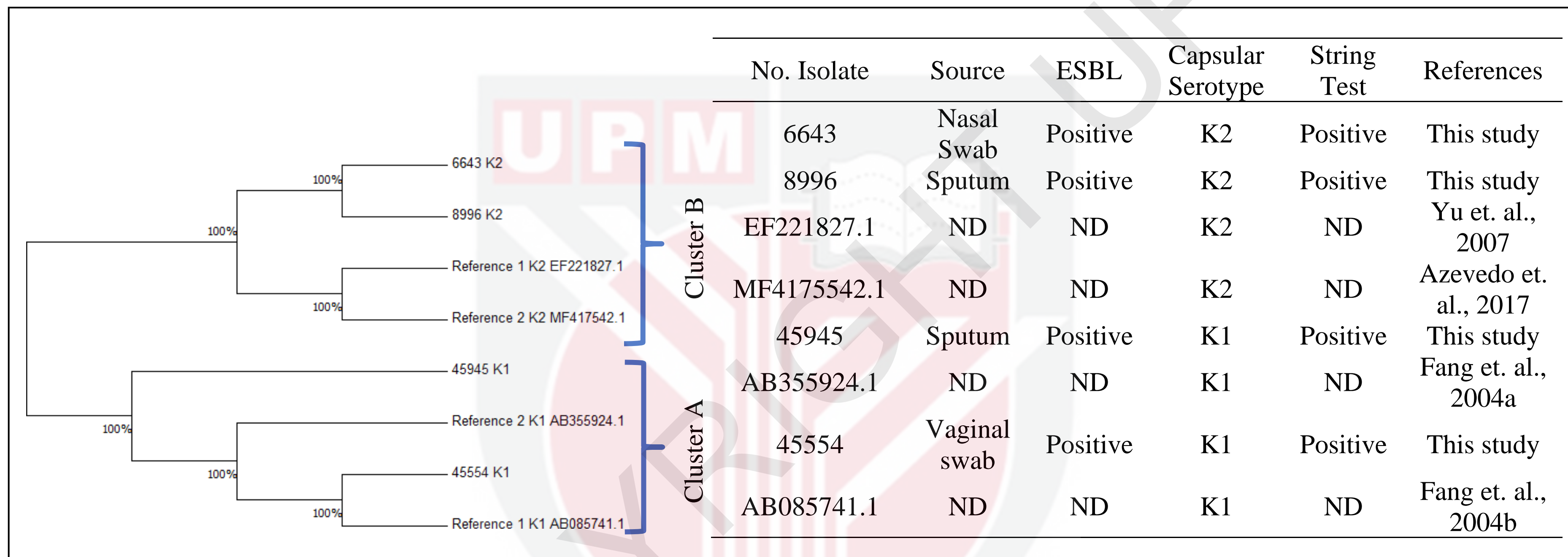

No. Isolate	Source	ESBL	Capsular Serotype	String Test	References
6643	Nasal Swab	Positive	K2	Positive	This study
8996	Sputum	Positive	K2	Positive	This study
EF221827.1	ND	ND	K2	ND	Yu et. al., 2007
MF4175542.1	ND	ND	K2	ND	Azevedo et. al., 2017
45945	Sputum	Positive	K1	Positive	This study
AB355924.1	ND	ND	K1	ND	Fang et. al., 2004a
45554	Vaginal swab	Positive	K1	Positive	This study
AB085741.1	ND	ND	K1	ND	Fang et. al., 2004b

Figure 4.5.1 shown the phylogenetic tree of the positive capsular serotype K1 and K2 on *K. pneumoniae* isolates and the tabulation of information of isolates detected. Note: ND; not detected.

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4.6 Statistical Analysis

Statistical analysis using Chi-Square, X^2 analysis was carried out to identify the significant associations between presence of K1 serotype and hypermucoviscosity test; presence of K2 serotype and hypermucoviscosity test; and presence of K1 and K2 serotypes and hypermucoviscosity test. The significant association was indicated can be if the p-value less than (<) 0.05 with 95% of confidence interval (CI). However, there is a not significant association between the presence of K1 and K2 serotypes and the hypermucoviscosity test toward to *K. pneumoniae* isolates (X^2 (1) = 0.799; p = 0.372) as represent in Table 4.6.1 and Table 4.6.2. Similarly, there also not significant association between K1/K2 and Non – K1/K2 serotypes with hypermucoviscosity test on *K. pneumoniae* isolates (X^2 (1) = 1.686; p = 0.194) as shown in Table 4.6.3

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Table 4.6.1 shows the Chi-square table of association between the presence of K1 serotype with hypermucoviscosity test on *K. pneumoniae* isolates. N = 40 isolates.

	Hypermucoviscosity Test		X_2	dff.	p-value
	Negative	Positive			
K1 Serotype	n (%)	n (%)			
Absent	11 (27.5)	27 (67.5)	0.799	1	0.372
Present	0 (0)	2 (5.0)			

Table 4.6.2 shows the Chi-square table of association between the presence of K2 serotype with hypermucoviscosity test on *K. pneumoniae* isolates. N = 40 isolates

	Hypermucoviscosity Test		X_2	dff.	p-value
	Negative	Positive			
K2 Serotype	n (%)	n (%)			
Absent	11 (27.5)	27 (67.5)	0.799	1	0.372
Present	0 (0)	2 (5.0)			

Table 4.6.3 shows the Chi-square table of association between the presence of K1/K2 and Non – K1/K2 serotype with hypermucoviscosity test on *K. pneumoniae* isolates. N = 40 isolates.

	Hypermucoviscosity Test		X_2	dff.	p-value
	Negative	Positive			
Capsular Serotype	n (%)	n (%)			
Non – K1/K2	11 (27.5)	25 (62.5)	1.686	1	0.194
K1/K2	0 (0)	4 (10.0)			

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CHAPTER 5

DISCUSSION

5.1 Overview

In the Enterobacteriaceae family, *Klebsiella pneumoniae* is an important microorganism associated with nosocomial infections. *K. pneumoniae* is a gram-negative bacterium and appears mucoid - lactose fermenters on MacConkey agar. When grown on blood agar, *K. pneumoniae* showed no haemolysis or γ – haemolysis as described by Hall (2013). As shown in Chapter 4, Table 4.1.1, a range of biochemical tests needs to be carried out to confirm the identification of *K. pneumoniae*. The biochemical tests described were Triple Sugar Iron (TSI) test, SIM test to observe the mortality patterns and the production of sulphide and indole, urea test, oxidase test, and citrate test.

In comparison to the classical *K. pneumoniae*, hypervirulent *K. pneumoniae* isolates has been reported to cause more serious infections in both immunocompromised and healthy individual in the community settings. This may be due to their high resistant against in vitro killing and phagocytosis by serum, neutrophils and macrophages as reported in other studies. Moreover, there is an increasing of other invasive infections such as liver abscess, pneumoniae and sepsis, being reported worldwide caused by hypervirulent *K. pneumoniae* isolates (Choby et al., 2019). In this study, the bacterial isolates were originated from urine, nasal swab, tissues, and sputum. However, Siu et al. (2012) upheld that they carry out more clinical manifestations compared to the types of samples collected in the current study. In addition, more attention is related to the metastatic infection with underlying diseases

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that occur in patients presented, especially liver abscess. The study carried out rapid detection of multiplex PCR test for the confirmation of the capsular serotypes K1 and K2 kvKP. Hence, more evidence is needed to relate the sites of infections with the occurrence of *K. pneumoniae* K1 and K2 serotypes for future study.

Based on the age range, majority of the patients were between 81 to 90 years old. No data was collected on early age on 0 to 10 years old range patient. This reason showed that the older patients had a higher risk of being infected by *K. pneumoniae* isolates than younger patients, especially infants or children. Laupland & Church (2014) and Guo et al. (2017) agreed that the older person is easily infected with this isolate due to comorbid illness and immunosuppressed compared to younger patients. However, some points of view from Khaertynov et al. (2017) and Podschun & Ullmann (1998) expressed that an increasing number of these isolates involved outbreaks and close contact in the hospital. Otherwise, raise issues on AMR, especially multidrug resistance *K. pneumoniae,* and unexpected infection occurs in neonatal patients such as meningitis and sepsis. In brief, all age ranges can be infected, especially the potential host suitable and survive for these isolates; indeed, alert from hospital staff to prevent it from being exposed to the community.

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5.2 Distribution and Occurrence of *Klebsiella pneumoniae* ESBL in clinical isolates

This study showed that all samples were EBSL producing *K. pneumoniae* isolates. Potentially, this study is believed to have some interest in other types of AMR such as multidrug resistance, carbapenem resistance, and others as highlighted in the previous studies (C. Lee et al., 2017). In Malaysia, *K. pneumoniae* isolates have been increasingly reported as the cause of antimicrobial resistance (AMR) together with *Escherichia coli, Staphylococcus aureus* and other microbes, as described by Meer et. al. (2019). Similarly, various types of were AMR found in the population in Malaysia as stated by Ministry of Health Malaysia (2019). In addition, the ESBL producing *K. pneumoniae* isolates were reported by Mobasseri et al. (2020) study. However, this study was limited about the presence of ESBL detection by qualitative of each antibiotic used. It gives the answer relative between in-hospital and the AMR issues in this study.

This study was showed that presence of capsular serotypes K1 and K2 among these isolates collected. It means this study was determined their objective. However, the amount of K1 and K2 serotypes are equal. Remya et al. (2018) highlighted that the prevalence of K2 was higher than K1 serotypes. Nonetheless, other observation form C. Liu et al. (2018) stated that K1 serotypes (n = 18/73; 24.7%) was higher compared to K2 serotypes (n = 9/73; 12.3%). Alternatively, this study professed that will must on going at around our hospital in Malaysia on future. With hopes, it makes more information and discovery for our good health systems.

Hypermucoviscosity phenotypes can detect their characteristics by string test where it must pass more than 5 mm length stretched out. This study was tabulated the positive string test was more shows hypermucoviscosity phenotypes than negative.

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Interestingly, in Taiwan, 88.8% of isolates collected from patients with extrahepatic abscesses had the *K. pneumoniae* hypermucoviscosity phenotypes (Ku et al., 2008). Similarly, 41.5% of *K. pneumoniae* bacteremia caused *K. pneumoniae* hypermucoviscosity phenotypes (C. H. Lee et al., 2006). Moreover, in their previous study, they used this same method for known the hypermucoviscosity phenotypes and classified hvKp. However, Catalán-nájera et al. (2017) believed hypermucoviscosity and hypervirulent; both are different phenotypes defining hypermucoviscosity by a positive string test. At this moment's Lin et al. (2020) agreed that the accuracy in the definition of hypervirulent might not be hypermucoviscosity. Some suggest improving the definition of hypervirulent by using detection of *rmpA* genes for the presence of combined hypervirulent and hypermucoviscosity phenotypes as supported by several previously studied (Harada & Doia, 2018; Wyres et al., 2020). Still, this study is aware that this method is one indicator before further steps because it low budgets, cuts time-consuming, and handles easily.

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5.3 PCR Analysis as Rapid Detection Method for the Detection of K1, K2, and Other Serotypes

This study found two of K1 and K2 serotypes respectively in 40 isolates. And, in this believes they have several interest serotypes in non – K1/K2 isolates. This study added one additional primer, K16S (around 150 pb), as the internal control for identifying the *K. pneumoniae* isolates. The study conducted by Remya et al. (2018) showed that six out of 370 *K. pneumoniae* isolates were detected positive for K2 serotype using Multiplex PCR. On the other hand, only one K1 serotype was observed in this study. However, no internal control was included in this analysis.

Another study by Zhao et al. (2020) identified five *K. pneumoniae* serotypes by PCR. These include K1, K2, K5, K54, and K64, among the hvKp isolates. The highest serotypes reported in their study were K1 (25.5%), K2 (20%), K5 (2%), K54 (4%), and K64 (8%). Similarly, I. R. Lee et al. (2016) highlighted K1 was the most common (64.3%, 45/70), followed by K2 (20%, 14/70), K5 (5.7%, 4/70) and K57 (2.9%, 2/70). K16, K28, K63 using allele-specific PCR.

In conclusion, the PCR method is a better method for detecting the capsular serotypes. In addition, it can be the gold standard if detection of the interested primer were classified as causing hvKp at one time only, especially by Multiplex PCR.

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5.4 Phylogenetics Tree Analysis

In this study, K1 and K2 serotypes were separated into two clusters by phylogenetics tree analysis. This analysis was based on the DNA sequencing of capsular serotypes genes; *magA* and *K2A* sequences. However, the presence of other virulence genes such as *kfu* (ferric uptake transporter), *iuc* (aerobactin siderophore), *iro* (salmochelin siderophore) and *irp* (yersiniabactin siderophore) genes were not determined in this study. Therefore, the genetics background of K1 and K2 serotypes of these isolates were not investigated. Previous studies showed that other analyses such as Whole Genome Sequencing (WGS) (Lin et al., 2020), Multilocus Sequencing Types (MLST) (Yu et al., 2007) and Pulse Field Gel Electrophoresis (PFGE) patterns (Yeh et al., 2007) maybe used to compare the genetics background between different *K. pneumoniae* serotypes.

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CHAPTER 6

CONCLUSION

6.1 Conclusion

In conclusion, this study was showed the high occurrence of non-K1/K2 (90%) as compared to K1 and K2 serotypes (10%) were detected by Multiplex PCR analysis. This means the starting point for discovering capsular serotypes in *K. pneumoniae* isolates, especially in clinical consents. K1 and K2 serotypes were separated into two clusters by phylogenetic tree analysis. However, the genetic relatedness between each other these isolates were not determined. Interestingly, this study highlighted that Multiplex PCR may be utilized as a rapid and specific analysis for the detection method for the detection of K1 and K2 in *K. pneumoniae* isolates.

6.2 Recommendations for future studies

Further surveillance of *K. pneumoniae* isolates from a larger number of clinical isolates is important to provide a better understanding and evaluation of this organism's genotypic characteristics and resistance mechanisms. Also, it increases another capsular serotype primer to explore and investigate the occurrence of these isolates related diseases and AMR. Some suggestions in phylogenetics tree analysis by other alternative methods such as MLST can be performed to determine the genetic background of ESBL producing *K. pneumoniae* clinical isolates.

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APPENDICES

A. LIST OF THE ISOLATES' INFORMATION

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NO	Identification number	Gender	Age	Biological specimen	Hypermucoviscosity testing (String test) (mm)	HvT (+, -)	Capsular serotype K1 (1000b)	Capsular serotype K2 (600b)	Comment
1	47325	Female	61	Tissue	4	-	-	-	
2	47257	Female	15	Sputum	5	+	-	-	
3	47166	Female	47	Urine	8	+	-	-	
4	47113	Male	31	Sputum	>10	+	-	-	
5	47012	Male	72	Sputum	5	+	-	-	
6	46349	Female	71	Sputum	10	+	-	-	
7	46329	Female	55	Body fluids	>10	+	-	-	
8	46259	Male	80	Urine	7	+	-	-	
9	46075	Male	19	ETT	>10	+	-	-	
10	45945	Male	50	Sputum	10	+	+	-	
11	45837	Female	89	Sputum	10	+	-	-	2000b
12	45554	Female	71	Vaginal swab	>10	+	+	-	
13	45467	Male	60	Sputum	10	+	-	-	3000b
14	45466	Male	72	Sputum	7	+	-	-	
15	45446	Male	33	Sputum	7	+	-	-	

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16	45445	Female	48	Sputum	6	+	-	-	2000b
17	45406	Male	84	Sputum	7	+	-	-	
18	45239	Male	47	Blood	6	+	-	-	
19	45150	Male	31	Sputum	6	+	-	-	
20	45147	Male	70	Sputum	4	-	-	-	
21	42828	Male	19	Sputum	1	-	-	-	
22	42706	Female	80	Sputum	6	+	-	-	
23	42595	Male	85	Sputum	0	-	-	-	
24	42434	Female	91	Sputum	2	-	-	-	
25	42375	Male	80	Sputum	5	+	-	-	
26	42302	Female	91	Nasal swab	7	+	-	-	
27	42297	Female	54	Sputum	0	-	-	-	2000b
28	42250	Female	45	Sputum	3	-	-	-	
29	41901	Male	79	Sputum	4	-	-	-	
30	41780	Female	81	Nasal swab	10	+	-	-	
31	41743	Male	51	Sputum	1	-	-	-	
32	41677	Male	58	Sputum	>10	+	-	-	
33	41608	Female	52	ETT	5	+	-	-	
34	8996	Male	80	Sputum	8	+	-	+	

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35	8047	Male	64	Sputum	>10	+	-	-	
36	7460	Female	50	Sputum	0	-	-	-	
37	7193	Male	63	Tissue	5	+	-	-	2000b
38	7013	Male	60	Urine	6	+	-	-	
39	6643	Male	47	Nasal swab	8	+	-	+	
40	6107	Female	15	Tracheal aspirate	0	-	-	-	

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