UNIVERSITI PUTRA MALAYSIA

MOLECULAR TYPING OF HYPERVIRULENT GENES IN Klebsiella pneumoniae RECOVERED FROM CLINICAL ISOLATES

AININ FARAH HANNAN BINTI ROSLI

lp
FPSK2 2020 37

ABSTRACT

MOLECULAR TYPING OF HYPERVIRULENT GENES IN *Klebsiella pneumoniae* RECOVERED FROM CLINICAL ISOLATES

Ainin Farah Hannan Rosli[1], Nurshahira Sulaiman[1], Mohd Nasir Mohd Desa[1], Siti Norbaya Masri[2]

Department of Biomedical Sciences[1], Department of Medical Microbiology and Parasitology[2], Faculty of Medicine and Health Sciences, Universiti Putra Malaysia

Introduction: *Klebsiella pneumoniae* is a gram-negative, rod-shaped bacterium, non-motile, appears as mucoid and can be found in soil, human's skin and intestine. Infections due to hypervirulent *Klebsiella pneumoniae* capsular serotypes K1 and K2 are increasingly reported in patients diagnosed with liver abscess, meningitis, and nosocomial infection. This may be attributed by the presence of polysaccharide capsule, adhesions and aerobactin production. Furthermore, the treatment for *K. pneumoniae* infection is challenging with its ability to produce extended-spectrum beta-lactamases (ESBLs) which caused resistant against almost all beta-lactam antibiotics except carbapenems. **Objective:** The current study is conducted to detect the presence of capsular serotypes K1 and K2 hypervirulent genes in *K. pneumoniae* from the ESBLs clinical isolates. **Hypothesis:** The strains of *K. pneumoniae* with ESBL gene isolates from clinical specimen are expected to have hypervirulent genes. **Methodology:** Ethics clearance is obtained from UPM Medical Research Ethics Committee. A total of 44 *K. pneumoniae* ESBL producing isolates were obtained from Hospital Pakar Sultanah Fatimah. Hypermucoviscosity test (String test) was carried out to observe the formation of viscous string of K. pneumonia isolates with more than 5mm in length. Extraction of the DNA was done using a commercial extraction kit. Multiplex Polymerase Chain Reaction (PCR) was used to detect the presence of K1 and K2 capsular genes. Visualization of the bands on 1.7% agarose gel for the capsular serotypes K1 and K2 were observed with the presence of 1283bp and 531bp of product size respectively. **Results**: Biochemical test analysis showed that all the isolates were lactose fermenters and gamma hemolysis. Out of 44 isolates, 35 isolates (79.5%) showed hypermucoviscous phenotype. However, only a small proportion of these isolates were positive for K1 (5%) and for K2 (10%) serotypes detected by Multiplex PCR. **Conclusion**: 79.5% of positive hypermucoviscosity phenotype does not significantly associated with K1/K2 serotypes. 82% of the ESBL K. pneumoniae clinical isolates were associated with non-K1/K2 serotype. This might be associated to other 76 capsular serotypes.

Keywords: Klebsiella pneumoniae, hypervirulent, extended-spectrum beta-lactamases (ESBLs), Multiplex PCR

© COPYRIGHT UPM

ABSTRAK

PENILAIAN MOLEKUR TERHADAP HIPERVIRULEN GENETIK KLEBSIELLA PNEUMONIAE DARI ISOLAT KLINIKAL

Ainin Farah Hannan Rosli[1], Nurshahira Sulaiman[1], Mohd Nasir Mohd Desa[1], Siti Norbaya Masri[2]

Jabatan Sains Bioperubatan[1], Jabatan Mikrobiologi dan Parasitologi Perubatan[2], Fakulti Perubatan dan Sains Kesihatan, Universiti Putra Malaysia

Pendahuluan: *Klebsiella pneumoniae* ialah sejenis bakteria gram negatif, ia tidak mempunyai mekanisma pergerakan, berbentuk bujur, hadir dalam keadaan mucoid dan kebiasaannya bakteria ini hidup di dalam tanah, pada permukaan kulit, and usus manusia. Jangkitan yang disebabkan oleh hipervirulen *K. pneumoniae* serotaip K1 dan K2 dilaporkan semakin meningkat di kalangan pesakit yang menghidapi abses hati, meningitis dan jangkitan nosokomial. Ini mungkin disebabkan oleh factor virulen seperti kehadiran kapsul, lekatan yang kuat pada permukaan tisu, dan penghasilan aerobaktin.Rrawatan untuk merawat jangkitan yang berpunca daripada *K. pneumoniae* adalah lebih mencabar dengan kehadiran enzim Extended Spectrum Beta Lactamase (ESBLs) kerana ia menyebabkan tindakbalas kebal terhadap antibiotic "extended-spectrum". **Objektif:** Tujuan kajian ini dijalankan adalah untuk mengesan kehadiran kapsular gen K1 dan K2 di kalangan isolate ESBL *K. pneumoniae.* **Hipotesis:** Serotaip *K. pneumonie* K1 dan K2 dijangka dapat dikesan di kalangan isolate ESBL *K. pneumoniae* menggunakan teknik Multiplex PCR. **Metodologi:** Kajian ini telah mendapat kelulusan daripada Jawatankuasa Etika Universiti Melibatkan Manusia (JKEUPM). Sejumlah 44 isolat *K. pneumoniae* ESBL telah diperoleh dari Hospital Pakar Sultanah Fatimah. Ujian hipermukoviskositi dijalankan untuk memerhati penghasilan benag likat melebihi 5mm daripada isolat K. pneumoniae. Pengekstrekan DNA dibuat dengan menggunakan kit komersial pengekstrekan. Multipleks tindakbalas rantai polimerase (PCR) telah digunakan untuk mengesan kehadiran saiz produk gen K1 (1283bp) dan K2 (531bp) pada gel agarose (1.7%)/ **Keputusan:** Analisis ujian biokimia telah menunjukkan bahawa semua isolat adalah penapai laktosa dan gamma hemolisis. Tigapuluh lime daripada 44 isolat (79.5%) daripadanya menunjukkan fenotipik hipermukoviskositi. Dalam kajian ini, hanya 5% dikesan sebagai serotaip K1 dan 10% dikesan sebagai serotaip K2 melalui ujian Multiplex PCR. **Konklusi:** 79.5% positif fenotaip hipermukoviskositi adalah tidak signifikan dengan serotaip K1/K2. 82% dari ESBL *K. pneumoniae* telah dikesan sebagai serotaip bukan-K1/K2 di mana ia mungkin merangkumi dalam kalangan 76 kapsular serotaip.

Kata kunci: Klebsiella pneumoniae, hipervirulen, extended-spectrum beta-lactamases (ESBLs), Multipleks PCR

© COPYRIGHT UPM

ACKNOWLEDGEMENT

First of all, thanks to Allah S.W.T for His mercy and guidance in giving me full strength in completing this final year project. I finally managed to finish up this research with great enthusiasm and determination even facing with some difficulties.

Next, I would like express my special thanks of gratitude to my beloved supervisor, Dr. Nurshahira Sulaiman for providing her invaluable guidance, comments, and suggestions throughout the course of the project. I truly appreciate her help as without her, I would not be able to complete this study. I would also like to extend my gratitude to my co-supervisors, Associate Prof. Dr. Mohd Nasir Mohd Desa and Dr Siti Norbaya Masri for giving me the opportunity to perform this research and sharing the knowledge in the microbiology fields.

Not only that, I would like to thank Mr. Sabri, staff of Applied Microbiology Laboratory for being helpful in preparing all materials and instruments that had been used throughout my study. My sincere thanks also go to post-graduate students, Mr Abdul Rahman and Miss Diana for helping, supporting me continuously and giving guidance to me since the first day of my study until the end.

In addition, I would also like to give my appreciation to my parents for their unconditional understanding, prayers and sacrifice as well as giving their financial and moral support all the time. I am also thankful to my fiance, Muhammad Mujib Khairul Amri for supporting me and keeping me motivated whenever I felt down and pressured. Finally, a big thank you for everyone that has been involved in this study either directly or indirectly and given me support and strength to complete this study. Thank you.

© COPYRIGHT UPM

TABLE OF CONTENTS

Contents

ABSTRACT .... **Error! Bookmark not defined.**
ABSTRAK .... **Error! Bookmark not defined.**
ACKNOWLEDGEMENT .... **Error! Bookmark not defined.**
APPROVAL .... **Error! Bookmark not defined.**
DECLARATION .... **Error! Bookmark not defined.**
TABLE OF CONTENTS .... **Error! Bookmark not defined.**
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF ABBREVIATIONS xii
CHAPTER
1.0 INTRODUCTION 1
1.1 Background 1
1.2 Objectives 2
1.2.1 General objective: 2
1.2.2 Specific objectives: 2
1.3 Hypothesis 2
2.0 LITERATURE REVIEW 5
2.1 The morphology of *Klebsiella pneumoniae* 5
2.2 Methods used to screen hypervirulent 6
2.2.1 Antimicrobial Susceptibility Testing (AST) 6
2.2.2 Virulence genetic characteristics of *K. pneumoniae* 6
2.2.3 Hypermucoviscosity Test (HV Test) 7
2.2.4 Capsular Swelling Test 7
2.2.5 Complement C3 8
2.3 Symptoms of hypervirulent *Klebsiella pnumoniae* which cause to liver abscess 8
2.4 Virulence factors that cause *Klebsiella pneumoniae* invades into deep organs 8
2.4.1 Capsular polysaccharide 8
2.4.2 Aerobactin production 9
2.4.3 *Klebsiella pneumoniae* with Extended Spectrum Beta-Lactamase (ESBL) gene 9
2.5 Molecular detection using Multiplex PCR 9

© COPYRIGHT UPM

3.0 METHODOLOGY 11
3.1 Sample collection 11
3.2 Bacteria Identification 11
3.3 Bacteria cultivation 14
3.3.1 Lysogeny Broth (LB Agar) 14
3.3.2 MacConkey Agar 14
3.4 Preservation and Sub-culturing 16
3.5 Hypermucoviscosity Testing (String Test) 17
3.6 DNA extraction 18
3.7 PCR Assay for K1 and K2 Genes Detection 23
4.0 RESULT 29
4.1 Bacterial Strains and Morphological Identification of *Klebsiella pneumoniae* 29
4.2 Hypermucoviscosity Test (String test) of *K. pneumoniae* 32
4.3 Detection of capsular serotype K1 and K2 32
4.4 Statistical analysis 33
5.0 DISCUSSION 37
5.1 Overview 37
5.2 Occurrence of ESBL *K. pneumoniae* in clinical isolates 37
5.3 Hypermucoviscosity testing (String test) of *Klebsiella pneumoniae* 38
5.4 Identification of capsular serotype K1 and K2 38
5.3 Diseases associated with *K. pneumoniae* serotypes K1 and K2 39
5.4 PCR analysis as rapid detection method of K1 and K2 serotypes 39
6.0 CONCLUSION 40
6.1 Conclusion 40
6.2 Recommendation for future study 40
REFERENCES 42
APPENDICS 48

© COPYRIGHT UPM

LIST OF TABLES

Table	Page
Table 2.1.1: Media culture and *Klebsiella pneumoniae* colony characteristics	**5**
Table 2.5.1: Molecular detection of capsular serotype K1 and K2	**10**
Table 3.7.1: DNA Sequences of K1 and K2	**23**
Table 3.7.2: Multiplex PCR components for K1 and K2 capsular serotype detection	**23**
Table 3.7.3: Cycling condition for Multiplex PCR	**24**
Table 4.4.1: Population of patients based on age	**33**
Table 4.4.2: Descriptive statistic	**35**
Table 4.4.3: Association between hypermucoviscosity and K1 serotype	**35**
Table 4.4.4: Association between hypermucoviscosity and K2 serotype	**35**
Table 4.4.5: Association between hypermucoviscosity and K1/K2 serotypes	**36**

© COPYRIGHT UPM

LIST OF FIGURES

Figure	Page
Figure 2.2.3.1: Structure of *Klebsiella pneumoniae*	**7**
Figure 3.1.1: *Klebsiella pneumoniae* from clinical samples	**11**
Figure 3.2.1: Gram staining	**13**
Figure 3.3.2.1: Klebsiella pneumoniae colonies on MacConkey agar 5% Blood sheep agar	**15**
Figure 3.3.2.2: Klebsiella pneumoniae on blood agar	**16**
Figure 3.4.1: MacConkey and glycerol solution	**17**
Figure 2.2.3.3: Structure of *Klebsiella pneumoniae*	**7**
Figure 3.1.1: *Klebsiella pneumoniae* from clinical samples	**11**
Figure 3.4.1: Gram staining	**13**
Figure 3.3.2.1: Klebsiella pneumoniae colonies on MacConkey agar 5% Blood sheep agar	**15**
Figure 3.3.2.2: Klebsiella pneumoniae on blood agar	**16**
Figure 3.4.1: MacConkey and glycerol solution	**17**

© COPYRIGHT UPM

Figure 3.6.1: Autoclave all centrifuge tubes for DNA preparation 20

Figure 3.6.2: The DNA extraction kit including readymade buffers such as BW, BL, TW and AE solution 21

Figure 3.4.3: Column tube in balance position in centrifuge machine 22

Figure 3.7.1: Cycling condition for Multiplex PCR 24

Figure 3.7.2: Samples in PCR machine column 25

Figure 3.7.3: PCR machine 25

Figure 3.7.4: Samples run in gel electrophoresis 27

Figure 3.7.5: Bands view under UV Transilluminator 28

Figure 3.7.6: Visualization of K1 nad K2 bands on agarose gel 28

Figure 4.1.1: Morphology of Klebsiella pneumoniae under microscope 29

Figure 4.1.2: The growth of Klebsiella pneumoniae on A) Lysogeny Broth (LB) agar, B) MacConkey and C) Blood agar 30

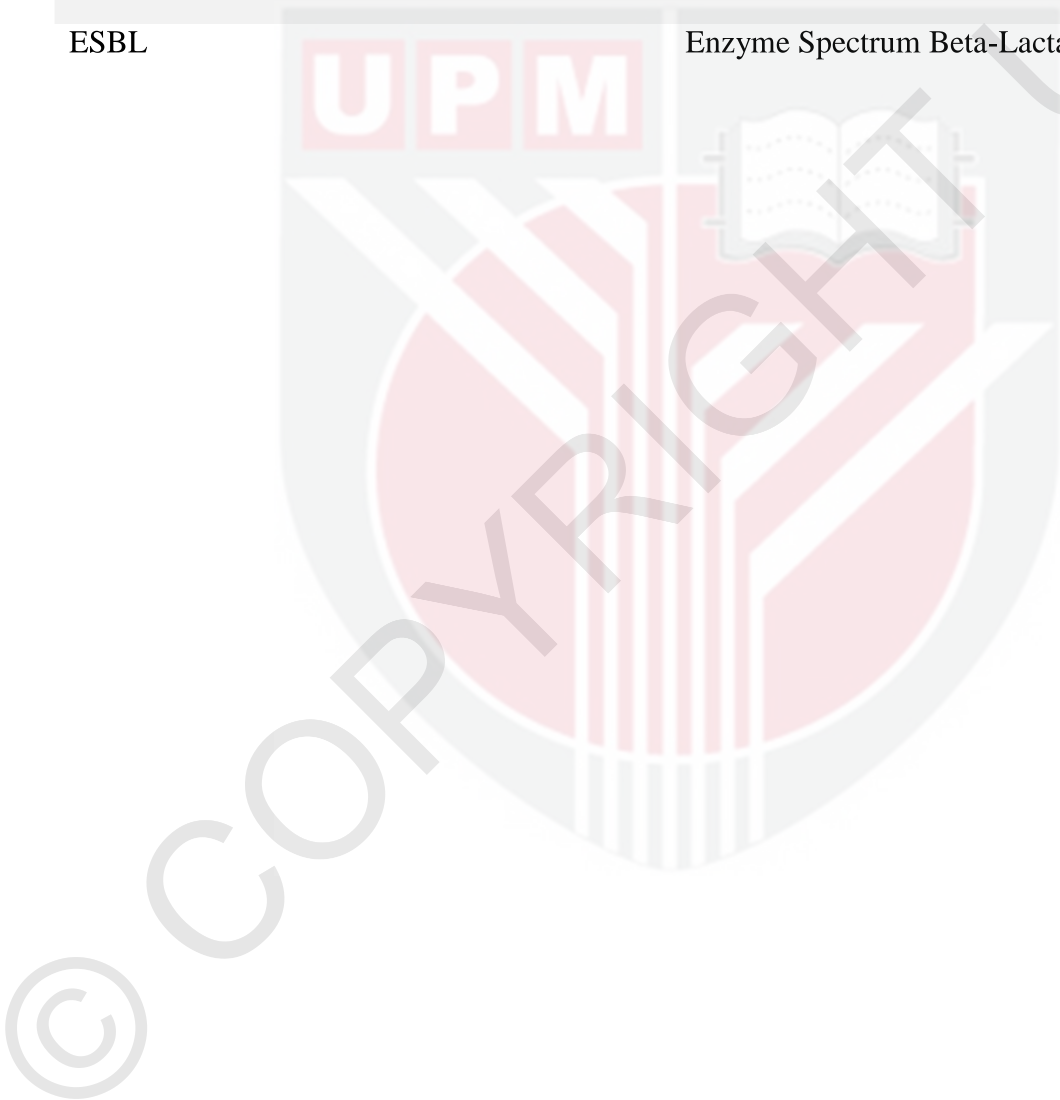
Figure 4.1.3: Hypermucoviscosity Test showing the string more than 5mm 31

Figure 4.3.1: Visualization of K1 and K2 bands on agarose gel 33

© COPYRIGHT UPM

LIST OF ABBREVIATIONS

K. pneumoniae	*Klebsiella pneumoniae*
HvKp	Hypervirulent *Klebsiella penumoniae*
KLAS	*Klebsiella pneumoniae* liver abscess
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
SPSS	Statistical Package for the Social Sciences
ESBL	Enzyme Spectrum Beta-Lactamase



© COPYRIGHT UPM

CHAPTER ONE

INTRODUCTION

1.1 Background

Klebsiella pneumoniae is a gram-negative bacterium which capsulated with rod shape. It appears with mucoidal characteristic once it has been cultured on Mac Conkey agar. *Klebsiella pneumoniae* is a pathogen of human respiratory system which can be transmitted through person-to-person contact. These bacteria can be found in soil, human skin and intestine. People who had been infected by *K. pneumoniae* may experience some symptoms such as clamminess and blueness skin, low blood pressure, loss of appetite, fatigue muscular, pain on joints and headaches. Most patients have cough with sputum and phlegm, shortness of breath and pleuritic chest pain as the *K. pneumoniae* attack their lungs.

The diseases that caused by *K. pneumoniae* might be due to their virulence factors. *K. pneumoniae* having virulence factors makes them called as hypervirulent *Klebsiella pneumoniae.* Virulence factor is generally defined as molecules that produced by *K. pneumoniae* to enhance their achievement to colonization into the host. Some common virulence factors are polysaccharide capsule, adhesions and aerobactin production. Polysaccharide capsule is an extracellular structure made up of long polysaccharide chains for bacterial capsule. It protects the bacteria cells from environmental dangers such as phagocytosis and antibiotics. Hypervirulent *Klebsiella pneumoniae* (hvKp) have polysaccharide capsule with larger size compared to classiscal *Klebsiella pneumoniae* (cKp). We can find serotype K1 and K2 at hypercapsule area in polysaccharide capsule. By detecting these two serotypes, we can indicate that *K. pneumoniae* is a hvKp. Next, adhesion is the cell-surface components. It helps *K. pneumoniae* to attach to different host cells which a major cause to infections especially nosocomial infections. Nosocomial infections are infections that have been caught in a hospital and potentially cause by organisms with

© COPYRIGHT UPM

resistant to certain antibiotics. Besides, aerobactin production only present in hyKp. It will cause to increasingly resistant to antimicrobials via acquisition of mobile elements carrying resistance determinants.

Apart from that, according to the previous study, Helmi U. et al, 2016, had detected 141 from 161 clinical samples with *K. pneumoniae* isolates that have Extended Spectrum Beta-Lactamase (ESBL) gene. It is an enzyme that resistant to most penicillins, cephalosporins and monobactam but not to carbapenems. Due to resistant to most of antibiotics, *K. pneumoniae* causes to bloodstream infection associated with pneumonia, urinary tract infection, meningitis and etc. Thus, the main purpose of this research is to continue with further experiment to detect hypervirulent genes in *K. pneumoniae* with ESBL.

SENTRY Antimicrobial Surveillance Program was conducted a study from year 1997 to 2016 about Distribution of resistant phenotypes by infection sources. Castanheira et all, 2019 had done a study to make a comparison among *Klebsiella pneumoniae, Escherichia coli* and few bacteria with ESBL gene towards few main infection sources. There were three out of four infection sources had reached the highest rank in *K. pneumoniae* with ESBL such as skin and skin structure, urinary tract, and pneumonia in hospitalized patients.

Klebsiella pneumoniae is increasingly reported with its ability to cause infections and diagnosed with liver abscess, pneumonia, meningitis, and nosocomial infection which proven by many previous researches. Primary *Klebsiella penumoniae* liver abscess complicated with metastatic meningitis or endophthalmitis is a globally emerging infectious disease and this remains unclear (Fang C. et al, 2004). Besides, infections because of hvKp are increasingly recognized worldwide and its ability to cause organ and life-threateing disease in healthy individuals from the community merits concern (Sellick J. et al, 2018). *Klebsiella pneumoniae* liver abscess (KLAS) is an emerging invasive infection caused by highly

© COPYRIGHT UPM

virulent community-acquired strains of *K. pneumoniae* displaying hypermucoviscosity (Vila A. et al, 2011). Hence, this study is important to identify the hypervirulent of ESBL *Klebsiella pneumoniae* which indicated by detection of capsular serotypes K1 and K2.

© COPYRIGHT UPM

General objective:

This study aims to genotype the *K. pneumoniae* ESBL producers for capsular serotypes K1 and K2 by multiplex PCR.

Specific objectives:

1. To observe the distribution of *Klebsiella pneumoniae* K1, K2 or non-K1/K2 isolates in the clinical samples using multiplex PCR
2. To determine the hypermucoviscosity phenotype of *Klebsiella pneumoniae* using String test
3. To identify the distribution of capsular serotypes K1 and K2 using molecular approaches

© COPYRIGHT UPM

CHAPTER TWO

LITERATURE REVIEW

2.1 The morphology of *Klebsiella pneumoniae*

Klebsiella pneumoniae is a short, plump, and have straight rod shape (bacillus) bacterium. It has about 1–2 μm × 0.5–0.8 μm (micrometer) of size. *K. pneumoniae* is arranged singly, in pairs, or in short chains and sometimes in clusters. It is generally comes with mucoid which gummy or wet looking colonies. It is gram-negative bacteria which can be found in soil, human's skin and intestine. It can be transmitted through person-to-person contact. *K.pneumoniae* is very pathogenic to human respiratory system.

The genus is named for German physician and bacteriologist Edwin Klebs. *Klebsiella pneumoniae*, also called Friedländer's bacillus, was first described in 1882 by German microbiologist and pathologist Carl Friedländer. *K. pneumoniae* is best known as a pathogen of the human respiratory system that causes *pneumoniae.*

Table 2.1.1: 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	Ye5llow coloured colonies

© COPYRIGHT UPM

2.2 Methods used to screen hypervirulent

Hypervirulent *Klebsiella Pneumoniaee* (hvKp) is an evolving pathotype that is more virulent than classical K. pneumoniae (cKp). hvKp usually infects individuals from the community, who are often healthy. Infections are more common in the Asian Pacific Rim but are occurring globally. There are some methods that commonly used by scientists to detect hvKp.

2.2.1 Antimicrobial Susceptibility Testing (AST)

AST also called as Disk Diffusion Test which defined as an antibiotic susceptibility test to determine of what choice of antibiotics should be used to treat infection. Following the incubation, the diameter of the inhibition zone for each antibiotic will be measured and interpreted as susceptible, intermediate and resistant. Ujir et al, 2014 did a study on the distribution of ESBL genes and activities in clinical isolates of *K. pneumoniae* at a district hospital in Malaysia. All K. pneumoniae isolates were tested against antibiotics from cephems, groups, monobactam, penicillins, carbapenems, beta-lactam/beta-lactamase inhibitor, aminoglycosides, fluoroquinolones and folate pathway inhibitor groups (Ujir et al, 2014).

2.2.2 Virulence genetic characteristics of *K. pneumoniae*

The pneumonia is caused by *K. pneumoniae* strains at were encoded following percentage of virulence genes as FIM-H 85 (88.5%), mrkD 80 (83.4%), magA 22 (22.9%), K2 25 (26.0%), rmpA 62 (64.6%), uge 74 (77.0%), kfu 33 (34.3%), and aerobactin 63 (65.6%). Virulence genes of *K. pneumoniae* such as K1, K2, rmpA, uge, kfu and aerobactin were strongly associated with hvKp than cKp. There is no significant difference was found in FIM-1 and MrKD3 genes (Shah et al, 2017).

© COPYRIGHT UPM

2.2.3 Hypermucoviscosity Test (HV Test)

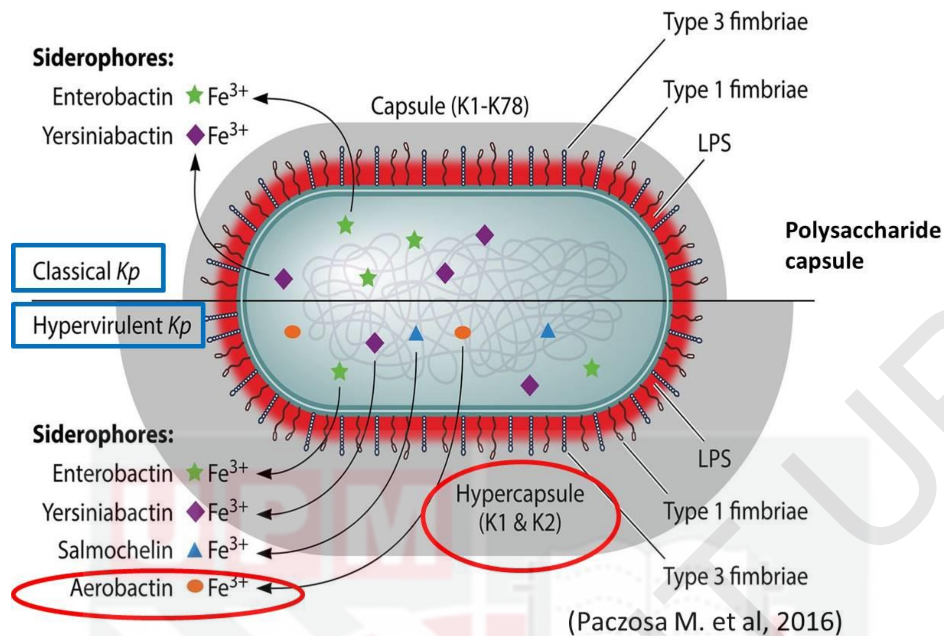


Figure 2.2.3.1: Structure of Klebsiella pneumoniae

According to the Figure 1, hypermucoviscous phenotypes are encapsulated strains of *K. pneumoniae* commonly in K1 and K2 serotypes that generate larger amounts of extracapsular polysaccharide constituting a mucoviscous web that protects these strains from phagocytosis by neutrophils and from serum killing by complement (Vila A et al, 2011). The positive result will be demonstrated by the formation of viscous string more than 5mm in length indicating of high hypervirulent of *Klebsiella pneumoniae*.

2.2.4 Capsular Swelling Test

The capsular swelling test is done to identify *K. pneumoniae* capsular serotypes my few methods such as anti-capsular type sera, counter-current immunoelectrophoresis from pure isolates, or polymerase chain reactions (PCRs) (Wang et al, 2019). Serotyping is conducted by the capsular swelling technique after inoculation of agar and incubation at 37 °C.

© COPYRIGHT UPM

2.2.5 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.

2.3 Symptoms of hypervirulent *Klebsiella pnumoniae* which cause to liver abscess

A liver abscess is a mass filled with pus inside the liver due to *K. pneumoniae* which having higher rates cases in the Far East. Pyogenic liver abscess is the most often polymicrobial that accounts to 80% of hepatic abscess cases. Hypervirulent *K. pneumoniae* (hvKp) is an evolving phenotype that is more virulent than classical *K. pneumoniae* (cKp). K1 and K2 have been reported to have a significant association with increased virulence and septicemia-related infection rates, as well as liver abscesses. hvKp infection frequently occurs at multiple sites or subsequently metastatically spreads, frequently requiring source control. hvKp has an increased ability to cause central nervous system infection and endophthalmitis, which require rapid recognition and site-specific treatment (Russo et al, 2019).

2.4 Virulence factors that cause *Klebsiella pneumoniae* invades into deep organs

2.4.1 Capsular polysaccharide

According to Figure 2.1, the researchers could detect K1 and K2 serotypes in larger size of capsular polysacsharide of hvKp. Several researchers have identified whether the K1 and/or K2 capsule types enhanced virulence compared to non-K1/K2 types. There were several researches have been reported that hypervirulent genes were metastaticaly spread was very common in the K1/K2 groups. hvKp is able to enhance the serum resistant and biofilm production (Russo et al,. 2019).

© COPYRIGHT UPM

2.4.2 Aerobactin production

Aerobactin production only establishes in hvKp that make it specific virulence factor. Both cKp and hvKp have enterobactin and yersiniabactin but only hvKp has aerobactin. Aerobactin makes *K. pneumoniae* becomes increasingly resistant to antimicrobials via acquisition of mobile elements. Extensively drug-resistant cKp strains acquire hvKp-specific virulence determinants, resulting in nosocomial infection (Russo et al,. 2019).

2.4.3 *Klebsiella pneumoniae* with Extended Spectrum Beta-Lactamase (ESBL) gene

Beta-lactam antibiotic is one of the common antibiotics classes used in treating bacterial infection in hospital. Beta-lactam antibiotic act by interfering with the formation of peptidoglycan therefore inhibit cell wall formation. Bacteria have been constantly exposed to heavy selective antimicrobial pressure and empirical usage of antibiotics, especially in clinical setting (Lee et al., 2013). In order to survivebacteria have counteract bactericidal effect of beta-lactams. Consequently, it involves bacterial defense system has created a beta-lactamase enzyme as major beta-lactam resistant mechanism. Beta-lactamase act by cleaving beta-lactam ring thus deactivates beta-lactam antibiotics (Washington W.J et al., 2006).

2.5 Molecular detection using Multiplex PCR

Previous Multiplex PCR have targeted a few virulence genes *K. pneumoniae* such as magA, mpA, entB, ybtS, kfu, iutA, mrkD, allS, and many more. In this study, detecting K1 and K2 capsular serotypes will be the main targets as both them have been commonly reported of having virulence properties compared to other genes. All around the world are extensively susceptible to antibiotics but contains the virulence factors related to hypervirulent *K. pneumoniae* K1 and K2 (Catalan et al., 2019). Both capsules K serotype show hypervirulent activity which causing septic meningitis of abscess (Yu et al., 2007).

© COPYRIGHT UPM

Table 2.5.1: Molecular detection of capsular serotype K1 and K2

Primer	Primers DNA sequences	Size of product (bp)	References
K1 **magA**	Forward: GGTGCTCTTTACATCATTGC Reverse: GCAATGGCCATTTGCGTTAG	Size (bp): 1243 Capsular serotype K1	Wang et al,. 2019
K2 K_2A	Forward: GGTGCTCTTTACATCATTGC Reverse: GCAATGGCCATTTGCGTTAG	Size (bp): 643 Capsular serotype K2	
K1 **magA**	Forward: GGTGCTCTTTACATCATTGC Reverse: GCAATGGCCATTTGCGTTAG	Size (bp): 1283 Capsular serotype K1	Compain et al,. 2014
K2 K_2A	Forward: CATAAGAGTATTGGTTGACAG Reverse: CTTGCATGAGCCATCTTTCA	Size (bp): 461 Capsular serotype K2	
K1 **magA**	Forward: GGTGCTCTTTACATCATTGC Reverse: GCAATGGCCATTTGCGTTAG	Not stated	Yu et al., 2007
K2 K_2A	Forward: CAACCATGGTGGTCGATTAG Reverse: TGGTAGCCATATCCCTTTGG	Not stated	

© COPYRIGHT UPM

CHAPTER THREE

METHODOLOGY

3.1 Sample collection

Figure 3.1.1: ***Klebsiella pneumoniae*** **from clinical samples**

In this study, there were 44 collected samples in Microbiology laboratory which originally from Hospital Pakar Sultanah Fatimah. All samples were collected from this hospital from year 2009 to 2012. All samples were having ESBL-gene which previously carried out by Mohd Helmi Bin Ujir under his Master program which entitled 'Distribution of ESBL genes and activities in clinical isolates of *Klebsiella pneumoniae* at a district hospital Malaysia'. The ethical approval was obtained from UPM Medical Reseach Ethics Committee with the reference number UPM/TNC/RMC/1.4/18.1 (JKEUPM) F2 (See appendix I for approval letter).

3.2 Bacteria Identification

All *K. pneumoniae* isolates were identified through gram staining method. This method is purposely to determine the Gram stain of bacterial sample. It is commonly used to identify the chemical make up of the cell wall of bacteria. The cell wall can strain either positive or negative, according to its chemistry. First of all, the *K. pneumoniae* isolates were

© COPYRIGHT UPM

cultured on LB Broth agar and aerobically incubated overnight at 37°C. Then, a colony of *K. pneumoniae* was taken and put on glass slide and mixed with a drop of distilled water. Let it dry before proceeding with some stains. Next, apply a primary crystal violet stain in order to heat-fixed smear of bacterial culture. The glass slide as rinse thoroughly and added iodine stains to bind to crystal violet and enhance it in cell. After done rinsing the glass slide, rapid decolorization with ethanol was applied to interact with the lipids of the cell membrane. Lastly, apply safranin as a counterstain to pick up the Gram-negative bacteria. *Klebsiella pneumoniae* basically possess a thin layer of peptidoglycan between two membranes. The glass slide was ready to be examined under microscope.

© COPYRIGHT UPM

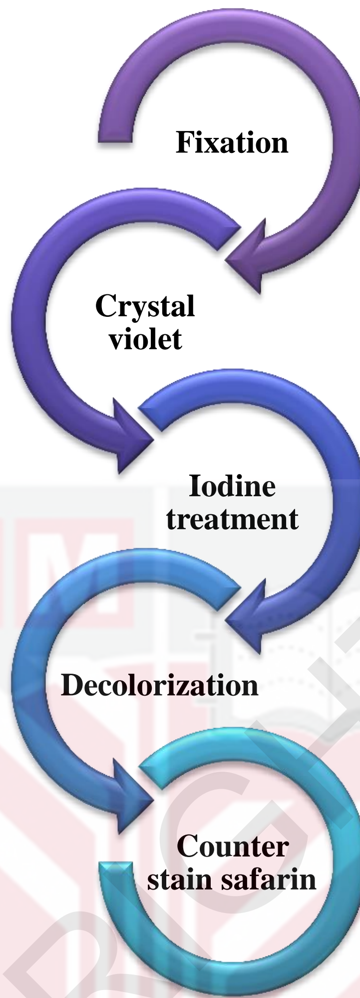


Figure 3.2.1: Gram staining

© COPYRIGHT UPM

3.3 Bacteria cultivation

3.3.1 Lysogeny Broth (LB Agar)

LB agar is also known as nutrient agar as it provides nutritionally rich medium for growth of bacteria. 20 gram of LB powder was weighed and mixed with 500ml of distilled water a bottle. The bottle then was shook 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 to sterile and kill micro flora. Agar medium to be poured into plates is sterilized in a flask, and then poured afterward.

3.3.2 MacConkey Agar

The MacConkey agar was used as selective and differential media to differentiate between Gramnegative and Grampositive-bacteria. It contains protease, peptone, lactose monohydrate, bile salts and sodium chloride which inhibit the growth of Grampositive 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.

© COPYRIGHT UPM

Figure 3.3.2.1: Klebsiella pneumoniae colonies on MacConkey agar 5% Blood sheep agar

The colonies then were subcultured onto blood agar supplemented with 5% sheep blood 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 hemolysis which referring to the lack of haemolytic activity. In other words, the *K. pneumoniae* does not hemolyse the blood agar.

© COPYRIGHT UPM

Figure 3.3.2.2: Klebsiella pneumoniae on blood agar

3.4 Preservation and Sub-culturing

Brain Heart Infusion (BHI) 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. 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.

© COPYRIGHT UPM

Figure 3.4.1: MacConkey and glycerol solution

3.5 Hypermucoviscosity Testing (String Test)

The string test was carried out to observe the formation of a mucoviscous string of > 5 mm. A positive string test with *K. pneumoniae* strains were designated as hypervirulence, (Fang et al., 2004). Firstly, colonies grown overnight on 5% Sheep Blood agar were placed on a glass slide using a sterile loop and mixed with 3% potassium hydroxide (KOH). When exposed to 3% KOH, *K.pneumoniaee* undergwent cell lysis thereby releasing its viscous nucleic acid, which form a string when drawn up. The measurement of viscous string more than 5mm demonstrates *K.pneumoniaee* 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.

© COPYRIGHT UPM

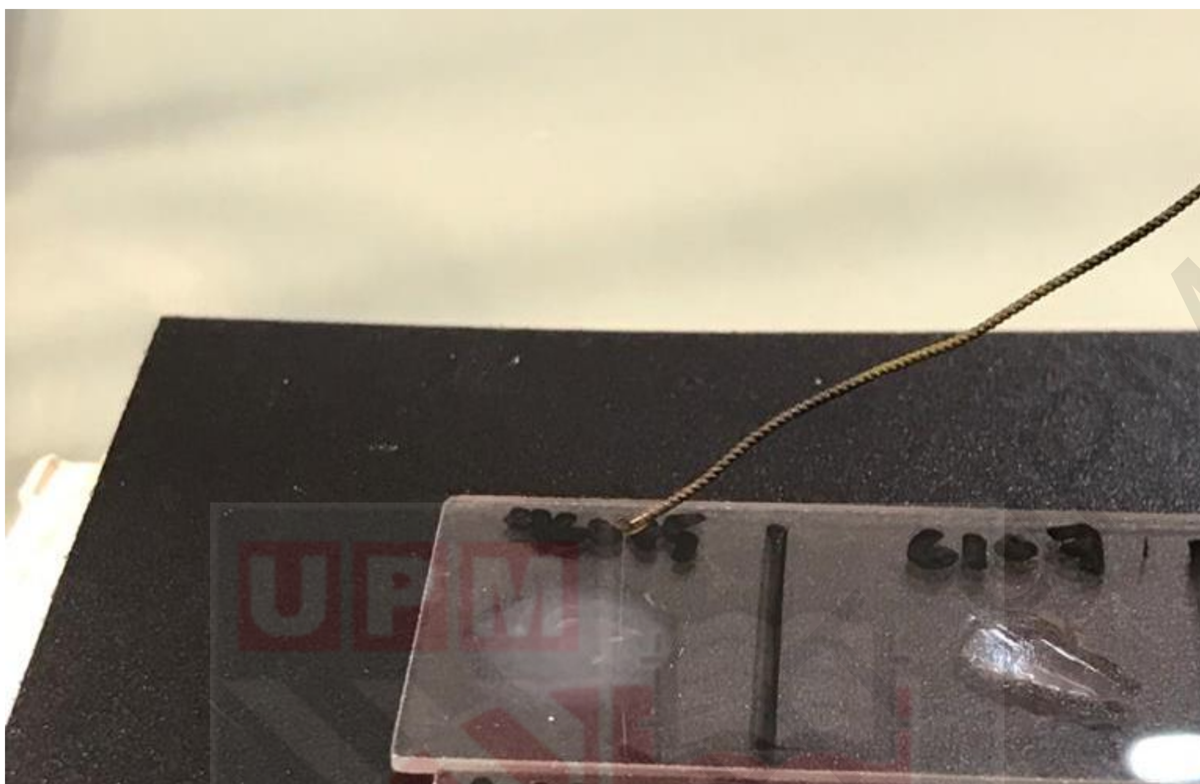


Figure 3.5.1: Hpermucoviscosity test

3.6 DNA extraction

Commercial DNA extraction kit (GeneAll biotech, Korea) was used to extract DNA from the bacteria isolates. This kit includes several ready use buffers (CL, BL, BW, TW and AE). Firstly, few colonies of *K. pneumoniae* isolates grown on 5% blood agar were suspended in 200µl LB Broth in 1.5ml microcentrifuge tube by using a sterile wire loop. The bacterial suspension was then centrifuged for 1 minute at 15000 RPM. The supernatant was discarded and the pelleted bacterial cells were left in the microcentrifuge tube. Then, the cell pellet was completely suspended in 200µl of buffer CL. Proteinase K solution and buffer BL were added into the cell suspension with volume of 20µl and 200µl respectively. The mixture was mixed completely by vigorous vortex. Proteinase K was added to digest the contaminating proteins. Next, the cell suspensions were incubated at 56°C for 15 minutes.

© COPYRIGHT UPM

After the incubation period, 200µl of buffer BL was added to the cell suspension. The tube was vortexed before proceeding with incubation at 70°C for 10 minutes. Then, 200µl of absolute ethanol was added into the cell suspension and mixed thoroughly. Ethanol was added into this solution to force the precipitation of nucleic acid out of the solution. After that, the mixture was carefully transferred into SV column by decanting and centrifuged for 1 minute at 15000 RPM. The collection tube was then replaced with a sterile collection tube. Before the next centrifugation, 600µl of buffer BW was added into the column. Then, replaced the collection tube and the new one was reinserted. A volume of 700µl TW buffer was added into the column and centrifuged for 1 minute at 15000 RPM. The pass-through in the collection tube was discarded and SV column was reinserted back. In order to remove the residual wash buffer, the column was centrifuged again for 1 minute at 15000 RPM. The column was then placed into a new 1.5ml microcentrifuge tube. The tube was incubated at room temperature for 1 minute after adding the 200µl of AE buffer. After that the tube was centrifuge for 1 minute at 15000 RPM to elute the DNA from the column into the microcentrifuge tube. For long-term storage, the microcentrifuge tube containing the extracted DNA was stored at -20°C.

© COPYRIGHT UPM

Figure 3.6.1: Autoclave all centrifuge tubes for DNA preparation

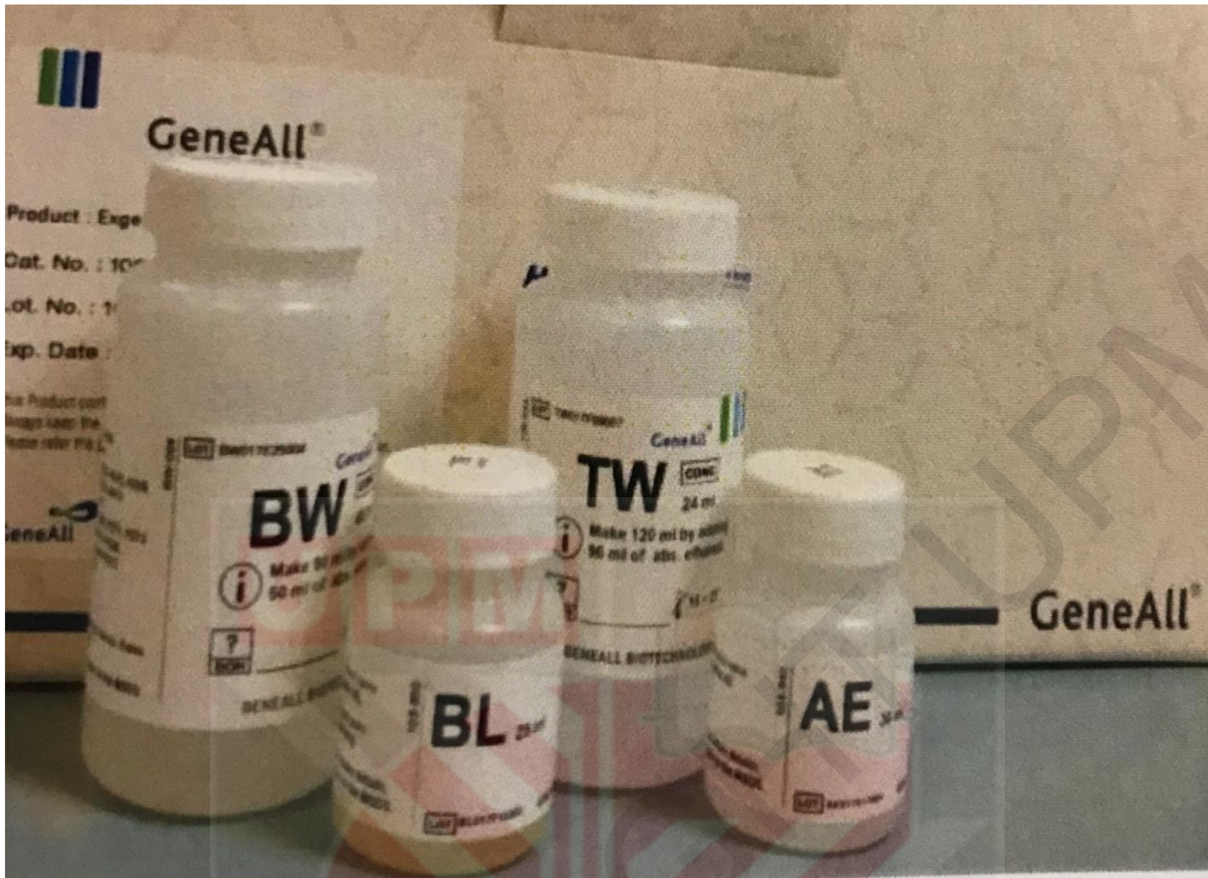


Figure 3.6.2: The DNA extraction kit including readymade buffers such as BW, BL, TW and AE solution

© COPYRIGHT UPM

© COPYRIGHT UPM

Figure 3.4.3: Column tube in balance position in centrifuge machine

3.7 PCR Assay for K1 and K2 Genes Detection

Polymerase Chain Reaction (PCR) is the common method used to produce multiples copies of DNA templates. 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 (5"GGTGCTCTTTACATCATTGC-3") and K_2A (5"-GGTGCTCTTTACATCATTGC-3"). The whole reaction is taken place in a thermal cycler machine according to the set up as shown in Table 2 below:

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 Reverse: GCAATGGCCATTTGCGTTAG	1283
K_2A (K2)	Forward: GGTGCTCTTTACATCATTGC Reverse: GCAATGGCCATTTGCGTTAG	531

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

Components	Volume per 25µl reaction (µl)
MyTaq HS^{TM} DNA Polymerase Master Mix (Sydney University, Save Sight Institute, Sydney, Australia)	10
K1 Forward	0.7
K1 Reverse	0.7
K2 Forward	0.7
K2 Reverse	0.7
Nucleus-free water	11.6

© COPYRIGHT UPM

DNA template	2

Table 3.7.3: Cycling condition for Multiplex PCR

Target gene	Cycling conditions					
***magA* (K1)**	95°C 2 minutes	95°C 30 seconds	58°C 2 minutes	72°C 10 seconds	72°C 5 minutes	Repeated for 25 cycles
K_2A (K2)	95°C 2 minutes	95°C 30 seconds	58°C 2 minutes	72°C 10 seconds	72°C 5 minutes	Repeated for 25 cycles

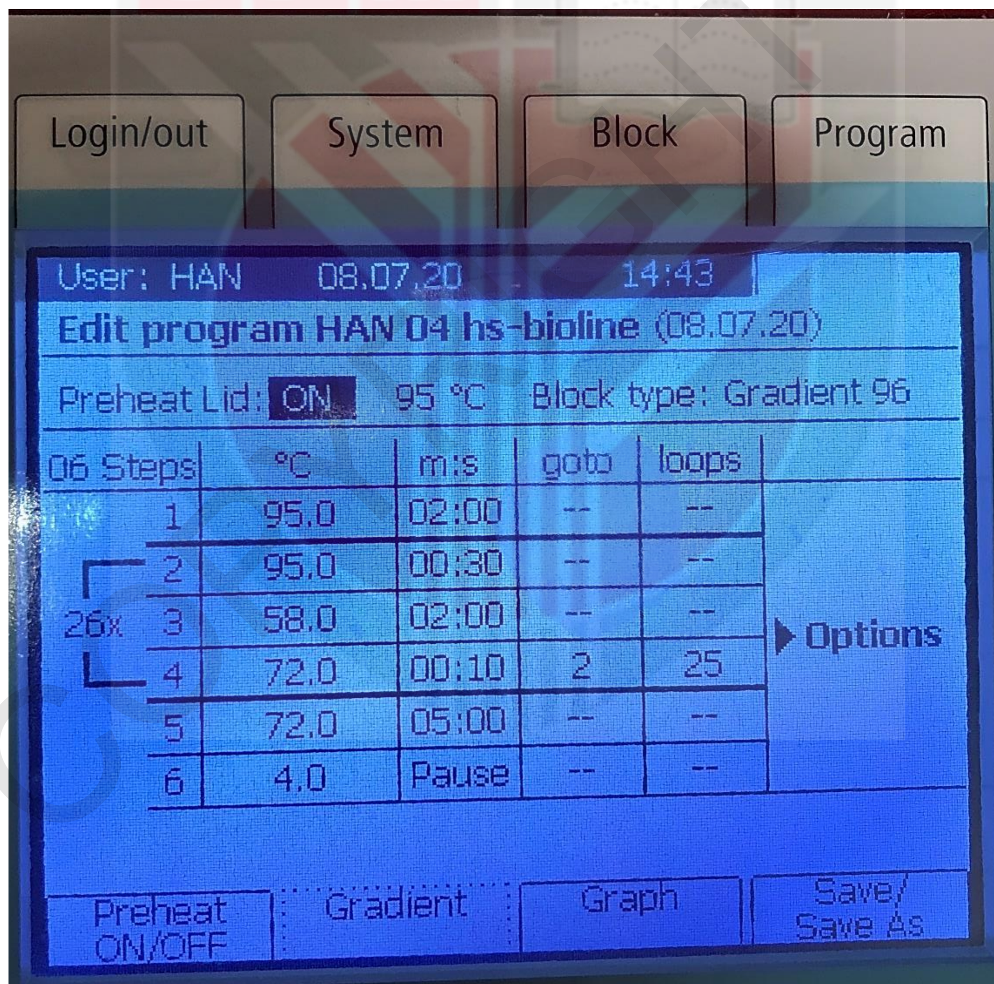


Figure 3.7.1: Cycling condition for Multiplex PCR

© COPYRIGHT UPM

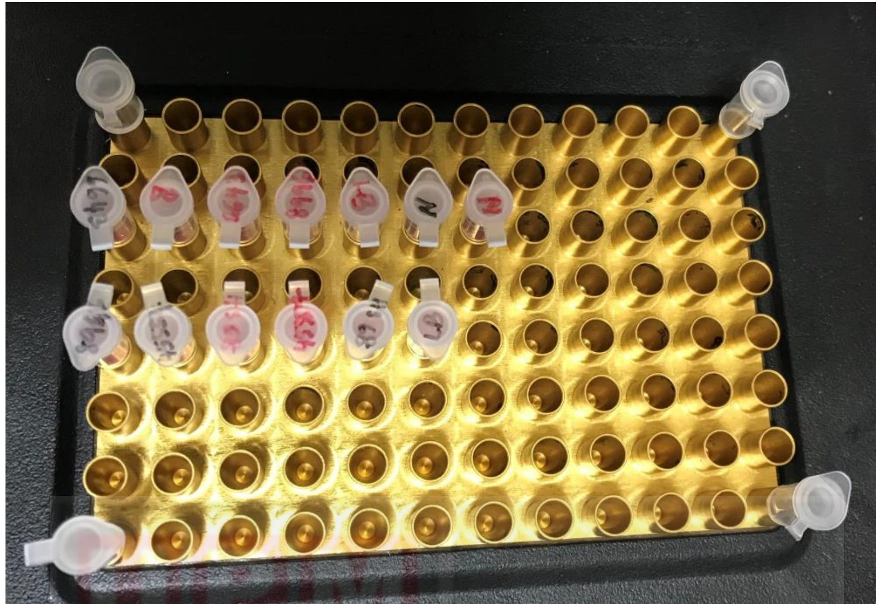


Figure 3.7.2: Samples in PCR machine column

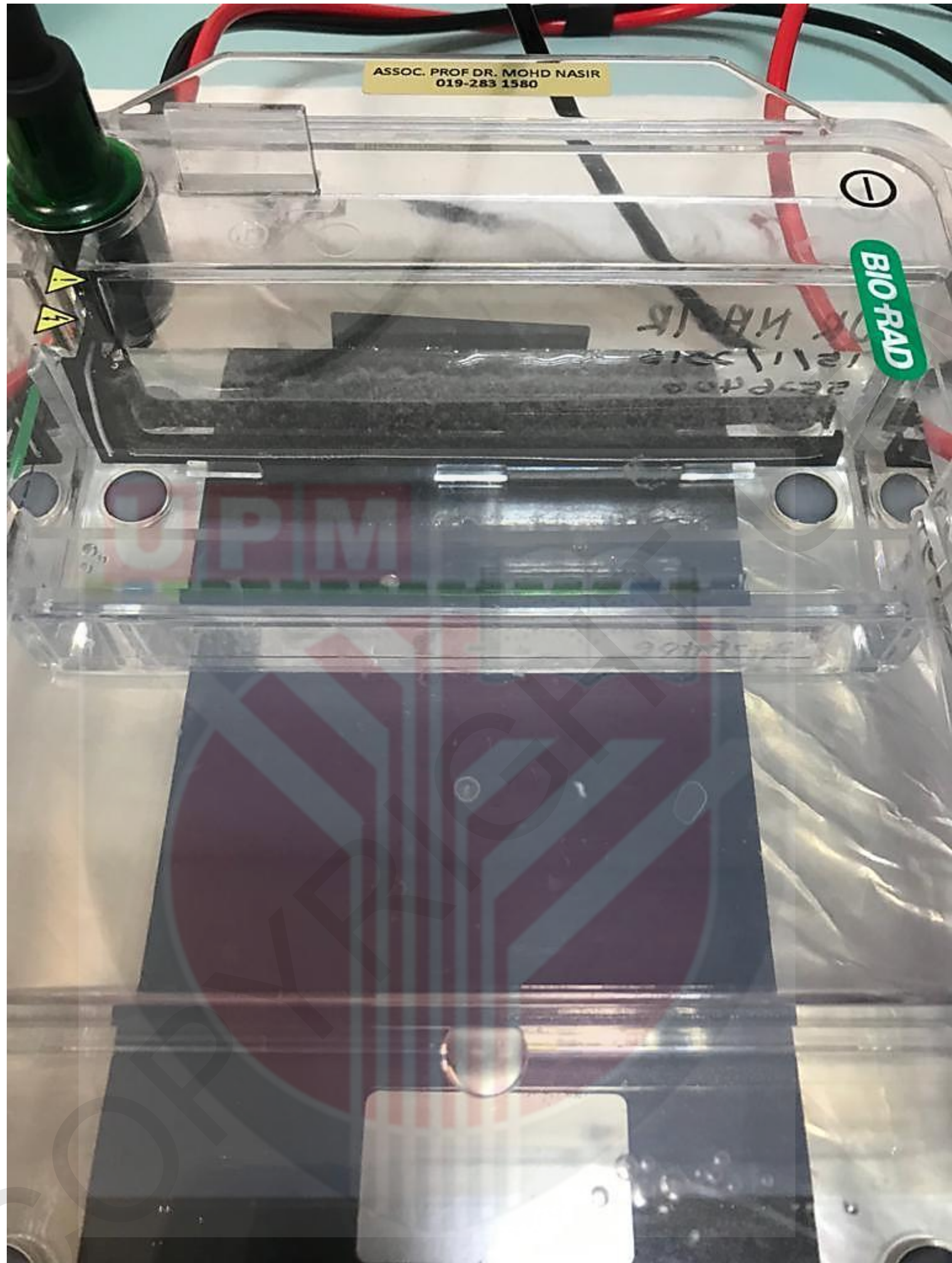
Figure 3.7.3: PCR machine

The PCR products were resolved through 1.5% agarose gel in 1x TBE buffer at 60v for 85 minutes. The product was run for gel electrophoresis to analyse and separate the PCR

© COPYRIGHT UPM

product according to their respective sizes and charges by using electric field. Five microliters 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 considered as hypervirulent K. pneumoniae if the bands appear at 1283bp and 533bp (Wang C et al., 2019).

© COPYRIGHT UPM



© COPYRIGHT UPM

Figure 3.7.4: Samples run in gel electrophoresis

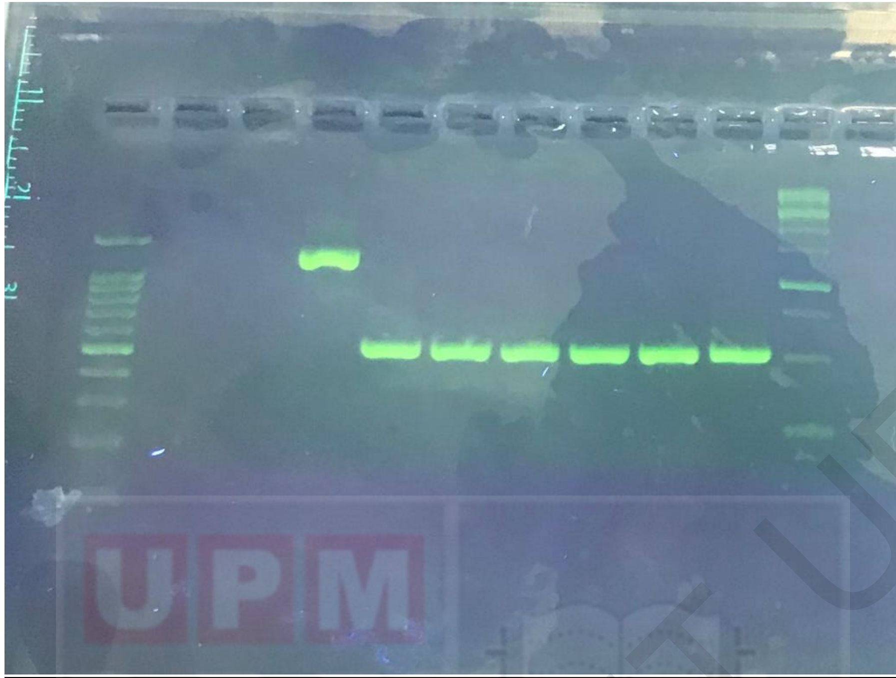


Figure 3.7.5: Bands view under UV Transilluminator

Figure 3.7.6: Visualization of K1 nad K2 bands on agarose gel

CHAPTER FOUR

RESULT

4.1 Bacterial Strains and Morphological Identification of *Klebsiella pneumoniae*

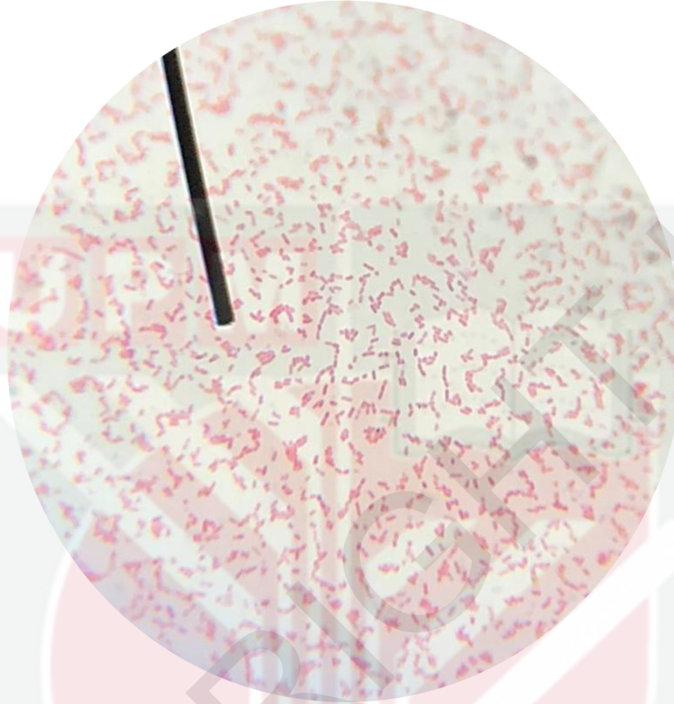


Figure 4.1.1: Morphology *of Klebsiella pneumoniae* under microscope

The morphology of *K. pneumoniae* could be seen in with encapsulated rod-shape. The pink colour of *K. pneumoniae* indicated as gram negative bacteria. During gram staining procedure, the crystal violet dissociates its solution into crystal violet and chloride ions which then penetrated by interacting with negatively charges components of gram negative bacteria. Interaction between crystal violet and iodine were forming large complex within inner and outer layers of cells. Decolorization of gram negative bacteria made it lost its purple colour and remained with pink colour.

© COPYRIGHT UPM

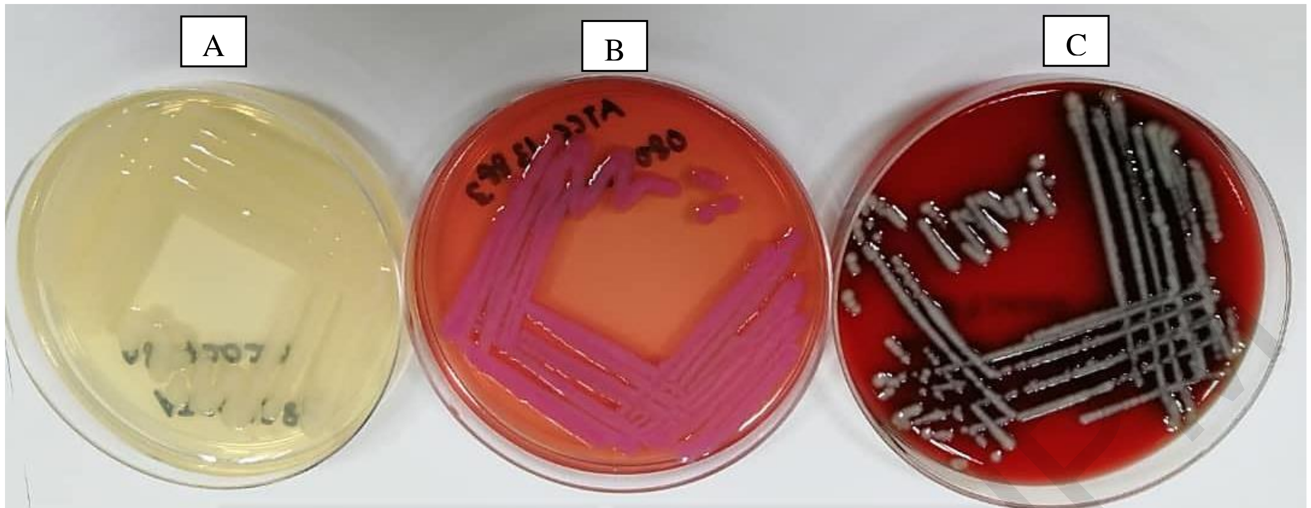


Figure 4.1.2: The growth of Klebsiella pneumoniae on A) Lysogeny Broth (LB) agar, B) MacConkey and C) Blood agar

A) *Klebsiella pneumoniae* on LB agar: LB agar is generally growth promotion which consisting peptides, vitamins, minerals and trace elements. Yeast extracts also used to as organic compounds for bacterial growth. K. pneumonia colonies appeared white, cream and fairly circular in shape.

B) *Klebsiella pneumoniae* on MacConkey: Lactose is the solely containing carbohydrate. *K. pneumoniae* is a Lactose-fermenting bacterium which it produced colonies that are varying shades of red, owing to the conversion of the neutral red indicator dye which below pH 6.8 from the production of mixed acids. Colonies of non-lactose fermenting bacteria appear colourless or transparent characteristic. Crystal violet and bile salts are incorporated in MacConkey to prevent their growth of gram positive bacteria.

c) Klebsiella pneumoniae on blood agar: *K. pneumoniae* is a gamma-hemolysis which is it lacks of hemolysis when cultured on blood agar. The colonies appeared with whitish colour.

© COPYRIGHT UPM

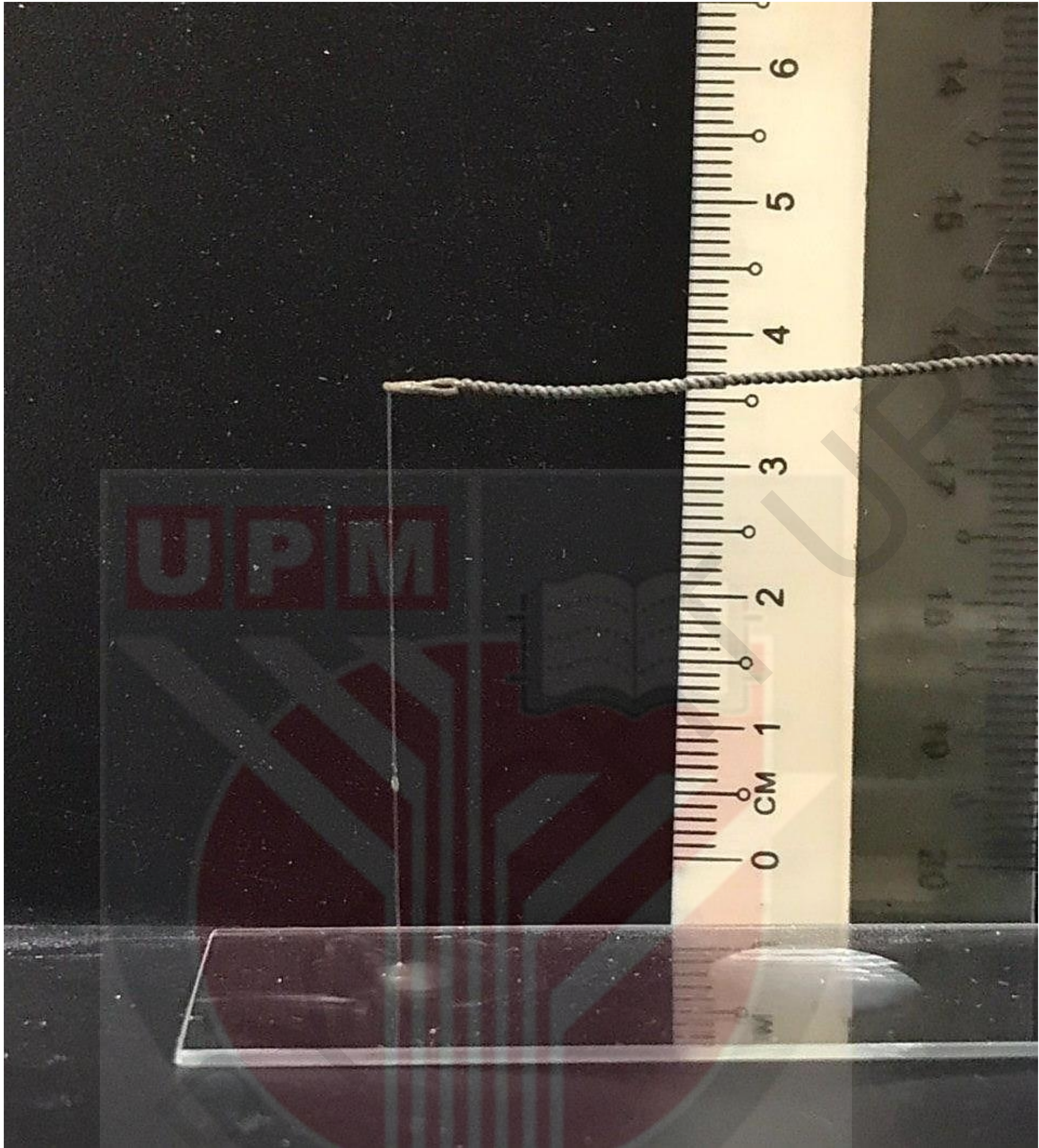


Figure 4.1.3: Hypermucoviscosity Test showing the string more than 5mm

The **Figure 4.1.3** above showed that string of one of *K. pneumoniae* isolates is around 30mm roughly and obviously far away beyond 5mm. A string could be stretched up after mixing a loop of *K. pneumoniae* colony with 3% KOH solution. The mixture turned to cloudy colour as the colony had been hydrolysed by KOH. This cloudy characteristic also demonstrated *K. pneumoniae* as gram negative bacteria. Gram positive bacteria will have no change and mixture still colourless and original.

MacConkey agar was used to grow the *K.pneumoniae* isolated from collected clinical samples. It is suitable as selective and differential agar for the growth of *K. pneumoniae.*

4.2 Hypermucoviscosity Test (String test) of *K. pneumoniae*

Based on the results of the modified string test, hypervirulent phenotypes were identified in 25 (54.5%) of the 44 isolates. A significantly higher number of patients with cKP (P = 0.009) was detected. Neither age nor sex was associated with positive string test (both P > 0.05).

4.3 Detection of capsular serotype K1 and K2

Polymerase chain reaction (PCR) was performed on the 44 *K. pneumoniae* isolates to identify the presence of K1 and K2 genes. Based on the gel electrophoresis results, appearance of K1 is observed at 1283bp and K2 gene is at 531bp. Isolates showing these bands were categorized as *K. pneumoniae* isolates. From the total 44 *K. pnemoniae* samples that were screened K1 and K2 genes, one (2.3%) of them were detected as K1 gene while seven (16%) were detected as K2 gene. This showed that eight isolates (18%) were classified as K1/K2 serotypes and 34 *K. pneumoniae* isolates were classified as non-K1/K2 serotypes.

© COPYRIGHT UPM

PCR twelvethrun: 08.07.2020
Multiplex PCR
Mastermix: HS Bioline
Annealing temperature (Gradient): 58.0°C
Gene: K1 and K2

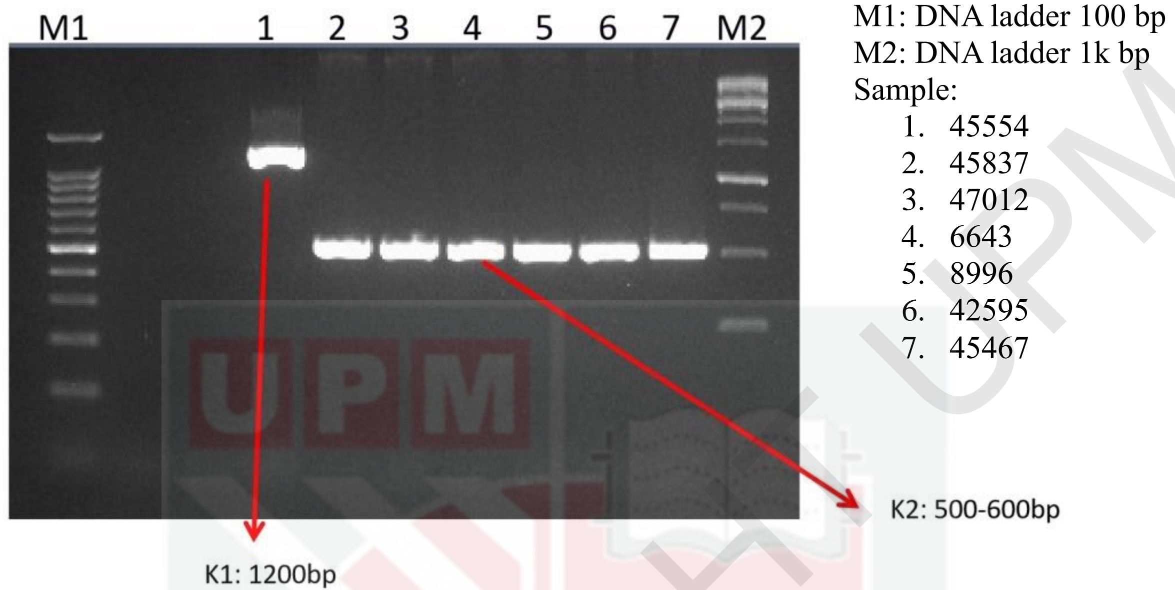


Figure 4.3.1: Visualization of K1 and K2 bands on agarose gel

4.4 Statistical analysis

The samples were collected from 2009 to 2012, and a total of 44 patients were diagnosed as suffering pneumonia, asthma, diabetes mellitus with the culture-positive *K. pneumoniae*. Out of them, 25 (56.8%) were males and 19 (43.2%) were females. Based on **table 4.4.1,** the mean age was 58.1 ± 21.0 years.

Table 4.4.1: Population of patients based on age

		Frequency	Percent	Valid Percent	Cumulative Percent
Age	15	2	4.5	4.5	4.5
	19	2	4.5	4.5	9.1
	31	3	6.8	6.8	15.9
	33	1	2.3	2.3	18.2

© COPYRIGHT UPM

45	1	2.3	2.3	20.5
47	4	9.1	9.1	29.5
48	1	2.3	2.3	31.8
50	2	4.5	4.5	36.4
51	1	2.3	2.3	38.6
52	1	2.3	2.3	40.9
54	1	2.3	2.3	43.2
55	1	2.3	2.3	45.5
58	1	2.3	2.3	47.7
60	2	4.5	4.5	52.3
61	1	2.3	2.3	54.5
62	1	2.3	2.3	56.8
63	1	2.3	2.3	59.1
64	1	2.3	2.3	61.4
66	1	2.3	2.3	63.6
70	1	2.3	2.3	65.9
71	2	4.5	4.5	70.5
72	2	4.5	4.5	75.0
79	1	2.3	2.3	77.3
80	4	9.1	9.1	86.4
81	1	2.3	2.3	88.6
84	1	2.3	2.3	90.9
85	1	2.3	2.3	93.2
89	1	2.3	2.3	95.5
91	2	4.5	4.5	100.0
Total	44	100.0	100.0	

COPYRIGHT UPM

Table 4.4.2: Descriptive statistic

Descriptive Statistics					
	N	**Minimum**	**Maximum**	**Mean**	**Std. Deviation**
Age	44	15.00	91.00	58.1136	21.03399
Valid N (listwise)	44				

Table 4.4.3: Association between hypermucoviscosity and K1 serotype

Hypermucoviscosity test	**K1**		**P value**
	Positive	**Negative**	**0.046**
Positive	0 (0%)	35 (100%)	
Negative	1 (11.1%)	8 (88.9%)	

Table 4.4.4: Association between hypermucoviscosity and K2 serotype

Hypermucoviscosity test	**K2**		**P value**
	Positive	**Negative**	**0.659**
Positive	6 (17.1%)	29 (82.9%)	
Negative	1 (11.1%)	8 (88.9%)	

According to **table 4.4.3 and 4.4.4**, *p* value for the association between hypermucoviscosity and K1 was 0.046 while K2 was 0.659. p value is less than 0.005. Thus, there is no association between hypermucoviscosity towards serotype K1 and K2. This means that the presence of hypermucovicosity is not the main indicator to detect K1 and K2 capsular serotypes.

© COPYRIGHT UPM

Table 4.4.5: Association between hypermucoviscosity and K1/K2 serotypes

Hypermucoviscosity test	Capsular Serotypes		P value
	K1/K2	**Non K1/K2**	**0.725**
Presence	6 (17.1%)	29 (82.9%)	
Absence	2(22.2%)	7(77.8%)	

Based on the **table 4.4.5,** p value of association between hypermucoviscosity and K1/K2 serotypes is 0.725 which is more than 0.05. Hence, there is no association between these two variables.

© COPYRIGHT UPM

CHAPTER FIVE

DISCUSSION

5.1 Overview

Out of 44 ESBL *K. pneumoniae* isolates, 35 were (79.5%) showed positive hypermucoviscous phenotype. Only a small proportion of these isolates were positive for K1 (5%, n=1) and K2 (10%, n=7) serotypes detected by Multiplex PCR. The study showed that approximately 18% of the ESBL *K. pneumoniae* from clinical isolates were associated with serotype K1/K2 confirmed by Multiplex PCR analysis.

5.2 Occurrence of ESBL *K. pneumoniae* in clinical isolates

A study was conducted by SENTRY Surveillance Antimicrobial, carried out by Castanheira., (2019) stated that the presumptive ESBL phenotype was mainly observed among *K. pneumoniae* (25.2%) than *E. coli* (10.1%) or *P. mirabilis* isolates (1.4%). From the same study, out of 678 *K. pneumoniae,* 146 isolates (31%) were detected as ESBL-producers which mainly located in Philippine, Singapore (18%), South African (9%), Japan (9%) and Hong Kong 8%). The association of ESBL-producer especially *K. pneumoniae* is quite aggressive in Asian countries. Apart from that, another study was conducted by Remya., (2018) showed that K2 serotypes was more prevalence among ESBL genes. Based on this study, seven out of 44 isolates were detected positive for K2 serotype which more than K1 serotype that only one was detected. Besides, *K. pneumoniae* were three times has higher risk than *E. coli* in nosocomial infection (Vading et al., 2018). Cell surface component of hvKp has adhesion which gives a major contributor towards nosocomial infections which known as infections that have been caught in a hospital and caused by organisms with resistant to antimicrobial agent.

© COPYRIGHT UPM

5.3 Hypermucoviscosity testing (String test) of *Klebsiella pneumoniae*

Hypermucoviscous phenotypes are encapsulated isolates of *K. pneumoniae* which mostly K1 and to a lesser extent to K2 that produce a lot of extra-capsular polysaccharide associating a mucoviscous web that defends these bacterium from phagocytosis by neutrophils and from serum killing by complement (F. Casella et al., 2009). Hypermucoviscous isolates demonstrate incremented of virulence in mice (R. McCabe et al., 2010), and grow in sticky colonies that yield long more than 5 mm mucous strings when mixed with KOH and touched with a bacteriological loop (hypermucoviscosity test).

5.4 Identification of capsular serotype K1 and K2

The capsule is crucial virulence factor of encapsulated pathogens, including *K. pneumoniae*. Capsular polysaccharides of *K. pneumoniae* are divided into at least 78 serotypes (Pan et al., 2008; Wyres et al., 2016). There were reports have shown that K1 and K2 serotypes are contributed with hvKP (Paczosa and Mecsas, 2016; Catalán-Nájera et al., 2017). Hypermucoviscous *K. pneumoniae* clinical isolates associated in China had various types of invasive infections such as pyogenic liver abscess, 42.9% were the K2 serotype and 23.8% were the K1 serotype (Guo et al., 2017). Apart from that, a study in China showed that 68.75% of the hypermucoviscosity-positive *K. pneumoniae* isolates have the K2 serotype (Zhao et al., 2016). K1 and K2 capsular serotypes were more common in hypermucoviscosity-positive strains than in hypermucoviscosity-negative strains in a study in South Korea. *K. pneumoniae* strains isolated from the urine of hospitalized patients showed that (Kim et al., 2017). K1 or K2 serotype (78%) mostly associated hypermucoviscous *K. pneumoniae* isolates from patients with bacteremia (Jung et al., 2013).

© COPYRIGHT UPM

5.3 Diseases associated with *K. pneumoniae* serotypes K1 and K2

Capsular polysaccharide has been shown to be an important virulent factor of *K. pneumoniae* infection pathogenicity. A study conducted by Fung CP et al., (2000) showed that among the 79 recognized capsular serotypes, K1 and K2 have been reported to have a significant association with increased virulence and septicemia-related infection rates, liver abscesses and endophthalmitis. In this study, 61% of sources are from sputum. This might be the reason why there is very little number of positive serotypes K1 and K2 compared to previous study. Next, serotypes K1 and K2 were predominantly accounting for 63.4% and 14.2% of all isolates recovered from patients with liver abscess (Fung et al., 2002). In this study, six from positive K2 serotypes were detected in sputum samples among patients with age above 60 years old. These patients were diagnosed with chronic disease.

5.4 PCR analysis as rapid detection method of K1 and K2 serotypes

A similar study conducted by Wang et al., (2019) showed that among the 16 *K. pneumoniae* isolates from liver abscesses, 50.0% (8/16) turned out to have serotype K1, 18.8% (3/16) serotype K2, and 31.3% (5/16) non-K1/K2 serotype. Another study carried out by Yu et al., (2007) stated that among 122 *K. pneumoniae* isolates, there were 45.9% (56) were detected as K1, 21.3% (26) as K2 and 32.7% (40) were identified as non-K1/K2. Thus choosing PCR analysis for detection of capsular serotypes is very reliable and give accurate result.

© COPYRIGHT UPM

CHAPTER SIX

CONCLUSION

6.1 Conclusion

In conclusion, *Klebsiella pneumoniae* strains can be detected by the hypermucoviscosity test. Biochemical and molecular characterization of hypermucoviscous phenotype associated genes is elementary. The multiplex PCR described in this study allows the rapid, reproducible, and sensitive detection of hypervirulence genes carried by ESBL *K. pneumoniae* isolates. In addition, the method is less time consuming and is suitable for screening virulent clones. This PCR will be useful for comparing the virulence profiles of large collections of *K. pneumoniae* organisms, including hvKP strains. It would be of particular interest to characterize particular groups of *K. pneumoniae* according to the source of isolation or the presence or absence of virulence factors in order to assess their correlation with clinical and epidemiological data. This multiplex PCR is anticipated to be valuable in epidemiological surveys of invasive infections due to *K. pneumoniae*. 79.5% of positive hypermucoviscosity phenotype does not significantly associated with K1/K2 serotypes. 82% of the ESBL *K. pneumoniae* clinical isolates were detected as non-K1/K2 serotype. This might be associated to other 76 capsular serotypes.

6.2 Recommendation for future study

It is highly recommended that collection of samples from larger sample size and from various places in order to obtain better prevalence and distribution of ESBL *K. pneumoniae* strain in the community. A part from that, it would be better if both ESBL *K. pneumoniae* and non-ESBL *K. pneumoniae* are used to reduce bias in research. Besides, other molecular typing method such as spa typing and Multilocus sequence typing (MLST) can be performed

© COPYRIGHT UPM

to determine the genetic background of *K. pneumoniae*. Lastly, evaluation study on molecular typing method can be done in order to identify the most sensitive and reliable molecular typing method purposely to help better understanding of molecular characteristic of *K. pneumoniae* strain.

© COPYRIGHT UPM

REFERENCES

Africa, S. (2012). *Biofilm formed by a hypervirulent (hypermucoviscous) variant of Klebsiella pneumoniaee does not enhance serum resistance or survival in an in vivo abscess model*. (June), 309–318.

Beigverdi R, Jabalameli L, Jabalameli F, Emaneini M. Prevalence of extended-spectrum-beta-lactamase-producing Klebsiella pneumonia: first systematic review and meta-analysis from Iran. J Glob Antimicrob Resist. 2019. https://doi.org/10.1016/j.jgar.2019.01.020 PMID: 30685458.

Bezdicek, M., Nykrynova, M., Plevova, K., Brhelova, E., Kocmanova, I., Sedlar, K., … Lengerova, M. (2019). Application of mini-MLST and whole genome sequencing in low diversity hospital extended-spectrum beta-lactamase producing Klebsiella pneumoniae population. *Plos One*, *14*(8), e0221187. https://doi.org/10.1371/journal.pone.0221187

Casella F, Finazzi L, Repetti V, et al. Liver abscess caused by Klebsiella pneumoniae: two case reports. Cases J 2009; 2: 6879

Catalán-Nájera, J. C., Humberto, B. C., Josefina, D. B., Alan, S. P., Rigoberto, H. C., García-Méndez, J., … Ulises, G. R. (2019). Molecular characterization and pathogenicity determination of hypervirulent Klebsiella pneumoniae clinical isolates serotype K2 in Mexico. *Diagnostic Microbiology and Infectious Disease*, *94*(3), 316–319. https://doi.org/10.1016/j.diagmicrobio.2019.01.013

Cerwenka H. Pyogenic liver abscess: differences in etiology and treatment in Southeast Asia and Central Europe. World J Gastroenterol 2010; 16: 2458-62

Compain, F., Babosan, A., Brisse, S., Genel, N., Audo, J., Ailloud, F., … Decré, D. (2014). Multiplex PCR for detection of seven virulence factors and K1/K2 capsular serotypes of

© COPYRIGHT UPM

Klebsiella pneumoniae. *Journal of Clinical Microbiology*, *52*(12), 4377–4380. https://doi.org/10.1128/JCM.02316-14

Dolejska M, Brhelova E, Dobiasova H, Krivdova J, Jurankova J, Sevcikova A, et al. Dissemination of IncFII(K)-type plasmids in multiresistant CTX-M-15-producing Enterobacteriaceae isolates from children in hospital paediatric oncology wards. Int J Antimicrob Agents. 2012; 40(6):510–5. https://doi.org/ 10.1016/j.ijantimicag.2012.07.016 PMID: 23043911

Fang, C. T., Chuang, Y. P., Shun, C. T., Chang, S. C., & Wang, J. T. (2004). A Novel Virulence Gene in Klebsiella pneumoniae Strains Causing Primary Liver Abscess and Septic Metastatic Complications. *Journal of Experimental Medicine*, *199*(5), 697–705. https://doi.org/10.1084/jem.20030857

Fung CP, Chang FY, Lee SC, Hu BS, Kuo BI, Liu CY, et al. A global emerging disease of Klebsiella pneumoniae liver abscess: is serotype K1 an important factor for complicated endophthalmitis? Gut. 2002;50:420–4. https ://doi. org/10.1136/gut.50.3.420

Fang CT, Lai SY, Yi WC, Hsueh PR, Liu KL, Chang SC. 2007. Klebsiella pneumoniae genotype K1: an emerging pathogen that causes septic ocular or central nervous system complications from pyogenic liver abscess. Clin Infect Dis 45:284–293. https://doi.org/10.1086/519262

Gilchrist CA, Turner SD, Riley MF, Petri WA, Jr., Hewlett EL. Whole-genome sequencing in outbreak analysis. Clin Microbiol Rev. 2015; 28(3):541–63. https://doi.org/10.1128/CMR.00075-13 PMID: 25876885; PubMed Central PMCID: PMC4399107

Gorrie CL, Mirceta M, Wick RR, Judd LM, Wyres KL, Thomson NR, et al. Antimicrobial-

© COPYRIGHT UPM

Resistant Klebsiella pneumoniae Carriage and Infection in Specialized Geriatric Care Wards Linked to Acquisition in the Referring Hospital. Clin Infect Dis. 2018; 67(2):161–70. https://doi.org/10.1093/cid/ciy027 PMID: 29340588; PubMed Central PMCID: PMC6030810.

Harada, S., Aoki, K., Yamamoto, S., Ishii, Y., Sekiya, N., Kurai, H., … Doi, Y. (2019). Clinical and molecular characteristics of Klebsiella pneumoniae causing bloodstream infections in Japan: occurrence of hypervirulent infections in healthcare . *Journal of Clinical Microbiology*, (August), 1–33. https://doi.org/10.1128/jcm.01206-19

Kaiser RM, Castanheira M, Jones RN, et al. Trends in *Klebsiella pneumoniae* carbapenemase-positive *K. pneumoniae* in US hospitals: report from the 2007-2009 SENTRY Antimicrobial Surveillance Program. Diagn Microbiol Infect Dis **2013**; 76:356–60.

Little ML, Qin X, Zerr DM, Weissman SJ. Molecular epidemiology of colonizing and disease-causing Klebsiella pneumoniae in paediatric patients. J Med Microbiol. 2014; 63(Pt 4):610–6. https://doi.org/10. 1099/jmm.0.063354-0 PMID: 24464691; PubMed Central PMCID: PMC4811641.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009; 25(16):2078–9. https://doi.org/10.1093/bioinformatics/btp352 PMID: 19505943; PubMed Central PMCID: PMC2723002.

Lin JC, Chang FY, Fung CP, Xu JZ, Cheng HP, Wang JJ, et al. High preva- lence of phagocytic-resistant capsular serotypes of Klebsiella pneumoniae in liver abscess. Microbes Infect. 2004;6:1191–8. https ://doi.org/10.1016/j. micin f.2004.06.003

Mohd Helmi, U., Mohd Desa, M. N., Taib, N. M., Tengku Jamaluddin, T. Z. M., & Masri, S.

© COPYRIGHT UPM

N. (2016). Multiple ambler class a ESBL genes among Klebsiella pneumoniae isolates in a Malaysian district hospital. *Tropical Biomedicine*, *33*(1), 109–119.

Niu, L., Zhao, F., Chen, J., Nong, J., Wang, C., Wang, J., … Hu, S. (2018). Isothermal amplification and rapid detection of Klebsiella pneumoniae based on the multiple cross displacement amplification (MCDA) and gold nanoparticle lateral flow biosensor (LFB). *PLoS ONE*, *13*(10), 1–15. https://doi.org/10.1371/journal.pone.0204332

Pitout JD. Infections with extended-spectrum beta-lactamase-producing *Enterobacteriaceae*: changing epidemiology and drug treatment choices. Drugs **2010**; 70:313–33.

Richardson LJ, Tong SY, Towers RJ, Huygens F, McGregor K, Fagan PK, et al. Preliminary validation of a novel high-resolution melt-based typing method based on the multilocus sequence typing scheme of Streptococcus pyogenes. Clin Microbiol Infect. 2011; 17(9):1426–34. https://doi.org/10.1111/j.14690691.2010.03433.x PMID: 21091832.

Rivero A, Gomez E, Alland D, Huang DB, Chiang T. K2 serotype Klebsiella pneumoniae causing a liver abscess associated with infective endocarditis. J Clin Microbiol 2010; 48: 639-41

Russo, T. A., & Marr, C. M. (2019). *crossm*. *32*(3), 1–42.

Schurch AC, Arredondo-Alonso S, Willems RJL, Goering RV. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene-based approaches. Clin Microbiol Infect. 2018; 24(4):350–4. https://doi.org/10.1016/j. cmi.2017.12.016 PMID: 29309930

Sellick, J. A., & Russo, T. A. (2018). Getting hypervirulent Klebsiella pneumoniae on the radar screen. *Current Opinion in Infectious Diseases*, *31*(4), 341–346.

© COPYRIGHT UPM

https://doi.org/10.1097/QCO.0000000000000464

Shah, R. K., Ni, Z. H., Sun, X. Y., Wang, G. Q., & Li, F. (2017). The determination and correlation of various virulence genes, ESBL, serum bactericidal effect and biofilm formation of clinical isolated classical klebsiella pneumoniae and hypervirulent klebsiella pneumoniae from respiratory tract infected patients. *Polish Journal of Microbiology*, *66*(4), 501–508. https://doi.org/10.5604/01.3001.0010.7042

Shon AS, Bajwa RP, Russo TA. Hypervirulent (hypermucoviscous) Klebsiella pneumoniae: a new and dangerous breed. Virulence. 2013; 4(2):107–18. https://doi.org/10.4161/viru.22718 PMID: 23302790; PubMed Central PMCID: PMC3654609.

Tong SY, Xie S, Richardson LJ, Ballard SA, Dakh F, Grabsch EA, et al. High-resolution melting genotyping of Enterococcus faecium based on multilocus sequence typing derived single nucleotide polymorphisms. PLoS One. 2011; 6(12):e29189. https://doi.org/10.1371/journal.pone.0029189 PMID: 22195020; PubMed Central PMCID: PMC3241712.

Vila, A. (2011). Appearance of Klebsiella Pneumoniae Liver Abscess Syndrome in Argentina: Case Report and Review of Molecular Mechanisms of Pathogenesis. *The Open Microbiology Journal*, *5*(1), 107–113. https://doi.org/10.2174/1874285801105010107

Wang, C. H., Lu, P. L., Liu, E. Y. M., Chen, Y. Y., Lin, F. M., Lin, Y. T., … Lin, J. C. (2019). Rapid identification of capsular serotype K1/K2 Klebsiella pneumoniae in pus samples from liver abscess patients and positive blood culture samples from bacteremia cases via an immunochromatographic strip assay. *Gut Pathogens*, *11*(1), 1–8.

© COPYRIGHT UPM

https://doi.org/10.1186/s13099-019-0285-x

Yu, W., Fung, C., Ko, W., Cheng, K., Lee, C., & Chuang, Y. (2007). Polymerase Chain Reaction Analysis for Detecting Capsule Serotypes K1 and K2 of Klebsiella pneumoniae Causing Abscesses of the Liver and Other Sites . *The Journal of Infectious Diseases*, *195*(8), 1235–1236. https://doi.org/10.1086/512686

Yu WL, Chan KS, Ko WC, Lee CC, Chuang YC. 2007. Lower prevalence of diabetes mellitus in patients with Klebsiella pneumoniae primary liver abscess caused by isolates of K1/K2 than with non-K1/K2 capsular serotypes. Clin Infect Dis 45:1529–1530. https://doi.org/10.1086/523006

© COPYRIGHT UPM

APPENDICS

Age	Age_group	Gender	Source2	Source	HVT	HVT2	K1	K2
52	2	2	2	4	10	1	2	2
58	2	1	1	1	10	1	2	2
51	2	1	1	1	6	1	2	2
81	2	2	2	2	6	1	2	2
79	3	1	1	1	5	1	2	2
54	2	2	1	1	1	2	2	2
45	2	2	1	1	5	1	2	2
91	3	2	2	2	5	1	2	2
80	3	1	1	1	10	1	2	2
91	3	2	1	1	7	1	2	1
85	3	1	1	1	1	2	2	2
80	3	2	1	1	3	2	2	2
19	1	1	1	1	4	2	2	2
70	3	1	1	1	5	1	2	2
31	2	1	1	1	10	1	2	2
47	2	1	2	3	5	1	2	2
84	3	1	1	1	10	1	2	2
48	2	2	1	1	6	1	2	2
33	2	1	1	1	12	1	2	2
72	3	1	1	1	5	1	2	2
60	2	1	1	1	8	1	2	2
89	3	2	1	1	10	1	2	1
50	2	1	1	1	6	1	2	1
71	3	2	1	1	5	1	2	2
15	1	2	1	1	3	2	2	1
61	3	2	2	5	2	2	2	2
55	2	2	2	6	6	1	2	2
31	2	2	2	3	7	1	2	2
62	3	1	2	3	5	1	2	2
72	3	1	1	1	15	1	2	1
31	2	1	1	1	5	1	2	2
47	2	2	2	7	5	1	2	2
47	2	1	1	1	10	1	2	2
80	3	1	2	7	10	1	2	2
71	3	2	2	8	3	2	1	2
19	1	1	2	4	10	1	2	2
15	1	2	2	9	1	2	2	2
64	3	1	1	1	6	1	2	2
47	2	1	2	2	15	1	2	1
63	3	1	2	5	10	1	2	2
60	3	1	2	7	7	1	2	2

© COPYRIGHT UPM

80	3	1	1	1	10	1	2	1
66	3	2	2	8	6	1	2	2
50	2	2	1	1	1	2	2	2

© COPYRIGHT UPM

Cultivation of *Klebsiella pneumoniae* isolates

Samples	Grow (1st)	Repeat (grow)	Die	Glycerol stock
41608		√		√
41677	√			√
41743		√		√
41780	√			√
41901		√		√
42247		√		√
42250	√			√
42302	√			√
42375		√		√
42434		√		√
42555			√	
42595	√			√
42706	√			√
42828		√		√
45147	√			√
45150	√			√
45198			√	
45239		√		√
45252			√	
45406		√		√
45442			√	
45445		√		√
45446	√			√
45466		√		√
45467	√			√
45554			√	
45837	√			√
45945		√		√
45949			√	
46075			√	
46300			√	
46349	√			√
47257	√			√
47325	√			√
46329	√			√
46349	√			√
46405	√			√
42332			√	
46476	√			√
47012	√			√
47113	√			√
47166	√			√

© COPYRIGHT UPM

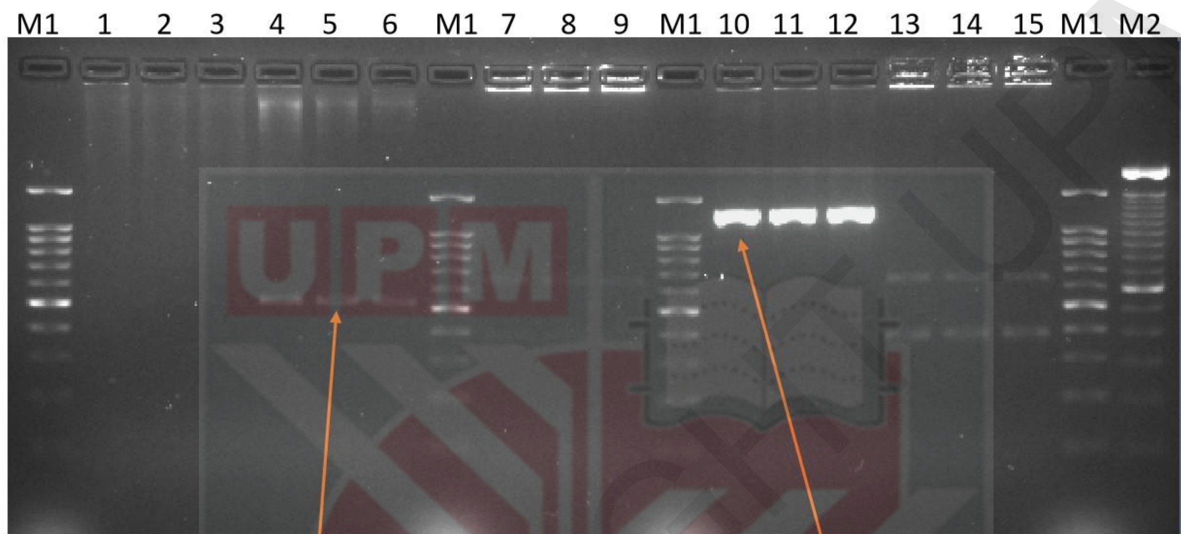
45960			√	
46297			√	
45256			√	
42242	√			√
42317			√	
42332			√	
42558			√	
45362			√	
42737			√	
46259	√			√
47001			√	
7331			√	
7098			√	
7328	√			
7467			√	
7478			√	
7332			√	
7341			√	
7329			√	
7460	√			
6127			√	
6270			√	
6107	√			
7172			√	
6375			√	
6125			√	
6122			√	
6050			√	
6141			√	
6115			√	
8047	√			
6142			√	
8182			√	
6367			√	
6160			√	
6484			√	
6643	√			
6341			√	
7259			√	
7855			√	
8136			√	
8362			√	
7920			√	
7907			√	

© COPYRIGHT UPM

6482			√	
6553			√	
7852			√	
7193	√			
7194			√	
7013	√			
8806			√	
8682			√	
8558			√	
9044			√	
8597			√	
8996	√			
8692			√	
8632			√	
8911			√	
8836			√	
8703			√	
8680			√	
8693			√	
8725			√	
9057			√	
8527			√	

© COPYRIGHT UPM

PCR tenth run: 03.03.2020
Multiplex PCR: Repeat
Mastermix: Bioline
Annealing temperature (Gradient): 57.5-58.0°C
Gene: K1 and K2
Sample:5



K2: 500-600bp

K1: 1200bp

M1: DNA ladder 100 bp
M2: DNA ladder 1k bp
Sample: 1-3 (47257)
Sample: 4-6 (45837)
Sample: 7-9 (45554)
Sample: 10-12 (6107)
Sample: 13-15 (7460)

1. 57.5
2. 57.8
3. 58.0
4. 57.5
5. 57.8
6. 58.0
7. 57.5
8. 57.8
9. 58.0

© COPYRIGHT UPM

Samples	Identification number	Gender	Age	Biological specimen	Lactose fermentation	Glyserol stocks	Hypermucoviscosity testing (String test)	Capsular serotype K1	Capsular serotype K2
1	41608	Female	52	ETT	√	√	10mm		
2	41677	Male	58	Sputum	√	√	10mm		
3	41743	Male	51	Sputum	√	√	6mm		
4	41780	Female	81	Nasal swab	√	√	6mm		
5	41901	Male	79	Sputum	√	√	5mm		
6	42297	Female	54	Sputum	√	√	1mm		
7	42250	Female	45	Sputum	√	√	5mm		
8	42302	Female	91	Nasal swab	√	√	5mm		
9	42375	Male	80	Sputum	√	√	10mm		
10	42434	Female	91	Sputum	√	√	7mm		+
11	42595	Male	85	Sputum	√	√	1mm		
12	42706	Female	80	Sputum	√	√	3mm		
13	42828	Male	19	Sputum	√	√	4mm		
14	45147	Male	70	Sputum	√	√	5mm		
15	45150	Male	31	Sputum	√	√	10mm		
16	45239	Male	47	Blood	√	√	5mm		
17	45406	Male	84	Sputum	√	√	10mm		
18	45445	Female	48	Sputum	√	√	6mm		
19	45446	Male	33	Sputum	√	√	12mm		
20	45466	Male	72	Sputum	√	√	5mm		
21	45467	Male	60	Sputum	√	√	8mm		
22	45837	Female	89	Sputum	√	√	10mm		+
23	45945	Male	50	Sputum	√	√	6mm		+
24	46349	Female	71	Sputum	√	√	5mm		
25	47257	Female	15	Sputum	√	√	3mm		+
26	47325	Female	61	Tissue	√	√	2mm		
27	46329	Female	55	Body fluids	√	√	6mm		
28	46405	Female	31	Blood	√	√	7mm		
29	46476	Male	62	Blood	√	√	5mm		
30	47012	Male	72	Sputum	√	√	15mm		+
31	47113	Male	31	Sputum	√	√	5mm		
32	47166	Female	47	Urine	√	√	5mm		

© COPYRIGHT UPM

33	42242	Male	47	Sputum	√	√	10mm		
34	46259	Male	80	Urine	√	√	10mm		
35	45554	Female	71	Vaginal swab	√	√	3mm	+	
36	46075	Male	19	ETT	√	√	10mm		
37	6107	Female	15	Tracheal aspirate	√	√	1mm		
38	8047	Male	64	Sputum	√	√	6mm		
39	6643	Male	47	Nasal swab	√	√	15mm		+
40	7193	Male	63	Tissue	√	√	10mm		
41	7013	Male	60	Urine	√	√	7mm		
42	8996	Male	80	Sputum	√	√	10mm		+
43	7328	Female	66	Vaginal swab	√	√	6mm		
44	7460	Female	50	Sputum	√	√	1mm		

© COPYRIGHT UPM