# DEVELOPMENT AND EVALUATION OF THERMOSTABILIZED MULTIPLEX POLYMERASE CHAIN REACTION FOR SIMULTANEOUS DETECTION OF *Klebsiella pneumoniae* AND *Haemophilus influenzae*

NUR AMALINA BINTI KHAZANI

UNIVERSITI SAINS MALAYSIA

May 2016

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By

NUR AMALINA BINTI KHAZANI

Thesis submitted in the fulfillment of the requirements
for the degree of
Master of Science

May 2016

## ACKNOWLEDGEMENTS

All praises to Allah, the most gracious and the most merciful for his mercy until I can complete this writing successfully. May peace and blessing be upon Prophet Muhammad, his family and companions.

First and foremost, I would like to express my sincere gratitude to my supervisor, Assoc. Prof. Dr Siti Suraiya Md Noor for the continuous support, constant supervision and guidance throughout the study. I am grateful to my co-supervisors, Assoc. Prof Dr. Chan Yean Yean and Dr Suharni Mohamad for their countless guidance and advice for inspiring me with their fruitful ideas, especially during laboratory work.

To my dear seniors and comrades, Nik Zuraina, Ain, Iman, Yasmin, Adila, Nurul, Ira, Jalilah, Afifah, Afiqah and Amira thank you for helping me a lot and sharing the precious moments in conducting this research. My appreciation also goes out to my other labmates, lecturers, and staffs from the Departments of Medical Microbiology and Parasitology, Universiti Sains Malaysia, who helped and assists me in various aspects of laboratory works and facilities during the course of this study. I am gratefully acknowledged to the Long Term Grant Research Scheme, Tabung Haji for the fund grant that have funded part of this study.

Last but not least, very special thanks to my family especially my father (Khazani Hassan), my mother (Salma Mohamad), my husband (Iqbal Alief Ramli), my son (Uzair) and my siblings (Auni, Ain, Akram and Hasnalinda) that has been my strongest motivation and inspiration. This thesis is dedicated to my family as token of appreciation and hope that I have made them proud. Jazakallahukhairankathira.

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| Symbols/ Abbreviations | Definition |
|---|---|
| % | Percentage |
| °C | Degree Celcius |
| µg | Microgram |
| µl | Microliter |
| λ | Lambda |
| AST | Antibiotic Sensitivity Testing |
| BA | Blood agar |
| BHI | Brain heart infusion |
| BLAST | Basic Local Alignment Search Tool |
| Bp | Base pair |
| CAP | Community acquired pneumonia |
| CBA | Chocolate blood agar |
| CDC | Centers for Disease Control and Prevention |
| CFU | Colony forming unit |
| CLSI | Clinical and Laboratory Standard Institute |
| Cm | Centimeter |
| $CO_2$ | Carbon dioxide |
| COPD | Chronic obstructive pulmonary disease |
| CSF | Cerebrospinal fluid |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxy-nucleotide tri-phosphate |
| EDTA | Ethylenediaminetetraacetic acid |
| ESBL | Extended spectrum beta-lactamase |
| ETEC | Enterotoxigenic *Escherichia coli* |
| EHEC | Enterohaemorrhagic *Escherichia coli* |
| *et al.* | Et alii (and others) |
| etc. | Et cetera (an so on) |
| Fg | Femtogram |
| G | Gram |
| *G* | Gravity |
| Hr | Hour |
| HCI | Hydrochloric acid |
| Hib | *Haemophilus influenzae* type b |
| HUSM | Hospital Universiti Sains Malaysia |
| IC | Internal control |
| ICU | Intensive care unit |
| L | Liter |
| LB | Luria bertani |
| LOD | Limit of detection |
| LPS | Lipopolysaccharide |
| LRTis | Lower respiratory tract infections |
| M | Molar |
| Mg | Milligram |
| $MgCI_2$ | Magnesium Chloride |
| MHA | Mueller- Hinton agar |

| | |
|---|---|
| MIC | Minimum inhibitory concentration |
| Min | Minute |
| Ml | Milliliter |
| mM | Millimolar |
| N | Sample size |
| NaOH | Sodium hydroxide |
| NaCI | Sodium Chloride |
| NCBI | National Center for Biotechnology Information |
| NS | Normal saline |
| Ng | Nanogram |
| OD | Optical Density |
| *Omp6* | Outer membrane protein 6 |
| PBS | Phosphate Buffer Saline |
| PCR | Polymerase Chain Reaction |
| Pg | Picogram |
| Pmol | Picomole |
| Psi | Pounds per square inch |
| Rcf | Relative centrifugal force |
| Rpm | Revolutions per minute |
| Rtis | Respiratory tract infections |
| Sec | Second |
| Spp. | Species |
| $T_a$ | Annealing temperature |
| $T_m$ | Melting temperature |
| *Taq* | *Thermus aquaticus* |
| TBE | Tris-Borate EDTA |
| U | Unit |
| USA | United States of America |
| UV | Ultra Violet |
| V | Volt |
| VP | Voges-Proskeur |
| WHO | World Health Organization |
| X | Times or multiple |

**PEMBANGUNAN DAN PENILAIAN UJIAN TINDAK BALAS RANTAIAN POLIMERASE MULTIPLEKS YANG STABIL SUHU BAGI PENGESANAN SERENTAK *Klebsiella pneumoniae* DAN *Haemophilus influenzae***

## ABSTRAK

*Klebsiella pneumoniae* dan *Haemophilus influenzae* adalah patogen penting yang berkaitan dengan pelbagai jenis jangkitan seperti pneumonia, jangkitan saluran pernafasan, meningitis dan juga septisemia. Namun, pengesanan melalui kaedah pengkulturan mengambil masa yang lama, serta kurang sensitif. Tindak balas rantai polimerase (PCR) konvensional pula masih memerlukan penyimpanan sejuk dan kakitangan terlatih untuk melaksanakan ujian ini. Kajian ini bertujuan untuk membangun dan menilai PCR multipleks stabil suhu bagi pengesanan serentak *K. pneumoniae* dan *H. influenzae.* Tiga set primer, terhadap gene *php K. pneumoniae, p6 H. influenzae* dan *glmM Helicobacter pylori* (kawalan dalaman) telah direka dan diuji di dalam kajian ini. Semua reagen PCR seperti enzim *Taq* polimerase DNA, $MgCI_2$, dNTPs, 10 X larutan penampan dan primer telah dioptimumkan dan dikeringkan ke dalam bentuk pelet dengan menggunakan penstabil enzim (trehalose). Kepekatan penstabil enzim dan *Taq* polimerase DNA juga telah dioptimumkan untuk multipleks stabil PCR. Analisis sensitiviti telah dinilai di peringkat genomik dan bakteria. Manakala, analisis pengkhususan multipleks stabil suhu PCR kemudiannya dinilai dengan menggunakan 30 bakteria berbeza. Kestabilan multipleks PCR ini telah diuji pada tiga suhu yang berbeza (4°C, 25°C, dan 37°C). Hasil kajian menunjukkan bahawa kestabilan ujian PCR ini adalah optimum apabila kepekatan 8% penstabil dan 200% *Taq* DNA polymerase digunakan. Had pengesanan (LOD) pada peringkat genomik untuk multipleks *K. pneumoniae* dan *H. influenzae* adalah 1

pg DNA. Sebaliknya, LOD pada peringkat bakteria multipleks PCR *K. pneumoniae* dan *H. influenzae* adalah pada 1 x $10^3$ CFU / ml. PCR multipleks stabil suhu ini telah menunjukkan 100% tanpa amplifikasi bakteria lain. Berdasarkan ujian kestabilan, multipleks stabil suhu PCR dianggarkan stabil untuk sekurang-kurangnya 3.02 bulan pada suhu 25°C. PCR multipleks stabil suhu untuk mengesan *K. pneumoniae* dan *H. influenzae* adalah mudah, spesifik, senang untuk dilaksanakan, kos yang efektif dan mengurangkan masa penyediaan PCR reagen. Oleh sebab itu, ujian ini mempunyai potensi untuk digunakan dalam rutin diagnosis, hospital dan pelbagai bidang lain.

**DEVELOPMENT AND EVALUATION OF THERMOSTABILIZED MULTIPLEX POLYMERASE CHAIN REACTION FOR SIMULTANEOUS DETECTION OF *Klebsiella pneumoniae* AND *Haemophilus influenzae***

## ABSTRACT

*Klebsiella pneumoniae* and *Haemophilus influenzae* are important pathogens associated with the various types of infections such as pneumonia, respiratory tract infections, meningitis and also septicemia. The identification of these pathogens using culture methods are time-consuming, and insensitive, while conventional polymerase chain reaction (PCR) still require cold-chain storage and trained personnel to perform the assay. Thus, the aim of this study is to develop and evaluate the thermostabilized multiplex PCR for simultaneous detection of *K. pneumoniae* and *H. influenzae*. Three sets of primer, *php* gene of *K. pneumoniae*, *p6* gene of *H. influenzae* and *glmM* gene of *Helicobacter pylori* (internal control) were designed and optimized in this study. All PCR reagents such as *Taq* DNA polymerase, $MgCI_2$, dNTPs, 10 X buffer and primers were optimized and lyophilized into a pellet form with an enzyme stabilizer (trehalose). The concentrations of enzyme stabilizer and *Taq* DNA polymerase were optimized for thermostabilized multiplex PCR. The analytical sensitivity of the assay was evaluated both at the genomic and bacteria levels. While, the analytical specificity of thermostabilized multiplex PCR was evaluated using 30 different bacteria. The stability of thermostabilized multiplex PCR was determine using accelerated stability test at three different temperatures (4°C, 25°C, and 37°C). The results showed that thermostabillized PCR was stable at the concentrations of 8% stabilizer and 200% *Taq* DNA polymerase. The limit of

detection (LOD) at genomic level for multiplex *K. pneumoniae* and *H. influenzae* was 1 pg of DNA. On the other hand, the LOD at bacterial level multiplex PCR of *K. pneumoniae* and *H. influenzae* was 1 x $10^3$ CFU/ ml. The thermostabilized multiplex PCRs demonstrated specificity of 100% with no amplification was observed with other bacteria strains. Based on the stability tests, the thermostabilized multiplex PCR was estimated to be stable for at least 3.02 months at 25°C. The thermostabilized multiplex PCR for detection of *K. pneumoniae* and *H. influenzae* is simple, specific, easy to perform, cost-effective and minimize the preparation time of PCR mixture. Hence, this assay has potential to be used in routine diagnosis, hospital settings and fields.

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of studies

*Klebsiella pneumoniae* and *Haemophilus influenzae* are classified under Gram negative bacteria which commonly colonize the human respiratory tract, especially in the nasopharynx and become part of normal respiratory flora (Kofteridis *et al.*, 2009; Farida *et al.*, 2013). Both pathogens are important and can lead to various types of healthcare associated infections such as pneumonia, respiratory tract infections (RTIs), meningitis and also blood stream infections (CDC, 2012). Most infections of *H. influenzae* come from direct extension of the nasopharynx to the lower respiratory tract. On the other hand, the infections of *K. pneumoniae* are usually found in the lung (pneumonia) and blood (bacteremia) (Custovic *et al.*, 2014).

Respiratory diseases, especially RTIs are the main causes of mortality and morbidity around the world. RTIs become the main public health concern affecting both children and adults particularly the lower respiratory tract infections (LRTIs) in both developing and developed countries (Boloursaz *et al.*, 2013; Ezeonwu *et al.*, 2014). It has been reported that the microbiological causes for LRTIs are bacterial pathogens such as *K. pneumoniae* and *H. influenzae* (Ramana *et al.*, 2013). Both pathogens were also reported to cause diseases during the Hajj season (Mansouri *et al*., 2012; Gautret *et al.*, 2016). Community-acquired pneumonia (CAP) becomes one of the common LRTIs with high morbidity and mortality. *H. influenzae* was reported as the second most important bacterial pathogen of pneumonia and meningitis after *Streptococcus pneumoniae* (Boloursaz *et al.*, 2013). In addition, RTIs serves as a

major contributor among intensive care unit (ICU) patients and the common pathogens that affect them are *K. pneumoniae* followed by *Proteus mirabilis* and *Pseudomonas aeruginosa* (Custovic *et al.,* 2014). Another study showed that *K. pneumoniae* is the prevalent etiological agent which leads to pneumonia after *S. pneumoniae (*Farida *et al.,* 2013).

### 1.1.1 *Klebsiella pneumoniae*

*K. pneumoniae* is classified under *Klebsiella* genus of Enterobacteriaceae and exist in the soil and in water as free living microorganisms (Kowalski, 2012). It is commonly considered as an opportunistic pathogen and can be asymptomatically found in the intestinal and respiratory tracts and skin of healthy individual but can also give infection in hospitalized patients (Holt *et al.,* 2015). *K. pneumoniae* is responsible for the majority of infections in human followed by *K. oxytoca*, *K. ozaenae* and *K. rhinoscleromatis* (Caputo *et al.,* 2015).

This pathogen is the common source of community and hospital-acquired infections, especially in respiratory tract and bloodstream infections. *K. pneumoniae* has been reported as an important cause of hospital-acquired infections, especially among patients in the neonatal ICU and mortality rates can be as high as 70%. (Gupta *et al.,* 2003). Moreover, the importance of *K. pneumoniae* as an invasive pathogen seems to be increasing due to the emergence and progressive spread of multi drug resistance specifically extended-spectrum β-lactamase (ESBL) (Meatherall *et al.,* 2009; Chandel *et al.,* 2011).

#### 1.1.1.1 Morphology

This Gram negative rod shaped bacterium is facultative anaerobes, non-motile and belongs to the normal flora of the intestines, mouth and skin. This species exhibits mucoid growth, size range from 0.3 to 1.0 mm in width and 0.6 to 6.0 mm in length and usually gives positive tests for lysine decarboxylase and citrate. This bacterium is surrounded by a capsule, which acts as a physical barrier to evade the host of immune response.

#### 1.1.1.2 Taxonomic Classification

Listed below is the hierarchy taxonomic classification for *K. pneumoniae.*

Kingdom : Bacteria

Phylum : Proteobacteria

Class : Gammaproteobacteria

Order : Enterobacteriales

Family : Enterobacteriaceae

Genus : *Klebsiella*

Species : *K. pneumoniae*

### 1.1.2 *Haemophilus influenzae*

*Haemophilus influenzae* which is also known as Pfeiffer's bacillus was described in 1892. Pfeiffer found an association between the sputum of patient and the clinical syndrome that causes influenza during the outbreak. The influenza was confirmed as *H. influenzae* and it was found to cause the secondary infection in 1933 (CDC, 2015). This pathogen is widely spread among human. The human upper respiratory tracts are known as the reservoir of various communities of commensal pathogens

like *H. influenzae* and the colonization of this bacterium in the nasopharynx occasionally results in diseases. *H. influenzae* are opportunistic pathogens in which they live in the host without causing diseases. However, they will take advantage when other factors such as viral infection are present (Sydnor and Perl, 2011). Usually, non-typeable *H. influenzae (*NTHi) is harbored as a normal flora. However, a minority of healthy individuals may accidentally be exposed to the colonization of *H. influenzae* type b (Hib) strains in the upper respiratory tract (Clementi and Murphy, 2011).

Some strains of *H. influenzae* possess a polysaccharide capsule representing the major virulence factor and antigen of this bacterial species (Ulanova, 2013). There are six capsular serotypes (a-f) of *H. influenzae*, in which serotype b is the most common pathogen for invasive diseases. The infection cause by this type can lead to meningitis, pneumonia, epiglottitis, bacteremia and cellulitis (CDC, 2014). However, due to the emergence and successful implementation of Hib vaccination program, serotype b become low and serotype a is increasingly found to cause invasive infections. Moreover, serotype f also emerged as a cause of invasive infections but is less common than serotype a (Ulanova, 2013). *H. influenzae* that lack capsular polysaccharides is known as non-typeable *H. influenzae*. NTHi is commonly present as the normal flora of the upper respiratory tract, less invasive and is an uncommon cause of opportunistic RTIs (Kaneko *et al.*, 2014). Nevertheless, it is one of the top causes of respiratory infections in human causing acute otitis media (AOM), bronchitis, chronic obstructive pulmonary disease (COPD) (Apisarnthanarak and Mundy, 2005).

#### 1.1.2.1 Morphology

*H. influenzae* is a rod-shaped, non-motile and facultative anaerobe bacteria. This bacteria grown on chocolate agar at 37°C in an enriched $CO_2$ incubator and requires two growth factors which are factor X (haemin) and V factor (NAD). The colony of this pathogen looks like convex, smooth, pale and grey colour. Gram-stained shows Gram-negative coccobacilli with no specific arrangement.

#### 1.1.2.2 Taxonomic Classification

Listed below is the hierarchy taxonomic classification for *H. influenzae.*

Kingdom : Bacteria

Phylum : Proteobacteria

Class : Gammaproteobacteria

Order : Pasteurellales

Family : Pasteurellaceae

Genus : *Haemophilus*

Species : *H.influenzae*

## 1.2 Epidemiology

The aetiological bacteria of RTIs are different in developed and developing countries. Pneumonia is stated as one of the main causes of death among newborn infants. Approximately 922,000 deaths in children were estimated due to pneumonia in 2015 (WHO, 2015). The significant pathogens associated with pneumonia in the developed countries are *S. pneumoniae*, *Mycoplasma pneumoniae*, and *H. influenzae.* However, the implemented of pneumococcal vaccination program decrease the rates of pneumonia among children in the United States (Boloursaz *et al.,* 2013). In

contrast, 1.9 million children died from respiratory infections all over the world especially in Southeast Asia and Africa (WHO, 2009). CAP was reported as the major cause of adult mortality in Asian countries (Peto *et al.,* 2014). *S. pneumoniae, Burkholderia pseudomallei* and *H. influenzae* were the most common pathogens reported in Cambodia during the period of 1995 to 2012 (Goyet *et al.,* 2014). Moreover, *K. pneumoniae* was particularly detected in CAP in Thai adults and Cambodian children. India (53.9%) was the largest contributor of etiologic pathogens associated with the hospital acquired pneumonia (HAP). However, Pakistan (55%) and China (41%) were the major contributors to the ventilator associated pneumonia (VAP) (Chawla, 2008).

According to the Department of Statistics Malaysia (DOSM), pneumonia is one of the top five causes of death in Malaysia. In 2009, pneumonia was the 4th leading cause of death in the Ministry of Health (MOH) Hospitals in Malaysia (10.4% of hospital mortality) while respiratory disease was the 3rd leading cause of hospitalization (MOH, 2010). RTIs significantly cause morbidity and mortality especially in low income countries. Children are susceptible to infections by various pathogens. Therefore, clinicians may face difficulties in determining the causative pathogen that infects them. The amount of studies conducted to determine the aetiology of LRTI among children is still limited. Despite the widespread vaccination program especially against Hib, children are still susceptible to the risk of severe lung infections (Nathan *et al.,* 2014). Approximately, thirty percent of patients visit to private and governments' clinic in Malaysia due to upper RTIs. On the other hand, a study conducted by Noor Shafina and co-workers (2015) showed that *Escherichia*

*coli* and *K. pneumoniae* are the most common pathogens in urinary tract infection (UTI) among children.

Respiratory tract infections (RTIs) have recently become the leading cause of morbidity and mortality in several parts of the world. RTIs involve both upper and lower respiratory tracts, and trigger the production of various diseases. The World Health Organization (WHO, 2010) reported that the acute lower respiratory tract infections and influenza are among the top ten leading causes of death. Prior studies have shown that different bacterial pathogens infected and produced various diseases. Generally, pathogens reported and associated with RTIs were *H. influenzae* and *K. pneumoniae* (Benedetto and Sevieri, 2013; Ngekeng *et al.,* 2015; Gadsby *et al.,* 2015). Infection cause by these types of bacteria leads to the production of severe health problems, especially among young children and immunocompromised persons, including, but not limited to, bronchitis, common cold, meningitis, otitis media and pneumonia.

The spread of RTIs is varies among different countries, perhaps due to differing cultures, geographical regions and health care systems (Zhang *et al.,* 2014). More serious diseases usually come from developing countries such as China, Indonesia, Malaysia and India. *H. influenzae* and *K. pneumoniae* have been reported as etiological agents in respiratory infections, and have become the most common causes of community-acquired pneumonia in adults in Japan (Fukuyama *et al.,* 2014). Previous studies have reported that *K. pneumoniae* ranks as a high cause of pneumoniae in Malaysia and Cambodia (Loh *et al.,* 2007; Rammaert *et al.,* 2012). Nathan *et al.* (2014) demonstrated that a significant pathogen isolated from a

nasopharyngeal swab among young children having RTI was *H. influenzae.* Considering the global burden of *H. influenzae*, vaccination programmes have been integrated into national immunization since the year 2000. However, in spite of vaccination, children remain to encounter severe lung infections (Nathan *et al.,* 2014). A similar study that also support this finding states that Hib is a major cause of bacterial meningitis and pneumoniae in India, Thailand, Malaysia and Indonesia (Lolekha *et al.,* 2000; Nur Erleena *et al.,* 2008). Apart from pneumoniae, Rehana *et al.* (2015) demonstrate that *K. pneumoniae* also found as significant cause for meningitis patients in Kelantan, Malaysia.

### 1.2.1 *Klebsiella pneumoniae*

*K. pneumoniae* is spread worldwide and is present in human, animal and the environment including forest, soil and water (Podschun and Ullmann, 1998; Cabral, 2010). The strains of *K. pneumoniae* are often opportunistic pathogens associated with RTIs and UTIs in hospitalized patients and compromised individuals (Cabral, 2010; Clegg and Murphy, 2016). The risk of infection and carriage rates of *K. pneumoniae* increases along with the increase in the period of stay in a hospital (Podschun and Ullmann, 1998; Sydnor and Perl, 2011). The bacteremia, UTI and nosocomial pneumonia (NP) rates in ICU located in Malaysia are 8.9, 4.7 and 20.5 per 1,000 patient-days, respectively. Notably, *Acinetobacter* species, *K. pneumoniae, P. aeruginosa* and MRSA were the predominant pathogens isolated from the nosocomial infection during these periods in the three ICU in Malaysia (Katherason *et al.,* 2008).

*K. pneumoniae* is known as a significant cause of hospital-acquired and community-acquired infection, especially among patients in the neonatal ICU. It becomes a major problem to patients and public health as it is a major cause of death, higher morbidity in hospitalized patients and leads to the increase of emotional stress (WHO, 2002). Recent study showed that methicillin-resistant *S. aureus* (MRSA) and *K. pneumoniae* are the most common isolated organisms in the ICU patients in Egypt (Eida *et al.,* 2015). On the other hand, hospital-acquired infections due to *K. pneumoniae, P. aeruginosa* and *Acinetobacter baumannii* were reported to be increasing in the USA (Peleg and Hooper, 2010).

Most of community-acquired infections occur in developing countries. Community acquired infection is a common disease and has become a major source of morbidity and death worldwide especially in the elderly and those with chronic disease (Yoon, 2014). *K. pneumoniae* was reported as the most prevalent community acquired pathogen in Egypt (Magdy *et al.*, 2013). It was also proven that this pathogen is involved in severe community acquired and nosocomial pneumoniae in Cambodia and Taiwan (Rammaert *et al.,* 2011; Chuang *et al.,* 2012). *K. pneumoniae* is also one of the causative agents of community-acquired meningitis and brain abscesses in Asia, largely in Taiwan (Ko *et al*., 2002). Rehana *et al.* (2014) found that coagulase negative *Staphylococcus, Acinetobacter* species, *S. aureus, Streptococcus* spp and *K. pneumoniae* are prevalent hospital associated pathogens and are the cause of meningitis in Kelantan. This pathogen was ranked as the top cause of adult pneumonia requiring hospitalization in Malaysia (Loh *et al*., 2007). The most frequent organism isolated in the sputum culture associated with

pneumoniae among Hajj pilgrims from Malaysia are *H. influenzae* (20.3%), *K. pneumoniae* (17.6%), *S. pneumoniae* (16.2%) and *S. aureus* (9.5%).

Moreover, the increase of the colonization rates of *K. pneumoniae* appears to be associated with the use of antibiotics in comparison to the factor of length of stay in the hospital (Podschun and Ullmann, 1998; Sydnor and Perl, 2011). Severe nosocomial and community acquired infections due to multidrug resistant bacteria lead to the difficulty in finding suitable antibiotics. Furthermore, the extensive usage of broad- spectrum antibiotics to treat patients has led to the emergence of multiple resistant strains and the development of multidrug- resistant strains which produce extended–spectrum beta-lactamase (ESBL) (Sikarwar and Batra, 2010). The extensive use of antibiotics has led to the development of ESBLs which provides resistance against antibiotics (Sheikh *et al*., 2015). Palasubramaniam *et al.* (2005) reported that the connection between ESBL *K. pneumoniae* with nosocomial outbreak in a paediatric oncology unit in a Malaysian public hospital may be due to the clonal spread of the organism or by the transfer of plasmids encoding ESBL genes. The stability of the plasmids encoding ESBL has led to the emergence of multiple resistant towards several classes of antibiotics.

### 1.2.2 *Haemophilus influenzae*

The most well-known strain of *H. influenzae* is the type b strain which is the leading cause of invasive bacterial disease in children before the introduction of the first licensed *H. influenzae* vaccine (Agrawal and Murphy, 2011; Price *et al*., 2015). *H. influenzae* type b is a leading cause of bacterial meningitis and pneumoniae especially in infants and young children (Thairu *et al.*, 2014). Moreover, pneumoniae

caused by *H. influenzae* type b strain has been reported to be more common than meningitis. The rates of Hib remain higher in American Indian and Alaska Native children (MacNeil *et al.*, 2011). Children infected by *H. influenzae* type b are among the responsible cases of bacterial meningitis admitted to University Malaya Medical Center (UMMC) (Nur Erleena *et al.*, 2008). However, it was reported that Hib in Thailand, Malaysia, India and Vietnam have low incidence of infection compared to the Western countries (Lolekha *et al.*, 2000). This finding was strengthened by Mc Neil (2015) who stated that *H. influenzae* type b contributes to a smaller proportion of bacterial meningitis in Malaysia.

The extensive use of the *H. influenzae* type B vaccine has triggered a great reduction in the prevalence of clinical disease arising from type b *H. influenzae* infection (King, 2012). As a result, the proportion of nasopharyngeal colonization among children showed a decrease in rate (Xu *et al.*, 2012). The epidemiological trends of Hib disease among children have since been shifted towards the increase of the disease among elderly and those with underlying conditions (Puig *et al.*, 2014). In Malaysia, the immunization associated with pneumococcal and influenza vaccines and RSV monoclonal antibodies is not extensively practiced due to economic reasons. Therefore, children are at the risk of severe lung infections even though vaccination of *H. influenzae* type b (Hib) has been incorporated since year 2000 (Nathan *et al.*, 2014). Hib has overshadowed the implications of other serotypes of *H. influenzae* in the etiology of bacterial infections. Serological types of *H. influenzae* such Hia can also cause significant morbidity and mortality. The highest incidence rates have been reported among the native population in North American

Indian (Ulanova, 2012). Moreover, the increasing incidence of two other serotypes such as Hie and Hif has been reported in England (Ladhani *et al*., 2012).

On the other hand, NTHi is the main cause of *Haemophilus* respiratory disease and is a common colonizer pathogen in the upper respiratory tract system in a healthy individual. NTHi can cause diseases such as sinusitis, otitis media, and cystic fibrosis in adults and sepsis in neonates (Foxwell *et al.,* 1998; Kosikowska *et al.,* 2015). Recent study from Schutter (2011) reported that majority of the sample from *H. influenzae* was identified as NTHi. The introduction of immunization with conjugate vaccine among children has caused NTHi to become common in the UK. Diseases such as meningitis and septicaemia are observed in neonates. Notably, misidentification towards *Haemophilus* species brings a wider effect for clinical diagnosis and assessment of disease outcomes from antibiotics (Price *et al*., 2015) Thus, it is important to differentiate *H. influenzae* from other species. The common strains involved in community acquired respiratory infections are *H. influenzae* or *S. pneumoniae* which are usually more susceptible to antibiotics.

## 1.3 Pathogenicity

### 1.3.1 *K. pneumoniae*

Pathogenicity refers to the ability of an organism to cause disease. The ability is characterized by the genetic component of the organism and their effects to the host. There are several pathogenicity factors that influence the ability of *K. pneumoniae* to cause diseases including pili, siderophores, capsule and cell surface lipopolysaccharides (LPS). Each of these factors plays specific roles in the pathogenesis and leads to the different types and modes of infection (Podschun and

Ullmann, 1998). Different patterns of virulence factors can be seen with the respect of the different infected sites and host defense (Podschun and Ullmann, 1998).

Moreover, geographic differences have also been recognized in affecting the range of diseases caused by *K. pneumoniae*. Studies conducted by Ko *et al.* (2002) reported that different regions are infected by different diseases. In Taiwan, meningitis and liver abscess infected by *K. pneumoniae* are the major health problems. As a comparison, 28 cases in Taiwan and 25 cases in South Africa, accounting for 29% and 62% of all cases of community-acquired *K. pneumoniae* bacteremia in Taiwan and South Africa were reported. The prevalence of serotypes K1/K2 of this pathogen colonizing the intestinal tract was reported to contribute to invasive liver abscess in Taiwan and Singapore. Moreover, Malaysia is the third leading rank of serotypes K1/ K2 found in stool after China and Taiwan (Lin *et al.*, 2012). *K. pneumoniae* was not common found in United States, Australia, Europe and Argentina which only four cases of community-acquired *K. pneumoniae* pneumonia have been found in two years out of nine large hospitals (Ko *et al.*, 2002).

Listed below are several factors associated with pathogenicity of *K. pneumoniae*.

1) Pili

   Pili, also known as fimbrae are non-flagellar and consist of polymeric globular protein subunits. Its main function is to attach the pathogens into the host cells (Vuotto *et al.*, 2014).

2) Siderophores

Iron is an important factor in bacterial growth. It functions as a redox catalyst in proteins participating in oxygen and electron transport processes. By increasing the intake of iron, the susceptibility towards *K. pneumoniae* infection will automatically increase (Podchun and Ullmann, 1998; Russo *et al.*, 2014).

3) Capsule

Capsule is important to the virulence of *Klebsiella* spp. (Schembri *et al*., 2004). It contains complex acidic polysaccharide and can be categorized into 77 serological types. The functions of capsular are to protect the bacterium from phagocytosis by polymorphonuclear granulocytes and avoids killing of the bacteria by bactericidal serum factors (Podschun and Ullmann, 1998; Ares *et al.*, 2016).

4) Cell surface lipopolysaccharide (LPS)

LPS is the most vital virulence factors of *K. pneumoniae* in triggering sepsis. LPS consists of lipid A, core and O-polysaccharide in order to attack complement-mediated killing.

### 1.3.2 *H. influenzae*

Pathogenicity of *H. influenzae* can be divided into two which are typeable (especially type b) and non-typeable forms. The typeable *H. influenzae* plays a role in systemic infection such as otitis media, pneumoniae and meningitis which involves self-defense (Lolekha *et al.*, 2000). The non-typeable form is usually associated with

respiratory diseases such as sinusitis and bronchitis (Kosikowska *et al.,* 2015). This type of strain rarely causes diseases outside the respiratory tract and can be considered as primary mucosal pathogens. *H. influenzae* has evolved rapidly in order to evade the host defense system including the ability to invade into local tissues (King, 2012).

Listed below are several factors associated with pathogenicity of *H. influenzae.*

1) Fimbriae

The function of fimbriae is to assist attachment to epithelial cells.

2) Lipooligosaccharide

It contributes to the invasion into many stages including producing inhibition of ciliary function and also loss or death of ciliary mucosal cell. It was claimed that lipooligosaccharide is present in non-capsulated strains (Pettigrew *et al.,* 2002).

3) Pili

Pili cause agglutination of red blood cells and promote attachment to respiratory tract epithelial cells. They are present only on a small portion of NTHi strains (King, 2012).

4) Outer membrane protein (OMP)

*H. influenzae* expresses between 10 to 20 OMPs and the combination of the expressed proteins varies between strains. OMP consists of porin proteins

which are P2, P5 and P6. P2 and P5 proteins facilitate the binding of bacteria to the mucus. P2 contributes to the virulence of *H. influenzae* type b, while P5 is involved in the invasion of the mucosal epithelium (Clementi and Murphy, 2011; Euba *et al*., 2015).

## 1.4 Detection Methods

Precise identification of microorganisms is vital for treatment of infection and better disease diagnosis. Different procedures are designed for identification and detection of microorganisms. Conventional methods such as Gram staining, culture and biochemical tests have become the gold standard for detection of bacteria. These methods rely on phenotypic identification of the causative organism and are effective towards the diagnosis of patients. In particular, culture methods use selective liquid or solid culture to grow, isolate and count the target bacteria (Jasson *et al*., 2010). Meanwhile, rapid methods for detection of microorganisms such as polymerase chain reactions (PCR) are used to increase the chances of detection.

### 1.4.1 Conventional Methods

#### 1.4.1.1 *Klebsiella pneumoniae*

##### 1.4.1.1.1 Culture

*K. pneumoniae* can be cultivated in many types of media such as nutrient agar, blood agar (BA) and MacConkey agar. Colonies appear mucoid, sometimes sticky with 3-4 mm diameter.

#### 1.4.1.1.2 Biochemical Tests

##### 1.4.1.1.2.1 Simmon Citrate Agar

Simmon Citrate Agar is a medium to differentiate members of Enterobacteriaceae. Bacteria are able to utilize citrate as a carbon source and the enzyme citrase will hydrolyze citrate into oxaloacetic acid and acetic acid. The production of $CO_2$ changes the pH indicator to blue.

##### 1.4.1.1.2.2 Methyl Red

This test is used to determine the acidity when an organism ferments glucose. The pH indicator (methyl red) is added into the culture broth. The red color is absent when the pH increases, indicating a negative result in which *K. pneumoniae* is present.

##### 1.4.1.1.2.3 Voges Proskauer (VP)

This test is used to determine the presence of acetoin. It is primarily used to differentiate between *E. coli* (VP-negative) from the *Klebsiella-Enterobacter* groups (VP-positive).

#### 1.4.1.1.3 VITEX identification

The identification process using a Vitex GN card is performed according to the guidelines of the manufacturer for ESBL detection.

#### 1.4.1.1.4 Antimicrobial susceptibility testing (AST)

##### 1.4.1.1.4.1 Disk diffusion test

AST is performed using Clinical and Laboratory Standard Institute (CLSI) disc diffusion method (CLSI, 2012). Antibiotic discs are placed onto the surface of Mueller Hinton Agar (MHA) and the plate is incubated at 37°C for 18-24 hours. The antibiotics tested are imipenem, meropenem and doripenem.

#### 1.4.1.1.4.2 Etest

Minimum inhibitory concentration (MIC) can be determined by Etest strips which consist of gradient concentrations of antibiotics on a plastic strip. E-test strips are placed onto the surface of MHA and the plate is incubated at 37°C for 18-24 hours. The concentration is measured by examining the zone edge intersects of the plastic strip.

### 1.4.1.2 *Haemophilus influenzae*

#### 1.4.1.2.1 Culture

The typical detection of *H. influenzae* is performed by culture. Microbiological differentiation of this organism from other species depends on the colony morphology, haemin and NAD (X and V factors). The culture of *H. influenzae* is performed on chocolate agar plate at 37°C in $CO_2$ enriched incubator. The colonies appear as smooth, pale, grey or transparent and convex in shape. Another method to identify *H. influenzae* is by inoculating it together with the *S. aureus* on BA. *Staphylococcus* produces the necessary blood factor essential for the growth of *H. influenzae.* Smaller colonies representing *H. influenzae* will grow around *S. aureus* (WHO, 2003).

#### 1.4.1.2.2 Biochemical Tests

##### 1.4.1.2.2.1 X and V factors

A single colony of *H. influenzae* from the chocolate agar is inoculated onto NA. Commercial X, V and XV factor discs are placed onto the plate and incubated at 37°C in $CO_2$ enriched incubator for 18-24 hours. *H. influenzae* will grow around the XV factor only.

#### 1.4.1.2.2.2 Catalase test

Catalase is present only in viable cultures so colony growth must be carried out for 18 to 24 hours culture. Positive catalase results are shown by vigorous bubbling which indicates the presence of catalase.

#### 1.4.1.2.3 Serotyping

Serotyping can differentiate between typeable strains (a to f) by using slide agglutination. However, this method is not applicable for non-typeable strains. Specific antisera can distinguish each strain due to different antibodies reaction with different somatic antigens. To perform this technique, one drop of normal saline is placed on a slide. The colony of *H. influenzae* is mixed thoroughly to reach homogenous suspension form. Then, one drop of specific antiserum is added and mixed thoroughly (CDC, 1998). Visible agglutination within one minute observation indicates a positive reaction.

#### 1.4.1.2.4 Antigen detection

Antigen detection is more sensitive compared to culture method since it relies on antigen rather than viable bacteria. Latex agglutination is a rapid and sensitive method used to detect Hib capsular polysaccharide antigen in CSF, serum, urine, pleural fluid or joint.

#### 1.4.1.2.5 Antimicrobial susceptibility testing

All *H. influenzae* isolates should be tested against a selected group of antimicrobial disk by using the disk diffusion and antibiotic gradient strip (E-test) testing methods. Disk diffusion provides the information on whether a strain is susceptible, intermediate or resistant. On the other hand, E-test provides the information on the MIC value of an antimicrobial agent.

### 1.4.2 Molecular methods

PCR is a great tool for identification of bacteria because it can amplify the target DNA through repeated cycles of DNA denaturation, annealing of the primer to the DNA and extension steps (Kalle *et al*., 2014). Therefore, the detection of bacteria from clinical samples becomes easier even though the bacteria are nonviable due to inappropriate storage, transportation, condition and antibiotic treatment. Currently, the simplicity of this assay has made PCR the most common technique used in molecular biology. This assay allows rapid detection and quantification of bacteria due to its speed, sensitivity and simplicity of use (Caliendo *et al.,* 2013).

#### 1.4.2.1 Monoplex PCR

Conventional PCR such as monoplex PCR is a technique where a single target sequence is amplified from a single DNA template by using a single primer set (Kalle *et al.,* 2014). The conventional PCR assay has been developed for detection of bacterial and viral pathogens with high sensitivity and specificity. The detection of *K. pneumoniae* carbapenemase (KPC) using PCR for rapid screening in an isolated hospital in Malaysia has been reported **(**Izzati *et al.,* 2014). Rapid detection to identify the emergence of this pathogen is important in order to reduce detection time as compared to culture time. A PCR technique using colonies can give results within four to six hours, with excellent sensitivity and specificity (Hrabak *et al.,* 2014). Many monoplex PCR assays are used to detect all of the families and subgroups associated with the family of beta- lactamase in *K. pneumoniae* (Queenan and Bush, 2007). Sensitive and rapid PCR techniques have been reported for the identification of *H. influenzae*. The application of PCR for rapid detection of *H. influenzae* in pneumonia patients has been developed by Abdeldaim and co-workers (2009). The

etiology of pneumonia among young children has been evaluated by the detection of Hib using PCR method (Hassan-King *et al.,* 1998). Moreover, the application of PCR is also being used for detection of meningitis incidence in Asia (Kennedy *et al.,* 2007).

**1.4.2.2 Multiplex PCR**

The conventional monoplex PCR has its limitation because it only provides the detection of single bacteria. Thus, this approach has been upgraded to allow immediate and simultaneous detection of several target genes in a single assay. Multiplex PCR amplifies two or more target DNAs using multiple sets of primers and is broadly used for detection and identification of *K. pneumoniae* and *H. influenzae*. Stralin *et al.* (2006) reported that multiplex PCR has been developed to identify bacterial pathogen in bronchoalveolar lavage that affects adult patients with lower respiratory tract infection (LRTI). Moreover, multiplex PCR has been used for detection of URTI, LRTI and meningitis caused by *H. influenzae, S. pneumoniae, M. Catarrhalis* and *N. meningitidis* (Post *et al.,* 1996; Tzanakaki *et al.,* 2005; Abdeldaim *et al.,* 2010). Most of the multiplex PCR developed describe the detection and identification of three common pathogens associated with meningitis and RTI. Multiplex PCR is also used to differentiate between *H. influenzae* and *H. parainfluenzae* in nasopharyngeal swabs among healthy children in Taiwan (Tian *et al.,* 2012). Ghasemi *et al.* (2013) developed a multiplex PCR approach for the identification of three genes associated with ESBL producing *K. pneumoniae* in ICU. Moreover, due to the emerging global implication of *K. pneumoniae* carbapenemase (KPC), Chen *et al.* (2014) has developed a multiplex PCR to distinguish between the two capsule polysaccharides causing diseases.

#### 1.4.2.3 Real-time PCR

The advent of new technologies such as real time PCR and microarray are becoming significant in this era. These approaches provide faster speed and greater sensitivity in the identification of bacteria. Detection of *H. influenzae* and *S. pneumoniae* by real-time PCR has been developed to increase the chances of detection rate and the accuracy of quantification (Corless *et al.,* 2001; Selva *et al.,* 2013). Kurupati and co-workers (2004) reported the detection of *K. pneumoniae* from blood culture bottles using real time multiplex PCR. Only one study on multiplex PCR of *K. pneumoniae* and *H. influenzae* has been reported to detect multiple respiratory pathogens by using microarray (Shen *et al.,* 2015). However, the application of these methods as diagnostic tools is challenging due to increasing costs, instruments availability, lack of access in the rural area, and lack of qualified trainer to perform these techniques (Espy *et al.,* 2006).

## 1.5 Treatments

### 1.5.1 *K. pneumoniae*

The rise of antibiotic-resistant strains of *K. pneumoniae* has made this pathogen difficult to treat. Some *Klebsiella* organisms are resistant towards multiple antibiotics including carbapenems which is known as the last drug option. Infections caused by KPC-producing bacteria can be difficult to treat because fewer antibiotics are effective against them. Clinicians are dependent on polymyxins and tigecycline for treatments. However, they are used irregularly due to their association with nephrotoxicity and neurotoxicity. Moreover, since more strains of *Klebsiella* spp. appear to develop and produce ESBLs, current antibiotics resistant seem to be increasing. These strains seem to be resistant towards carbenicillin, amoxicillin and

ceftazidime. However, ESBL can confer resistance to the third generation of cephalosporins such as cefotaxime, ceftriaxone and ceftazidime. A study from Lim *et al.* (2009) reported that majority of the *K. pneumoniae* strains which are resistant to more than three classes of antibiotics are susceptible towards imipenem.

The initial antibiotic selection for patients infected by *K. pneumoniae* depends on the local susceptibility patterns of causing bacteremia. *Klebsiella* infections that are susceptible can be treated using cephalosporin, carbapenems, aminoglycosides and quinolones (CDC, 2012). Severely ill patients diagnosed for bacteremia should be treated with a combination of aminoglycosides and cephalosporin. The combination of both antibiotics showed greater result compared to monotherapy antibiotic (Feldman *et al.,* 1990). Monotherapy treatment is likely to be effective against uncomplicated UTI. The usage of trimethoprim-sulfamethoxazole, trimethoprim alone, ofloxacin, nitrofurantoin, norfloxacin was found to be successful for the treatment of UTI patients. In the absence of ESBL production, cephalosporins would be the effective drug to treat meningitis (Paterson *et al.,* 2010).

### 1.5.2 *H. influenzae*

The treatment of *H. influenzae* is usually straightforward since this pathogen is sensitive towards most antibiotics (King, 2012). *H. influenzae* infection is regularly susceptible to a few antibiotics such as ceftriaxone, ampicillin, chloramphenicol and tetracyclines. Among cephalosporins, compounds such as cefuroxime, cefotaxime and ceftriaxone are highly sensitive. Penicillins are useful for the treatment of mucosal infections caused by non-typeable *H. influenzae.* The choice of antibiotic

and the length of treatment depend on the severity and the site of the infection. Usually, the treatment process of a patient infected by Hib takes up to ten days. Sometimes, cases associated with invasive disease which require hospitalization are best treated with the third-generation cephalosporin (CDC, 2015).

The first choice of antibiotic treatment for meningitis patients is ceftriaxone or cefotaxime which must be given intravenously. It is highly effective because these antibiotics are bactericidal for *H. influenzae*. For patients with meningitis who are older than two months old, dexamethasone is an effective treatment as it has been shown to decrease the rate of hearing loss and inflammatory response. Moreover, dexamethasone decreases the risk of mortality and neurologic of patients with community acquired meningitis. First-generation cephalosporins, such as cephalexin, cephradine and cefadroxil are used for treatment of lower respiratory tract infections and are considered powerful. However, with the increasing activity against *H. influenzae,* the treatment has been changed to the third generation of cephalosporins (Duke *et al.,* 2003).

As for epiglottis treatment, an artificial airway such as breathing tube is inserted into the windpipe. Administered antibiotics such as ceftriaxone and cefuroxime are given once the airway is secured. Other infections due to non-typeable *H. influenzae* can cause mucosal infections and can be treated with oral antibiotics. These include amoxilin (otitis media) and erythromycin (conjunctivitis). The treatment is switched to the usage of amoxicillin-clavulanate if the bacteria produce beta-lactamase. The duration of therapy for this disease is ten days (Pichichero, 2000)