DESIGN AND DEVELOPMENT OF MULTIPLEX REAL-TIME PCR (qPCR) FOR DETECTION OF *STREPTOCOCCUS PNEUMONIAE, KLEBSIELLA PNEUMONIAE* AND *HAEMOPHILUS INFLUENZAE*

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by

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In the name of ALLAH, the most Merciful, the most Compassionate. All praise and thanks be to Him, by whose blessings all goods can be accomplished. May peace be upon Prophet Muhammad, his families and companions. Alhamdulillah, the day I have been waiting for all the time has come. Here, I am grateful to have the opportunity to express my sincerest gratitude for those I will mention or not mention, and their kindness will always be remembered. First and foremost, my deepest gratitude to my dearest supervisor, Prof. Dr. Chan Yean Yean, for her continuous guidance, excellent supervision, expertise, support, and patience throughout this study. Thank you, Prof, for always lending me your precious time for my research project. I am extending my warmest gratitude to my lovely parents, Zahari Saleh and Nor Khapiza Khalid for their endless support, cares, and prayers. Thank you also for always providing me with my favourite food while I was writing my thesis. It gives me more energy and boosts my mood to finish my thesis writing. I love you. Next, I also would like to thank my friends, Noor Fardziatun, Alia, Rina, Jia Ying and Aisyah, for giving me emotional support and accompanying me for days and nights to complete my research. Not to forget my experienced seniors, Dr. Wardah, Dr. Nik, and Dr.Ira, for their endless guidance and knowledge. Special thanks to the staff in the Medical Microbiology laboratory for their kindness. I am truly blessed with the beautiful hearts around me. No words can describe the depth of gratitude I hold for every one of you. Last but not least, I want to thank me. I want to thank me for believing in me. I want to thank me for doing all this hard work. I want to thank me for having no days off. I want to thank me for never quitting. I want to thank me for trying to do more right than wrong and I want to thank me for fighting till the end. I love me.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

-	Negative or subtraction
%	Percentage
+	Positive or addition
×	Times or multiplication
÷ or /	Division or 'or'
°C	Degree celcius
µl	Microliter
µm	Micrometer
µM	Micromolar
ag/µl	Attogram per microliter
BHI	Brain-heart infusion
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CAP	Community-acquired pneumoniae
CFU	Colony forming unit
CI	Confidence interval
CO_2	Carbon dioxide
DALYs	Disability-adjusted life years
dH_2O	Distilled water
dNTPs	Deoxyribonucleotide triphosphate
dsDNA	Double stranded deoxyriboneucleic acid
ELISAs	Enzyme-linked immunosorbent assays
EPIC	Etiology of Pneumonia in the Community
fg/µl	Femtogram per microliter
fucK	Fuculose Kinase

g	gram
g	Gravitational force
GI	Gastrointestinal
HAP	Hospital-acquired pneumonia
HI	*Haemophilus influenzae*
Hib	*Haemophilus influenzae* type b
HIV/AIDS	Human immunodeficiency virus/ Acquired immunodeficiency syndrome
IAC	Internal amplification control
ICU	Intensive care unit
IDT	Integrated DNA technologies
IgA1	Immunoglobulin A1 protease
IV	Intravenous
kcal/mol	Kilocalorie per mole
KP	*Klebsiella pneumoniae*
LIS	Laboratory information system
LOD	Limit of detection
lytA	Autolysin A
mg/ml	Milligram per mililiter
$MgCL_2$	Magnesium chloride
ml	Mililiter
mm	Milimeter
n	Total
NaLC	Sodium chloride
NanA	Neuraminidase
NaOH	Sodium hydroxide
NAP	Nosocomial acquired pneumoniae
NCBI	National Center for Biotechnology Information

NHLBI	National Heart, Lung, and Blood Institute
NPC	Non-protein coding
NPV	Negative predictive value
NTHi	Non-typeable *Haemophilus influenzae*
NURTF	Normal upper respiratory tract flora
O_2	Oxygen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV13	Pneumococcal conjugate vaccine
phoE	Outer membrane phosphate porin precursor
Ply	Pneumolysin
PPV	Positive predictive value
PPV23	Pneumococcal polysaccharide vaccine
PspA	Pneumococcal surface protein A
Q	Quencher
qPCR	Real-time polymerase chain reaction
R	Reporter
rpoB	Beta subunit of RNA polymerase
RSV	Respiratory syncytial virus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SEM	Scanning electron microscope
SP	*Streptococcus pneumoniae*
sp	Species
Taq	Thermus aquaticus
TB	Tuberculosis
TBE	Tris-Borate-EDTA
TE	Tris-EDTA

T_m	Melting temperature
USM	Universiti Sains Malaysia
UTIs	Urinary tract infections
V	Nicotinamide adenine dinucleotide growth factor
VAP	Ventilator-associated pneumonia
WHO	World Health Organization
X	Hemin growth factor

LIST OF APPENDICES

REKA BENTUK DAN PEMBANGUNAN *REAL-TIME PCR* (qPCR) MULTIPLEKS UNTUK PENGESANAN *STREPTOCOCCUS PNEUMONIAE*, *KLEBSIELLA PNEUMONIAE* DAN *HAEMOPHILUS INFLUENZAE*

ABSTRAK

Bakteria normal flora boleh menjadi bahaya jika mereka menyerang bahagian badan selain habitat mereka. *Streptococcus pneumoniae*, *Haemophilus influenzae*, dan *Klebsiella pneumoniae* adalah flora saluran pernafasan atas yang normal yang boleh menyebabkan pneumonia. Senario ini merupakan cabaran yang ketara untuk kesihatan manusia terutamanya bagi pesakit immunocompromised. Pengesanan patogen yang pantas memberi rawatan yang berkesan. Oleh itu, kajian ini menyasarkan untuk mencipta dan membangunkan qPCR multipleks. Setelah berjaya mencipta primer, probe dan DNA sintetik untuk bakteria yang disasarkan, kepekatan primer, probe dan DNA sintetik untuk semua bakteria dan IAC telah dioptimum untuk membangunkan qPCR multipleks. Berdasarkan analisa, kepekatan primer yang optimum bagi bakteria yang disasar (*S. pneumoniae*, *K. pneumoniae* dan *H. influenzae*) dan IAC adalah masing-masing 0.7 µM dan 0.15 µM. Manakala, kepekatan optimum probe bagi *S. pneumoniae*, *K. pneumoniae*, *H. influenzae* dan IAC adalah masing-masing 0.2 µM, 0.1 µM, 0.3 µM, dan 0.05 µM. Tambahan pula, kepekatan optimum yang telah dipilih untuk DNA sintetik bakteria yang disasar dan IAC adalah masing-masing 50 fg/µl dan 1 fg/µl. Seterusnya, ujian sensitiviti analitikal telah dilakukan sebanyak tiga kali menggunakan DNA genom *S. pneumoniae*, *K. pneumoniae*, dan *H. influenzae* bermula dari 1 pg/µl hingga 100 ag/µl. Ujian sensitiviti menunjukkan nombor salinan untuk *S. pneumoniae*, *K. pneumoniae*, dan *H. influenzae* adalah masing-masing 227 salinan/µl, 9 salinan/µl, dan 51 salinan/µl. Kemudian, ujian analitikal spesifisiti telah dilakukan dengan menggunakan 32 bakteria klinikal asingan. Asai yang telah dibangunkan telah

berjaya mengenal pasti semua bakteria *S. pneumoniae, K. pneumoniae* dan *H. influenzae*, menunjukkan spesifikasi adalah 100%, sama seperti keputusan ketika BLAST. Ujian klinikal telah dilakukan dengan menggunakan 94 spesimen kahak yang telah diambil dari Makmal Mikrobiologi Perubatan dan Parasitologi USM, Kelantan, Malaysia. Daripada 94 spesimen kahak, 79 spesimen kahak dikesan positif oleh qPCR multipleks, manakala 32 spesimen kahak dikesan positif oleh kaedah kultur. Sensitiviti, spesifisiti, PPV, NPV dan ketepatan klinikal oleh qPCR multipleks yang telah dibangunkan adalah 95.24 – 100%, terutama sekali bagi *S. pneumoniae* dan *H. influenzae*. Walaubagaimanapun, sensitiviti, PPV, dan ketepatan klinikal untuk *K. pneumoniae* rendah, masing-masing 33.90%, 39.33%, dan 53.73%. Walaupun terdapat perbezaan yang ketara antara keputusan yang diberikan oleh qPCR dan kaedah kultur, tetapi keputusan yang dikeluarkan oleh elektroforesis gel agarosa dan jujukan Sanger adalah selari dengan keputusan oleh qPCR, membuktikan bahawa qPCR multipleks yang dibangunkan adalah boleh dipercayai dan diharap. Kesimpulannya, kajian ini telah berjaya membangunkan qPCR multipleks untuk pengesanan awal *S. pneumoniae*, *K. pneumoniae* dan *H. influenzae*. qPCR ini akan digunakan untuk penyelidikan pada masa hadapan bagi mengesan bakteria yang boleh menyebabkan community-acquired pneumonia.

DESIGN AND DEVELOPMENT OF MULTIPLEX REAL-TIME PCR (qPCR) FOR DETECTION OF *STREPTOCOCCUS PNEUMONIAE, KLEBSIELLA PNEUMONIAE* AND *HAEMOPHILUS INFLUENZAE*

ABSTRACT

Normal flora bacteria can become pathogenic once they invade a part of the body different from their regular habitat. *Streptococcus pneumoniae*, *Haemophilus influenzae,* and *Klebsiella pneumoniae* are normal upper respiratory tract flora that can cause pneumonia. The scenario poses a significant challenge to human health, especially in immunocompromised patients. Rapid detection of these pathogens facilitates effective therapies. Therefore, this study aims to design and develop multiplex qPCR. Following the successful design of primers, probes and synthetic DNA for the targeted bacteria, the concentrations of the primers, probes and synthetic DNA for all bacteria including IAC was optimised in order to develop a multiplex qPCR. According to the optimisation, the optimal concentrations of primers selected for the targeted bacteria (*S. pneumoniae*, *K. pneumoniae,* and *H. influenzae*) and IAC were 0.7 µM and 0.15 µM, respectively. Meanwhile, the optimal concentration of probes selected for *S. pneumoniae, K. pneumoniae, H. influenzae*, and IAC were 0.2 µM, 0.1 µM, 0.3 µM, and 0.05 µM, respectively. In addition, the concentrations of synthetic DNA selected for the targeted bacteria and IAC were 50 fg/µl and 1 fg/µl, respectively. Next, analytical sensitivity evaluation was conducted in triplicates using genomic DNA of *S. pneumoniae*, *K. pneumoniae*, and *H. influenzae* ranging from 1 pg/µl to 100 ag/µl. The sensitivity assessment revealed that the copies number for *S. pneumoniae, K. pneumoniae,* and *H. influenzae* were 227 copies/µl, 9 copies/µl, and 51 copies/µl, respectively. Subsequently, analytical specificity evaluation was conducted using 32 bacteria clinical isolates. The developed assay effectively

identified all isolates of *S. pneumoniae, K. pneumoniae* and *H. influenzae*, demonstrating a specificity of 100%, which corresponds with the BLAST result. A clinical evaluation test was conducted using 94 sputum samples collected from the Laboratory of Medical Microbiology and Parasitology USM, Kelantan, Malaysia. Out of the 94 sputum samples, 79 sputum samples were detected positive by the multiplex qPCR, while 32 sputum samples were detected positive by the culture method. The clinical sensitivity, specificity, PPV, NPV and accuracy of the developed multiplex qPCR were 95.24 – 100%, especially for *S. pneumoniae* and *H. influenzae*. However, the clinical specificity, PPV, accuracy for *K. pneumoniae* were low, 33.90%, 39.33%, and 53.73%, respectively. Despite significant discrepancies between the results obtained from qPCR and the culture method, the results from agarose gel electrophoresis and sequencing were consistent with the qPCR results, providing evidence that the developed multiplex qPCR is reliable. In conclusion, this study has effectively developed multiplex qPCR for the early detection of *S. pneumoniae*, *K. pneumoniae*, and *H. influenzae.* This qPCR will be employed in future research to detect bacteria responsible for community-acquired pneumonia.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Bacteria are unique unicellular microorganisms crucial in preserving the surrounding environment. Many are normal flora because they inhabit the human body without causing infection. Only a minority are accountable for causing disease and infection (Doron and Gorbach, 2008). Bacterial infections pose a significant and growing concern on a global scale, as severe bacterial infections can lead to hospitalisation and are linked to adverse effects (Liyanarachi et al., 2022). Infectious diseases caused by bacteria are the leading cause of mortality worldwide. They pose a substantial risk to human and animal populations' health and welfare, impeding productivity and substantially escalating economic losses (Vidic et al., 2017). Infectious diseases caused by bacteria include tuberculosis (TB), gonorrhoea, urinary tract infections (UTIs), pneumonia, and many more. However, an infectious disease that can caused by *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Klebsiella pneumoniae* is pneumonia (Uzoamaka et al., 2017).

Pneumonia is an acute respiratory illness that contributes to mortality and morbidity globally, particularly among the elderly, children, and those with comorbidities (Htun et al., 2019; World Health Organization (WHO), 2021). In 2021, pneumonia ranked Malaysia's third major cause of death (11.1%) (Department of Statistics Malaysia, 2022). The acute respiratory illness is significantly higher in underdeveloped countries compared to developed countries, with a burden that is 10 – 50 times greater (Alemayehu et al., 2019). Pneumonia is a disease that affects the

lungs. Lungs are a pair of spongy, air-filled, and pinkish-grey hue fundamental organs of the respiratory system that facilitate the gas exchange between the environment and the circulation. When breathing in, the oxygen (O_2) from the air passes through the nostrils and travels to the trachea. The trachea branches out to two bronchi. The O_2 then passes through the bronchi and is followed by the bronchioles. Finally, the O_2 will reach more than 300 million alveoli, where gas exchange occurs (Chaudhry and Bordoni, 2021; Haddad and Sharma, 2022).

Typically, the alveoli of a healthy person are filled with air. Meanwhile, the alveoli of pneumonia patients are filled with pus and fluid, making them feel pain when breathing and limiting their oxygen intake (WHO, 2021). There are some common categories of pneumonia; nosocomial-acquired pneumonia (NAP), also known as hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), and community-acquired pneumonia (CAP) (Lanks et al., 2019; Woodhead, 2013). *S. pneumoniae* and *H. influenzae* are common pathogens for CAP (Prina et al., 2015; Suaya et al., 2021), whereas *K. pneumoniae* is more common in HAP (Ashurst and Dawson, 2018). Severe pneumonia can lead to severe hypoxemic respiratory failure, which in turn may lead to septic shock and culminate in a fatal outcome due to multiple organ dysfunction (Ariani et al., 2013). The treatment for pneumonia includes supportive care, admission to the intensive care unit (ICU), and antibiotics such as macrolide, doxycycline, penicillin, and beta-lactam (Regunath and Oba, 2022; Sethi, 2022).

Identifying bacteria that cause illness is necessary before prescribing which antibiotic is appropriate for the patient. At the moment, the traditional methods to

detect bacteria include microscopic examination, bacteria culture, and immunological and biochemical testing. However, they are time-consuming, complex, low sensitivity, and require an expert to handle them, especially for cultivation and microscopic examination (Hu et al., 2020). An imperative component of an effective strategy to address infectious disease risks is employing a pathogen diagnostic assay that is rapid, selective and sensitive (Vidic et al., 2017).

Molecular diagnostic techniques are based on the amplification of nucleic acids, such as polymerase chain reaction (PCR), aim to bypass the traditional method and accelerate the diagnostic procedure. PCR enables the pathogen to be detected and identified concurrently, resulting in faster diagnoses compared to those obtained by culturing methods and eliminating the need for extra culture tests (Järvinen et al., 2009). Compared to conventional PCR, real-time PCR has become a promising assay to detect pathogens because it is fast (does not require agarose gel), sensitive and specific (Hu et al., 2020). A rapid and accurate identification of bacteria would permit the de-escalation of antibiotics, reducing the drug's adverse effect, cost, and probability of developing antibiotic resistance (Koo et al., 2021). Undoubtedly, early pathogen detection is vital for infection management and the development of more effective decision-making tools (Vidic et al., 2017).

1.2 Problem statement

Human beings have coexisted unfavourably with a multitude of microorganisms that have the capacity to induce infections and maladies. *S. pneumoniae, H. influenzae,* and *K. pneumoniae* are known bacteria that can cause pneumonia (Cillóniz et al., 2019; Slack, 2015). Antibiotic therapy was most frequently administered for pneumonia globally. The gold standard method for identifying bacteria involves cultivating bacteria from the clinical specimen to the media for an extended period according to strict protocols. It has become an issue since it is time-consuming and requires much work (Bhunia, 2014; Chen et al., 2022). Early detection of microorganisms is believed to pave the way for a rapid diagnosis and the immediate beginning of the necessary treatment. As a result, it improves patients' prognoses and lowers the disease's burden, the chance of death, and the economic loss possibility (Nii-Trebi, 2017).

The ability of PCR to replicate the deoxyribonucleic acid (DNA) notified the researchers about the efficient detection of selected bacteria. Therefore, PCR has been used to aid the detection of bacteria, and it is very promising because it is sensitive and specific (Chen et al., 2022). Many studies have focused on the identification of bacteria using real-time PCR because this assay is faster, more sensitive, and has better resolution (different bacteria use different coloured channels to plot graphs) than conventional PCR (Ramamurthy et al., 2011). Furthermore, many researchers have successfully developed real-time PCR, such as dye-based qPCR and probe-based qPCR, which are able to detect various pathogens simultaneously, including *S. pneumoniae, K. pneumoniae,* and *H. influenzae*. However, the developed real-time

PCR exhibited shortcomings in terms of low sensitivity and specificity. Hence, this study aims to improve the sensitivity and specificity of the assay. In addition, this assay will be employed by future study to detect common bacteria responsible for community-acquired pneumoniae, specifically *S. pneumoniae*, *K. pneumoniae* and *H. influenzae*.

1.3 Significance of the study

Pneumonia imposes a significant global health burden, surpassing ailments like cancer, diabetes, HIV/AIDS, malaria, and other diseases acknowledged as major global health concerns (Mizgerd, 2012; Quinton et al., 2018). The calculation of the disease burden involves assessing disability-adjusted life years lost. The staggering global impact of pneumonia is partly attributable to the fact that it takes more children's and elderly's lives globally (Cunha, 2001; Quinton et al., 2018). Pneumonia prognosis might be negatively impacted by a delay in administering antibiotics. Guidelines advocate for prompt initiation of antibiotics in pneumonia patients since multiple studies have indicated a higher survival chance when antibiotics are given within 4 hours of presentation (Kao et al., 2019; Lee et al., 2019). A study by Etiology of Pneumonia in the Community (EPIC) discovered that only 38% of pneumonia pathogens were detected when using traditional methods, including culture, antigen detection assay, and nucleic acid detection tests (Jain et al., 2015).

In recent years, molecular tools have gained prominence as the preferred diagnostic method for respiratory pathogens due to their exceptional sensitivity in identifying pathogens that are hard to isolate, possess low viability, or exist in minute quantities (Bhat et al., 2012). A study by Hu et al. (2020) has successfully developed a cheap dye based real-time PCR. The melting temperature that depends on amplicon size to detect numerous bacteria, including *S. pneumoniae, K. pneumoniae,* and *H. influenzae,* is subjective and less sensitive because the temperature may be shifted. In addition, previous study that used similar channel for many different pathogens, including *S. pneumoniae* and *H. influenzae* are less specific due to their inability to

differentiate which bacteria is detected (Edin et al., 2015). Furthermore, a recent study by Koo et al. (2021) has also successfully developed a multiplex qPCR that detected *S. pneumoniae*, *H. influenzae*, *K. pneumoniae*, and *Burkholderia pseudomallei* concurrently with the use of the different channels for each bacteria. However, it was found that the quality of some primers and probe used in the study was poor except for *K. pneumoniae*.

Therefore, this study aims to fill the gaps by developing a TaqMan real-time PCR (qPCR) assay with better quality primers and probes that simultaneously detect three bacteria (*S. pneumoniae*, *K. pneumoniae,* and *H. influenzae*). Apart from these three disease-causing targets, an additional internal amplification control (IAC) is incorporated to eliminate the risk of false negatives caused by PCR inhibitors and others. The crucial function of IAC is frequently overlooked and absent in numerous reported molecular assays. In addition, the developed assay will exhibit a high level of specificity as each targeted bacterium will be assigned its own dedicated channel. In summary, this study will develop a probe-based multiplex qPCR assay for the simultaneous detection of *S. pneumoniae, K. pneumoniae, H. influenzae,* and IAC with high sensitivity and specificity. It might be helpful in hospital settings where *S. pneumoniae*, *K. pneumoniae*, and *H. influenzae* always cause diseases.

1.4 General objective

The aim of this study is to develop a foolproof, sensitive and specific multiplex qPCR for detecting *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Haemophilus influenzae*.

1.4.1 Specific objectives

1) To design specific primers and probes for *Streptococcus pneumoniae, Klebsiella pneumoniae,* and *Haemophilus influenzae.*

2) To develop a multiplex real-time PCR to detect *Streptococcus pneumoniae, Haemophilus influenzae,* and *Klebsiella pneumoniae*.

3) To optimise the incorporation of internal amplification control (IAC) in the developed multiplex real-time PCR assay.

4) To perform analytical sensitivity and specificity of the developed multiplex real-time PCR assay.

5) To perform a clinical evaluation of the developed multiplex real-time PCR assay using sputum samples.

1.5 Flowchart of the study

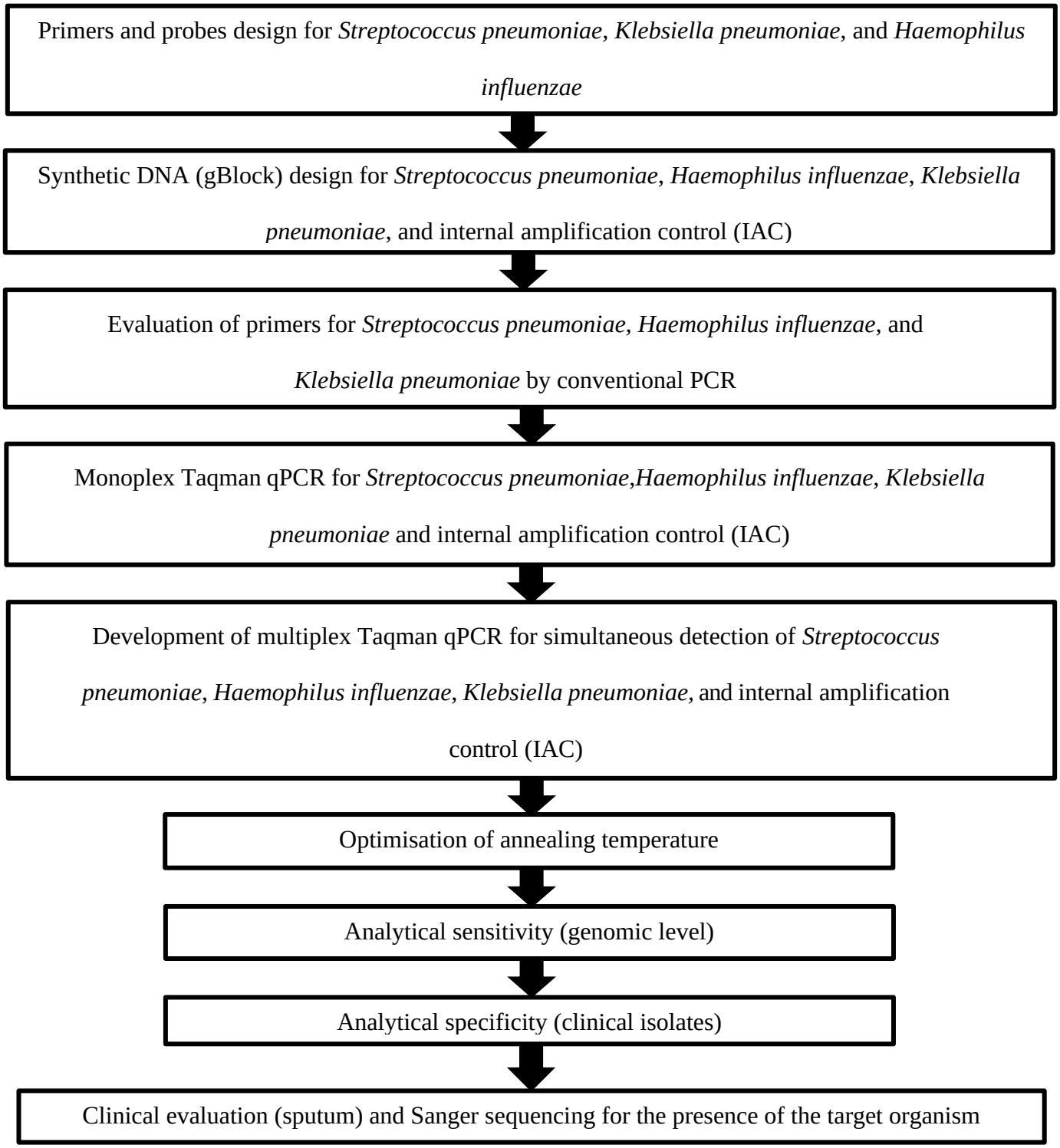


Figure 1.1: Workflow of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Overview on pneumonia

All vertebrates require a constant oxygen supply for metabolism and life. August Krogh referred to this requirement as “the call for oxygen,” while Max Kleiber referred to it as “the fire of life” (Hsia et al., 2016). The body will typically remove pathogens (bacteria, viruses, and fungus) from the air and prevent them from entering the lungs. When these pathogens enter the lungs, the immune system is activated, and a war between the body’s natural defense and pathogens happens, resulting in an inflammation of the alveoli. The inflammation of the alveoli causes the alveoli to fill with pus and fluid, which eventually leads to the symptoms of pneumonia (Figure 2.1) (National Heart Lung and Blood Institute (NHLBI), 2022).

Although there have been improvements in clinical management and new antimicrobials, pneumonia remains the top cause of morbidity and mortality globally. According to the Global Burden of Disease Study 2019, lower respiratory tract infections, including pneumonia, ranked as the fourth leading cause of disability-adjusted life-years (DALYs) in all ages. One DALYs refers to the loss similar as one year of full health. Children below 10 years and elderly aged more than 75 years have higher chances of suffering from lower respiratory infections (Vos et al., 2020). In 2021, pneumonia ranked as the third major cause of death in Malaysia (11.1%) and the primary cause of death in Wilayah Persekutuan Putrajaya (12.4%) (Department of Statistics Malaysia, 2022).

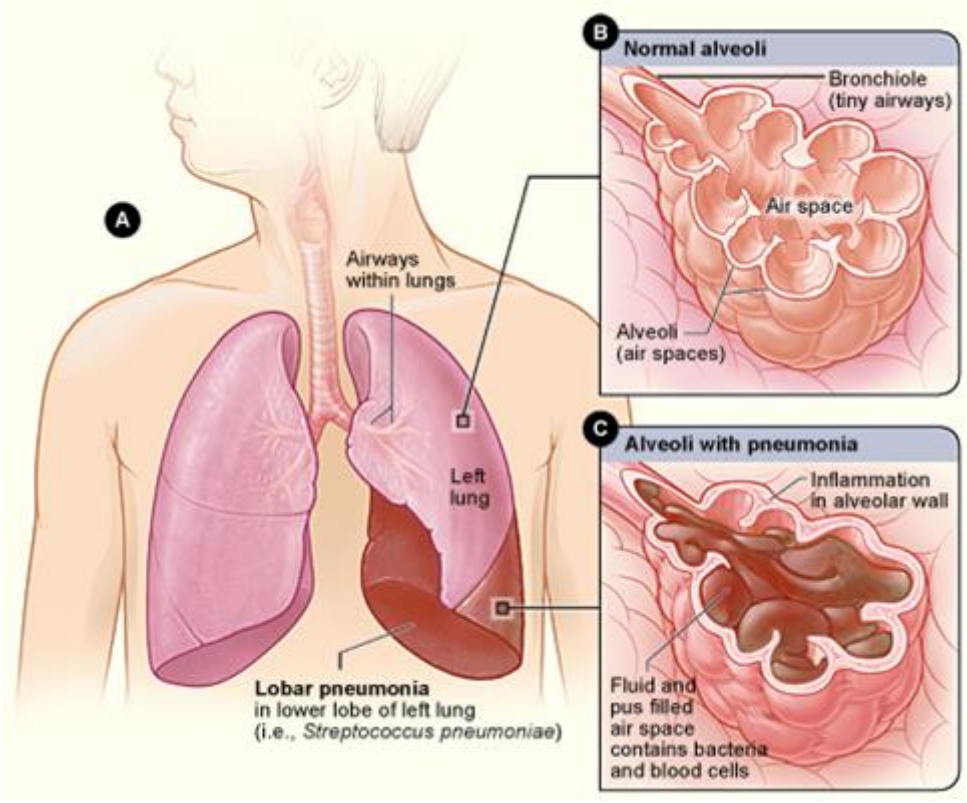


Figure 2.1: Pneumonia a) Pathogen infecting the person's left lung b) The condition of normal alveoli c) The condition of alveoli in pneumonia person (adopted from National Heart Lung and Blood Institute (NHLBI), 2022).

Pneumonia can be categorised into three types: nosocomial-acquired pneumonia (NAP), also known as hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), and community-acquired pneumonia (CAP). CAP is a type of infection that people acquire outside the hospital, and HAP is an infection that people acquire after being hospitalised for at least 48 hours. Meanwhile, VAP refers to the infection people acquire during ventilation (Lanks et al., 2019; Woodhead, 2013). Pneumonia is caused by more than 100 pathogens, including bacteria, viruses, and fungi. Examples of the pathogens are *S. pneumoniae*, *H. influenzae*, *K. pneumoniae*, *Aspergillus*, *Histoplasma capsulatum*, *Pneumocystis jirovecii*, rhinovirus, respiratory syncytial virus (RSV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Lim, 2022).

Streptococcus pneumoniae and *H. influenzae* commonly cause CAP (Carugati et al., 2020; Shoar and Musher, 2020; Torres et al., 2021), whereas *K. pneumoniae* commonly causes HAP (Ashurst and Dawson, 2024; Sattar et al., 2024). A patient with pneumonia manifests symptoms such as cough, shortness of breath, higher respiratory rate, sputum production, and chest pain. In addition, they may also show nonspecific systemic symptoms, such as fever, fatigue, muscular aches, and appetite loss (Halm and Teirstein, 2002; Raghavendran et al., 2007). Severe pneumonia may cause serious complications requiring admission to the intensive care unit (ICU) and multi-organ malfunction caused by inflammation (Ceccato and Torres, 2018; Morgan and Glossop, 2016). The treatments for pneumonia include antiviral (oseltamivir), antifungals (triazole, echinocandins, amphotericin B), antibiotics (macrolide, doxycycline, penicillin, and β-lactam), IV fluid and oxygen therapy (Mandanas et al., 2021; Regunath and Oba, 2022; Sethi, 2022).

2.1.1 Pathogenesis of bacterial pneumonia

The development of bacterial pneumonia usually commences with the invasion of pathogenic agents, such as *S. pneumoniae, H. Influenzae,* and *K. pneumoniae,* into the host's respiratory tract. Upon reaching the alveoli, the pathogen undergoes multiplication and subsequently triggers the host responses. The pathogen is capable of entering the host's lower respiratory tract by various means, including inhalation, micro-aspiration, direct inoculation, and hematogenous or contiguous dissemination from an adjacent focus. Direct inoculation by means of a penetrating thoracic injury or contagious dissemination from an infection site like mediastinitis is possible but uncommon (Mandell, 2015).

The hematogenous dissemination is possible to occur among intravenous drug abusers who have tricuspid endocarditis. Nonetheless, the primary possible pathways consist of the aspiration of the bacterial causative agent in small volumes into the host's oropharynx, which may occur during sleep, and the inhalation of contaminated droplets. Micro-aspiration is a phenomenon that can occur with notable frequency even in healthy individuals. However, the likelihood of pneumonia developing due to micro-aspiration is relatively low (Zahari et al., 2023). Once the bacteria successfully reaches the alveoli, the surfactant proteins and macrophages located in the alveoli will retain the bacteria at the bay. In the event of a failure of the host's defense mechanisms, the pathogen may proliferate and persist, inducing the host to initiate an inflammatory response. The response produced is responsible for the various clinical manifestations observed in patients diagnosed with pneumonia (Mandell, 2015). Alveolar capillary leakage may occur, resulting in the filling of alveoli with potential hypoxemia. Under

critical circumstances, secondary alterations in lung volume and compliance can lead to fatality (Chaudhuri et al., 2007).

Pneumonia typically develops in patients following various stages of tissue alterations. During the initial phase, the presence of proteinaceous exudate in the alveoli leads to the development of oedema. This is subsequently followed by a red hepatisation stage, which is characterised by the buildup of red blood cells. The subsequent stage is known as the grey hepatisation phase, characterised by the breaking down and lysis of erythrocytes accompanied by the deposition of fibrin and neutrophils. Subsequently, the resolution phase ensues, which encompasses the activities of macrophages, elimination of debris, and attenuation of inflammatory reactions (Mandell, 2015). The incidence of pneumonia is significantly influenced by the frequency of aspiration, pathogenic bacterial inoculum, quantity of aspirate, and the virulence of the aspirated bacteria in relation to the host's immune response (Zahari et al., 2023). Apart from mechanical safeguards, the innate and adaptive immune responses of the host are crucial in protecting against such occurrences (Reynolds, 1989).

2.2 Overview of *Streptococcus pneumoniae*

Streptococcus pneumoniae is a bacterium affecting the human respiratory tract and significantly contributing to morbidity and mortality worldwide. This pneumococcus typically resides in the nasopharyngeal region of the healthy individual but can also lead to fatal illnesses like otitis media, meningitis, sepsis, and pneumonia when it migrates to the middle ear, brain, blood, and lungs (Subramanian et al., 2019). Pneumonia is related to 60 to 87% of adult pneumococcal bacteremia (Jedrzejas, 2001). Clinical studies indicated that *S. pneumoniae* is the primary causative agent identified when isolating a bacterial organism (Iyer, 2023). It was noted that children have higher rates of pneumococcal carriage, ranging from 20 – 50% in richer resourced countries, compared to adults with rates of 5 – 20%. Whereas, in poor resource countries, the rates are significantly higher, where up to 90% of children and over half of adults are being colonised (Chao et al., 2015).

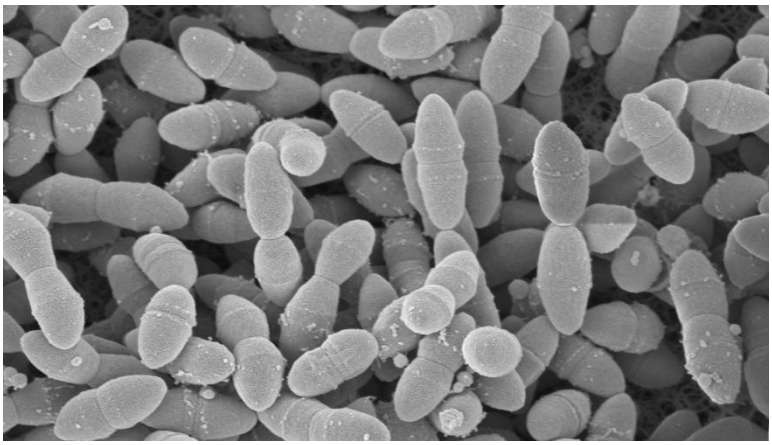
The transmission, colonisation, and invasion of *S. pneumoniae* rely on its capacity to elude or exploit the host's inflammatory and immunological responses (Weiser et al., 2018). The pneumococci transmission happens via close contact and aerosol. The colonisation in the body by pneumococci is regarded as a necessary condition for developing disease, although most colonised individuals do not exhibit symptoms. Hence, the adherence of *S. pneumoniae* to nasopharyngeal mucosal epithelial cells is a crucial stage in the progression toward causing disease (Marquart, 2021). This bacterium is composed of an exterior polysaccharide capsule that is negatively charged. The capsule is the primary virulence factor of the bacterium and is also responsible for determining its serotype. Examples of other virulence factors

are pneumolysin (*Ply*), pneumococcal surface protein A (PspA), immunoglobulin A1 (IgA1) protease, and neuraminidase (NanA) (Ahmed and Malik, 2022). They play crucial roles in facilitating important aspects of pneumococcal colonisation and/or invasion. Disrupting these processes reduces the pathogenicity of *S. pneumoniae* (Jedrzejas, 2001).

Young children, the elderly, and individuals with underlying diseases such as asplenia, chronic medical conditions, or immunosuppressive disease, mainly acquired immunodeficiency syndrome (AIDS), have notably elevated rates of disease caused by *S. pneumoniae*. At the moment, antibiotics remain the most efficacious therapeutic approach for *S. pneumoniae* infection (Ahmed et al., 2019). Unfortunately, the therapeutic efficacy of current antibiotics is progressively declining due to the global increase in the drug-resistant rate of *S. pneumoniae* caused by the extensive use of broad-spectrum antibiotics. On the other hand, current vaccines such as pneumococcal polysaccharide vaccine (PPV23) and pneumococcal conjugate vaccine (PCV13) only protect against serotypes that are included in the vaccine (Kim et al., 2017). Consequently, there is a demand to establish novel antimicrobials and vaccines for treating *S. pneumoniae* (Subramanian et al., 2019; Zou et al., 2023).

Streptococcus pneumoniae is an encapsulated, gram-positive (stained purple) bacteria that can be classified into more than 90 serotypes based on how it reacts with specific antibodies. It grows as cocci but is often present as diplococci (with a size of 0.5 to 1.25 µm) because of its lancet shape and in chains of different lengths (Figure 2.2 (a)). *S. pneumoniae* is a facultative anaerobe, fastidious, lactic acid fermenting organism, catalase-negative, non-motile, and does not produce spores. The growth of this bacteria can be enhanced by using 5% carbon dioxide (CO_2) or an anaerobic environment. The colonies of *S. pneumoniae* are initially raised but become flat with a depressed centre when cultured on blood agar. Furthermore, the colonies can display α-hemolysis where the medium surrounding them becomes green or brown due to the partial lyse of red blood cells in the blood agar (Figure 2.2 (b)) (Ahmed and Malik, 2022; Peter and Klein, 2008).

a)



b)

Figure 2.2: The morphology of *S. pneumoniae*; a) *S. pneumoniae* cells under scanning electron microscopy (SEM) ; b) *S. pneumoniae* colonies display as α-hemolysis on blood agar (adopted from Aryal, 2022; Robert Koch Institut, 2014).

2.3 Overview of *Klebsiella pneumoniae*

The first isolation of *Klebsiella pneumoniae* was made in the late 19th century and was known as Friedlander's bacterium (Paczosa and Mecsas, 2016). *K. pneumoniae* is an opportunistic pathogen in which humans are its primary reservoir. It can usually be found in the gastrointestinal (GI) tract and oropharynx without causing any harmful effects (Holt et al., 2015). The pathogen can also be found in environmental sources such as water, soil, vegetation, and industrial wastes (Sharma, 2015). Carrier rates for *K. pneumoniae* are significantly higher among hospitalised patients compared to the community. A study has found that the number of antibiotics administered to hospitalised patients is associated with carrier rates in the faeces as high as 77% (Esposito et al., 2018; Walter et al., 2018). However, once the bacteria successfully move to other sites in the body by evading the immune system, it can cause harmful diseases such as pneumonia, urinary tract infections, bloodstream infections, and sepsis (Holt et al., 2015).

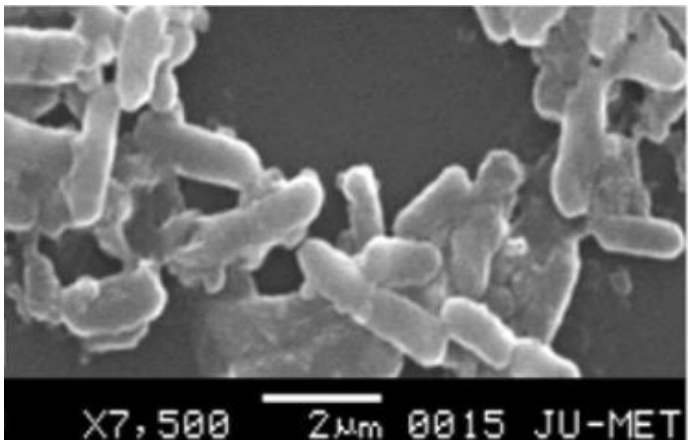
The ability of bacteria to infect and cause diseases to the host depends on the virulence factors. The bacterium's polysaccharide capsule is the primary virulence factor, enabling the bacteria to avoid opsonophagocytosis and serum killing by the host organism. So far, researchers have studied 77 various capsular types, and it has been observed that those *K. pneumoniae* without capsules are less harmful. Another virulence factor is the lipopolysaccharides at the outer surface of a gram-negative bacteria. The detection of lipopolysaccharides triggers an inflammatory response in the host and has been a significant cause of the complications seen in sepsis and septic shock. Fimbriae is another virulence factor that facilitates the attachment of the

bacteria to the host cells. Furthermore, siderophores are virulent factors that obtain iron from the host to enable the propagation of the infecting bacteria (Rønning et al., 2019; Tsereteli et al., 2018).

Pneumonia caused by *K. pneumoniae* can be categorised as community-acquired pneumonia (CAP) or hospital-acquired pneumonia (HAP), but infection of *K. pneumoniae* in CAP is uncommon in Western (Hirai et al., 2020; Munoz-Price et al., 2013). It is estimated that in Western culture, the CAP caused by this bacterium is approximately 3 to 5%, but in developing countries like Africa, this bacterium can be responsible for approximately 15% of all pneumonia cases (Ashurst and Dawson, 2024). However, other finding in Asia has reported that instead of *S. pneumoniae*, *K. pneumoniae* is also commonly responsible for causing CAP (Hirai et al., 2020; Rammaert et al., 2012). Furthermore, unlike Western, studies from Asia have shown that *K. pneumoniae* is more common cause of pneumonia compared to *S. pneumoniae* and *H. influenzae* (Chen et al., 2022; Morris et al., 2021). In the past, *K. pneumoniae* infections were severe, primarily in people who did not possess robust immune systems. However, with the emergence and spread of hypervirulent strains, the susceptible population has been expanded to include healthy individuals and immunosuppressed. Furthermore, the antibiotic resistance of *K. pneumoniae* strains has increased, making the treatment of infections caused by these strains tough (Paczosa and Mecsas, 2016).

Klebsiella pneumoniae is an encapsulated, gram-negative (stained pink), non-motile, lactose-fermenting, and facultative anaerobic bacteria. It is a rod-shaped bacterium with a size of about 0.3 to 1.0 µm in width and 0.6 to 6.0 µm in length (Figure 2.3 (a)). They can appear single, in pairs, short chains, or rarely in clusters. The colonies of *K. pneumoniae* appear mucoid and grey-white in colour when grown on blood agar. Furthermore, the colonies will display as γ-hemolysis on blood agar where no change occurs on the agar. This is because the bacterium is not a hemolysin producer. When no hemolysin is produced, no red blood cells are being lysed, resulting in no change observed on the blood agar (Figure 2.3 (b)) (Sharma, 2015).

a)



b)

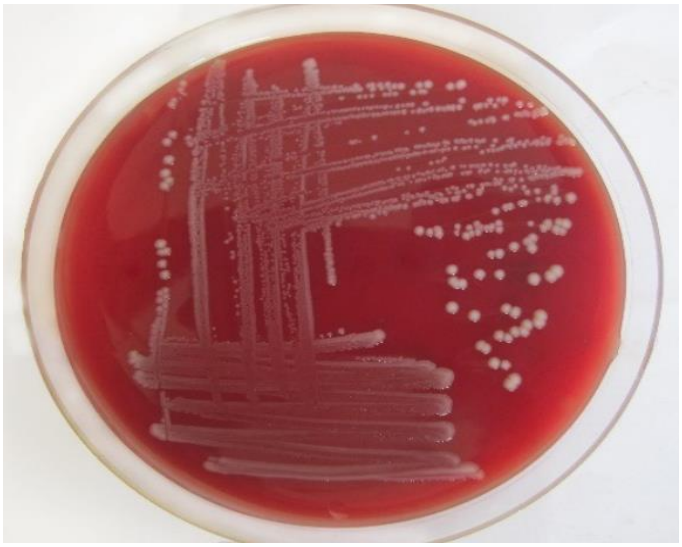


Figure 2.3: The morphology of *K. pneumoniae*; a) *K. pneumoniae* cells under scanning electron microscopy (SEM); b) *K. pneumoniae* colonies display as γ-hemolysis on blood agar (adopted from Sahu et al., 2013; Sharma, 2015).

2.4 Overview of *Haemophilus influenzae*

Haemophilus influenzae was mistakenly thought to be a source of influenza when it was first identified by Pfeiffer in 1982. It was the first bacterium whose genome has been fully sequenced. Most healthy individuals have *H. influenzae,* which acts as a commensal bacterium in their nasopharynx, where it can spread to cause respiratory tract infections as well as systemic infections. Infancy marks the start of *H. influenzae* colonisation in the upper respiratory tract. Throughout the first year of life, about 20% of infants are being colonised, and the percentage gradually increases. At ages 5-6 years and adults, more than 50% and at least 75% will be colonised by *H. influenzae*, respectively (King, 2012).

The pathogenesis of *H. influenzae* begins when it can attach to the respiratory mucosa. There are various factors that help *H. influenzae* to successfully attach to the mucosa, including adhesins, pili, Hia, and Hap proteins. Once it adheres to the mucosa, it will undergo various mechanisms to enhance its existence at the epithelial surface. The mechanisms include cleavage and neutralising immunoglobulin A1 (IgA1) by proteases, formation of microcolony, and antigenic drift. When *H. influenzae* is capable of invading the local tissue, it will try to survive in the respiratory tract intracellularly. The ability of this bacterium to survive in-vitro for a minimum of 72 hours within macrophages and epithelial cells has been demonstrated in various studies (Agrawal and Murphy, 2011; King, 2012).

Haemophilus influenzae is divided into two groups which are known as encapsulated (typeable) and unencapsulated (non-typeable) *H. influenzae* (NTHi) (Costa et al., 2022; Thofte et al., 2018). Encapsulated strain is reactive with typing antisera but not for NTHi (Agrawal and Murphy, 2011). There are six serotypes under encapsulated *H. influenzae* which are a, b, c, d, e, and f. However, among those serotypes, *H. influenzae* type b (Hib) is a predominant cause of severe illness, especially in children aged below five years (Farajzadeh Sheikh et al., 2021). Other serotypes like a, e, and f are less common than b, but c and d are rare. *H. influenzae* can be transmitted via direct contact with respiratory droplets from pharyngeal carriers. This bacterium can cause various severe infections, including meningitis, cellulitis, empyema, bacteremia and pneumonia. However, among all serotypes, Hib is the typical agent that causes pneumonia (Khattak; and Anjum, 2023).

The treatment for *H. influenzae* includes *β*-lactam antibiotics, mainly penicillin. However, the alteration of the penicillin-binding proteins and spread of plasmids carrying β-lactamase genes among *H. influenzae* has resulted in inefficacious treatment (Su et al., 2020). Other antibiotics used to treat this bacterium include extended spectrum cephalosporins, amoxicillin-clavulinic acid, trimethoprim-sulfamethoxazole, tetracyclines, quinolones and macrolide (King, 2012). Another treatment is vaccination, which is made to treat Hib infections. Before vaccinations, Hib caused most cases of bacterial meningitis in kids younger than 5 years old. More than 83% of those cases happened in kids younger than 2 years old. Although the Hib cases have declined but, the impact is still significant. In 2015, about 340 000 Hib cases were reported among children under 5 years old, with 76% being pneumonia and 29 600 being death cases (Farajzadeh Sheikh et al., 2021).