THERMOSTABILIZED MULTIPLEX PCR ASSAY FOR DETECTION OF SELECTED BACTERIA ASSOCIATED WITH RESPIRATORY TRACT INFECTIONS AMONG MALAYSIAN HAJJ PILGRIMS

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

µl	microliter
µm	micrometer
µM	micromolar
A	adenine
AGE	agarose gel electrophoresis
A&E	Accident and Emergency
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
ATCC	American Type Culture Collection
BHI	brain-heart infusion
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSC	biological safety cabinet
C	Cytosine
Ca^{2+}	calcium ions
$CaCl_2$	calcium chloride
CAP	community-acquired pneumonia
CDC	Centers for Disease Control
CFU	colony forming unit
CI	confidence interval
CLSI	Clinical and Laboratory Standards Institute
CO_2	carbon dioxide
df	degree of freedom

dH_2O	distilled water
dNTPs	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
et. al.	*et alia* (and others)
F	forward or sense primers
*fem*A	factor essential for methicillin
g	Gram
g	gravitational force
G	Guanine
G+C	guanine-cytosine
*glm*M	phosphoglucosamine mutase
HAP	hospital-acquired pneumonia
HCAP	healthcare-associated pneumonia
HCl	hydrochloric acid
HDU	high dependency unit
Hib	*H. influenzae* type b
HIV	human immunodeficiency virus
*hsp*65	heat shock protein 65
i.e.	*id est* (in other words)
IAC	internal amplification control
ICU	Intensive Care Unit
IgA	immunoglobulin A
kDa	kilodaltons
KPP	*Klinik Pakar Perubatan*
KRK	*Klinik Rawatan Keluarga*

KSA	Kingdom of Saudi Arabia
L	liter
LB	Luria-Bertani
LIS	laboratory information system
LOD	limit of detection
LPS	lipopolysaccharides
LRTIs	lower respiratory tract infections
M	molar
MALDI-TOF MS	Matrix-assisted laser desorption ionization–time of flight mass spectrometry
Mb	million base pair
mBar	millibar
MDR	multiple drug resistant
MERS-CoV	Middle East respiratory coronavirus
mg	milligram
Mg^{2+}	magnesium ions
$MgCl_2$	magnesium chloride
ml	milliliter
mm	millimeter
mM	millimolar
MRSA	methicillin-resistant strains of *Staphylococcus aureus*
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
n	frequency or total
N	grand total

NaCl	sodium chloride
NaLC	N-acetyl-L-cysteine
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
nm	nanometer
NPV	negative predictive value
NTHi	non-typeable *H. influenzae*
NURTF	normal upper respiratory tract microflora
O&G	Obstetrics and Gynecology
OD_{600}	optical density at 600 nm wavelength
OMPs	outer membrane proteins
*opr*L	outer membrane lipoprotein L
*p*6	outer membrane protein 6
PAC	positive amplification controls
PCR	polymerase chain reaction
pg	picogram
php	phosphohydrolase
ply	pneumolysin
ppm^2	people per square meter
PPV	positive predictive value
PVL	Panton-Valentine leucocidin
Q_{10}	acceleration factor of 10°C rise
R	reverse or antisense primers
RTIs	respiratory tract infections
SD	standard deviation

SEM	scanning electron microscope
spp.	species
T	thymine
T3SS	type three-secretion system
T_A	annealing temperature
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
URTIs	upper respiratory tract infections
USA	United States of America
USM	Universiti Sains Malaysia
UTIs	urinary tract infections
V	nicotinamide adenine dinucleotide growth factor
v	Volts
VAP	ventilator-associated pneumonia
WHO	World Health Organization
x	times or multiply
X	hemin growth factor
XDR	extensively drug resistant
ZN	Ziehl-Neelsen (staining)

ASAI PCR MULTIPLEKS STABIL HABA BAGI PENGESANAN BAKTERIA TERPILIH PENYEBAB JANGKITAN SALURAN PERNAFASAN DALAM KALANGAN JEMAAH HAJI MALAYSIA

ABSTRAK

Jangkitan saluran pernafasan (RTIs) merupakan masalah kesihatan yang paling umum dalam kalangan jemaah Haji. Bakteria-bakteria utama yang dikaitkan dengan RTIs ialah *Klebsiella pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae, Mycobacterium tuberculosis* dan *Pseudomonas aeruginosa*. Pengesanan pantas membolehkan rawatan yang efektif diberi kepada pesakit. Oleh itu, kajian ini dijalankan untuk membangun dan menilai sebuah asai bagi pengesanan serentak enam bakteria utama penyebab RTIs, berdasarkan reaksi rantaian polimerase (PCR) yang stabil terhadap haba. Kajian dimulakan dengan perekaan primer yang spesifik untuk setiap jenis bakteria sasaran, termasuk sebuah kawalan amplifikasi dalamam (IAC). Setiap set primer ini dianalisa bagi menentukan nilai spesifikasi dan sensitiviti masing-masing. Asai PCR multipleks telah dibina dengan menggunakan kepekatan primer dan komponen PCR yang telah dioptimum. Pada peringkat awal, tahap ketepatan asai ini dinilai ke atas pencilan bakteria dari sampel klinikal. Seterusnya, asai PCR ini menjalani pengeringan-beku dengan kehadiran trehalose sebagai gula penstabil. Penilaian kestabilan asai dilakukan pada suhu dan jangka masa yang berbeza. Dalam fasa terakhir, asai PCR ini diuji secara klinikal ke atas spesimen sputum dari Hospital Universiti Sains Malaysia, dan secara lapangan ke atas spesimen sputum dari jemaah Haji Malaysia. Hasil kajian mendapati bahawa kesemua set primer yang direka adalah spesifik terhadap bakteria sasaran. Kepekatan yang optima bagi setiap primer bakteria (0.4 µM) dan primer IAC (0.2 mM), $MgCl_2$ (2.5 mM), dNTPs (0.2 mM) dan enzim *Taq* DNA polimerase (0.75 unit) telah

digunakan dalam pembinaan asai PCR multipleks. Penilaian awal ke atas pencilan bakteria mendapati bahawa asai ini mencapai 100% nilai ketepatan terhadap bakteria sasaran dan bukan sasaran (*n* = 145) (spesifikasi analitikal), dan mampu mengesan serendah 10 pg DNA (200 sel bakteria) (sensitiviti analitikal). Pengeringan-beku ke atas asai ini telah dilakukan dengan campuran 6% trehalose ke dalam reagen PCR. Asai ini didapati stabil pada suhu bilik (25ºC) untuk tempoh sekurang-kurangnya enam bulan. Penilaian ke atas spesimen sputum klinikal (*n* = 200) mendapati bahawa tahap sensitiviti, spesifikasi dan ketepatan asai masing-masing mencapai 100%, 92% dan 95%. Manakala tahap sensitiviti, spesifikasi dan ketepatan ke atas spesimen sputum jemaah Haji (*n* = 202) masing-masing mencapai 100%, 92% dan 97%. Melalui kajian ini, bakteria utama yang dikesan daripada spesimen klinikal dan jemaah Haji masing-masing ialah *K. pneumoniae* dan *H. influenzae*. Kesimpulannya, ciri-ciri seperti cepat, mudah, stabil haba dan boleh dipercayai, membolehkan asai PCR multipleks ini diaplikasi sebagai sebuah alat diagnostik bagi pengesanan bakteria penyebab RTIs.

THERMOSTABILIZED MULTIPLEX PCR ASSAY FOR DETECTION OF SELECTED BACTERIA ASSOCIATED WITH RESPIRATORY TRACT INFECTIONS AMONG MALAYSIAN HAJJ PILGRIMS

ABSTRACT

Respiratory tract infections (RTIs) are the commonest health problem during the annual Hajj pilrimage. Common bacteria associated with RTIs include *Klebsiella pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae, Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*. Rapid detection of these pathogens could facilitate towards effective therapies. Therefore, this study aimed to develop and evaluate a thermostabilized polymerase chain reaction (PCR) assay for simultaneous detection of these six bacteria. The first step involved designing specific primers for the target bacteria and an internal amplification control (IAC). Each set of primers was evaluated to analyze for their specificity and sensitivity. A multiplex PCR was then developed by optimizing the concentration of primers and other components. Initial accuracy of the multiplex PCR was determined on clinical isolates. Subsequently, this assay had undergone lyophilization process in the presence of trehalose as the sugar-stabilizer. The assay stability was tested at different sets of temperature for different time-intervals. In the last stage, this assay was evaluated on the sputum specimens from Hospital USM and further evaluated at the field level using the specimens from Malaysian Hajj pilgrims. Results indicated that all the designed primers were specific to the respective target bacteria. The optimized concentrations of primers for bacteria (0.4 µM) and IAC (0.2 mM), $MgCl_2$ (2.5 mM), dNTPs (0.2 mM) and *Taq* DNA polymerase enzyme (0.75 unit) were used in the development of multiplex PCR assay. Initial evaluation on bacterial isolates showed that the assay was 100% accurate on both target and non-target bacteria (*n* =

145) (analytical specificity) with the lowest limit of detection was 10 pg DNA (200 bacterial cell) (analytical sensitivity). Lyophilization of this assay was successfully carried out in the presence of 6% trehalose in the PCR reagent. The assay was stable at the ambient temperature (25ºC) for at least six months. The sensitivity, specificity and accuracy of this assay were 100%, 92% and 95%, respectively on cinical sputum specimens (n = 200). Field evaluation on specimens from Malaysian Hajj pilgrims ensued the sensitivity and specificity of 100% and 92%, respectively, with the accuracy of 97%. From this study, two main bacteria detected from the clinical and Hajj sputum specimens were *K. pneumoniae* and *H. influenzae*, respectively. In conclusion, the rapidity, convenience, thermal-stable and reliable, could enable the application of this thermostabilized multiplex PCR assay to be used as a molecular diagnostic tool for the detection of six respiratory bacteria.

CHAPTER 1

INTRODUCTION

1.1 Hajj: The annual Muslim pilgrimage

Hajj is the annual Muslim pilgrimage to Mecca and specified holy sites in the Kingdom of Saudi Arabia (KSA). The religious pilgrimage is a compulsion for all physically and financially able Muslims, once in a lifetime. Hajj is performed in six days, starting from the eighth through the thirteenth of Dhul-Hijjah, the twelfth month of the Islamic lunar calendar. Every year, around two million of Muslims from more than 180 countries around the world gather in Mecca to participate in the rituals of Hajj. The steps in this six-day ritual are demonstrated in Figure 1.1.

The first rite of Hajj is entering "ihram", where the Hajj pilgrims declare their Hajj intention before entering Mecca and wearing plain garments of ihram cloth. Upon arrival at Mecca, pilgrims perform the arrival "tawaf", seven times counterclockwise circling of Kaaba, the black silk-clad stone structure. Pilgrims also perform "sa'ey", walking or running seven times between the hills of Safa and Marwah, and heading to the Mina encampment. On the next day, pilgrims take a journey to Arafat, to spend a day for reverent prayer (Al-Jazeera, 2017) and perform "wuquf", the grand climax of all rituals in Hajj. From the Arafat, pilgrims spend their night in Muzdalifah and return to Mina to perform symbolic stoning of the devil at the three pillars. As the symbolic of Hajj completion, pilgrims will perform "qurban" by slaughtering sacrificial animals and continue the rite of "tahallul" or head-shaving for the males. Pilgrims also perform stoning by throwing seven pebbles at the three pillars in Mina on the fourth and fifth day of Hajj, and heading back to Mecca before sunset. On the last day of Hajj, pilgrims perform the fare well tawaf before leaving Mecca.

Figure 1.1: The steps in the six-day Hajj pilgrimage (adopted from Al-Jazeera, 2017)

1.2 Health risks during the Hajj

Hajj pilgrimage is inevitably associated with various communicable and non-communicable health risks, due to the massive gathering of pilgrims who are closely surrounded in the confined area, doing the same thing at the same time (Shujaa and Alhamid, 2015). The crowd density of pilgrims during Hajj can reach about eight to nine people per square meter (ppm^2) (Shujaa and Alhamid, 2015), and at certain time to 12 ppm^2 during tawaf and closing to Kaaba (Rahman *et al.*, 2017). The massive gathering also could encourage disease transmission, especially of airborne pathogens. The crowd density at each confined area for Hajj rituals is shown in Figure 1.2.

In addition to the massive crowd, other challenges that could contribute to the health risks include extreme heat, extended stays at Hajj sites, strenuous activities, exhaustion and fatigue (Ahmed *et al.*, 2006; Rahman *et al.*, 2017). Furthermore, traffic congestions and inadequate of food are added health risks, while the advanced age of many pilgrims increase the morbidity and mortality risks (Ahmed *et al.*, 2006).

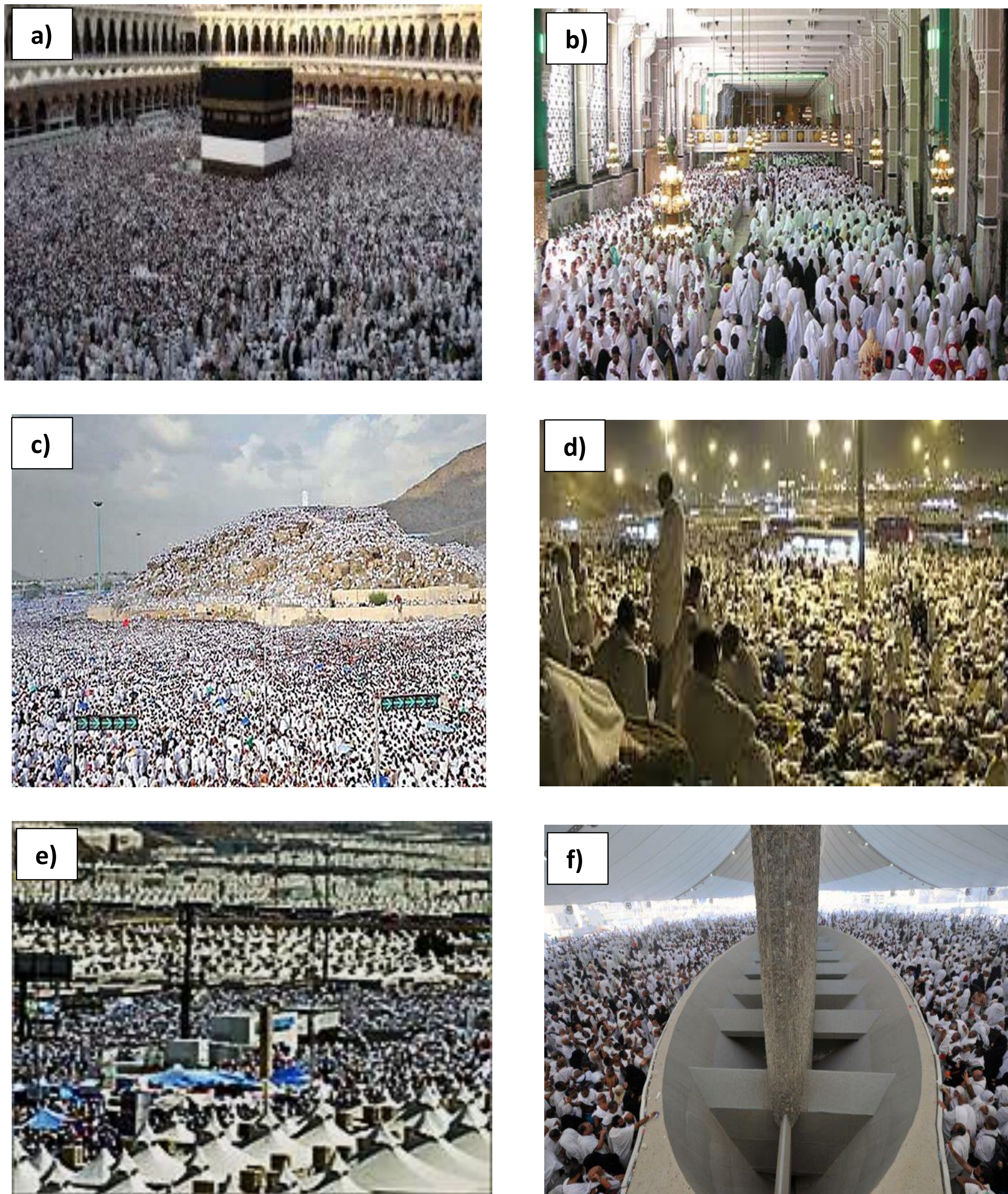


Figure 1.2: The crowd density at each confined area for Hajj rituals; a) “tawaf”; b) “sa’ey”; c) “wuquf”; d) night at Muzdalifah; e) Mina encampment; and f) stoning (adopted from Al-Jazeera, 2017; Huzaifa, 2017; Quayyum, 2018; Salam-Islam, 2016).

1.3 Respiratory tract infection

Respiratory tract infections (RTIs) have been reported to represent the top communicable diseases and accounted for the highest hospital admissions during Hajj (Alzeer, 2009). The severity of RTIs may vary from mild respiratory symptoms to severe pneumonia and tuberculosis of which requiring hospitalization or end up with death. Hajj is also challenged with the seasonal prevalence of influenza viruses. During the pandemic H1N1 in the year 2009, the mean prevalence of influenza was reported to be 2.1% among the arriving pilgrims and 3.6% among the Hajj returnees (Al-Tawfiq *et al.*, 2016). The 2012 Hajj season is also challenged by the emergence of Middle East respiratory coronavirus (MERS-CoV). MERS-CoV is potentially aggressive and may lead to serious outbreaks. However, no cases of MERS-CoV positive were reported among the 2012 Hajj pilgrims. These challenges indicate that Hajj is vulnerable to communicable diseases due to the massive condition. Moreover, among the major concern is the potential severe consequences of RTIs due to importation or exportation of the pathogens. The spread of pathogens among pilgrims and back to their home countries would contribute to globalization of respiratory infections (Shujaa and Alhamid, 2015).

Infections of the respiratory system are specifically determined through the symptoms and anatomic involvement. The anatomy of respiratory system consists of two major parts, which are the upper and the lower tracks (Figure 1.3). In parallel to its primary role for respiration, respiratory tract is prone to infectious agents, especially through the inhalation process. Direct contact with the external environment allows various particles and airborne microorganisms such as viruses, bacteria, fungi and parasites to enter the respiratory tract and cause infections on the sinuses, throat, airways or lungs.

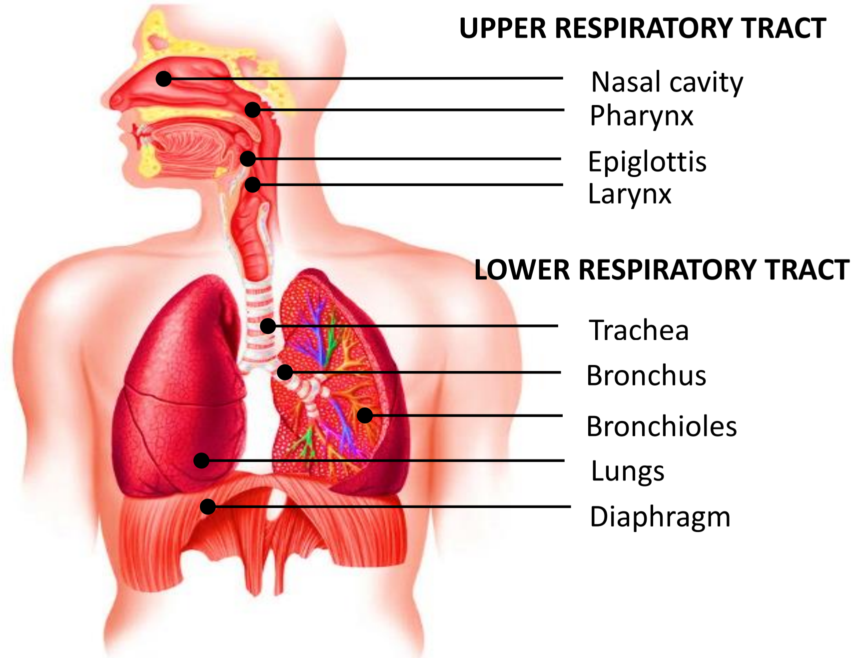


Figure 1.3: The anatomy of respiratory system consisting the upper and lower respiratory tracts (adapted from Calvetti and Bailey, 2018).

1.3.1 Upper respiratory tract infections (URTIs)

Upper respiratory tract infections (URTIs) involve any infections at the upper respiratory tract, which comprise of nasal cavity, pharynx, epiglottis and larynx. Symptomatic and localization of the infections could differentiate URTIs into several types, including sinusitis, rhinitis, otitis media, tonsillitis, epiglottitis, pharyngitis and laryngitis. The suffix "–itis" is from the Greek word that means "inflammation of". Besides the localization of infections, URTIs also may comprise other infections, such as coryza (colds), influenza, pertussis and diphtheria.

The primary clinical manifestation of URTIs includes the presence of respiratory symptoms, such as cough, sore throat, runny nose, hoarseness of voice and difficulty in breathing. These symptoms are generally due to secretory toxins from the pathogens or inflammatory response from the immune system. Most of URTIs are usually self-limiting and benign. URTIs are common in general population and have been the leading purpose for visiting physicians and absenteeism from work or school. This results in significant impact on public health.

Viruses have been noted as the major etiologic agents for URTIs although minority of the infections are due to bacteria. There are various number of viruses with multiple viral family and antigenic types responsible for URTIs. These include human rhinovirus (more than 100 serotypes) (Jacobs *et al.*, 2013), influenza virus (three types) (Hampson and Mackenzie, 2006), parainfluenza virus (four types), respiratory syncytial virus (two major subtypes with multiple genotypes) (Vandini *et al.*, 2017), adenovirus (more than 70 types) (Liu *et al.*, 2018) and coronavirus (six types) (Jonsdottir and Dijkman, 2016). However, despite the necessity of providing appropriate treatment, physicians commonly face difficulties in distinguishing

between viral and bacterial-URTIs because the present symptoms are generally similar (Zoorob *et al.*, 2012). In most cases of URTIs, microbiological diagnosis is rarely warranted, except for otitis media, pharyngitis and epiglottitis, which are typically caused by Gram-negative bacteria, group A beta-hemolytic streptococci and *Haemophilus* species, respectively. Hence, this has resulted in high prescription of antibiotics for URTIs among the outpatient settings (Schroeck *et al.*, 2015).

1.3.2 Lower respiratory tract infections (LRTIs)

Lower respiratory tract infections (LRTIs) affect the area in trachea and lungs. These infections, which include bronchitis, bronchiolitis and pneumonia, are less common than URTIs, but are more likely to cause morbidity and mortality, especially in developing and under developing countries (Bellos *et al.*, 2010). Most of the fatality and severe illness episodes of RTIs are due to pneumonia and other acute lower RTIs. Around 4.2 million deaths of LRTIs occurred worldwide among all age groups; with 1.8 million of these are children between age one to 59 months (Bellos *et al.*, 2010; WHO, 2008). Meanwhile, pneumonia during pregnancy and in elderly groups have been associated with increased morbidity and mortality compared to normal adults (Goodnight and Soper, 2005; Chong and Street, 2008).

Pneumonia can further be classified into community- and hospital-acquired infections. Community-acquired pneumonia (CAP) is defined as an acute infection of the pulmonary parenchyma that is acquired from the community, for instance, during the massive gatherings (Mandell *et al.*, 2007; Wiersinga *et al.*, 2012). CAP is usually a self-limiting disease, wherein most of the patients are treated as outpatients. However, CAP is also a potentially serious illness that can be associated with substantial morbidity and mortality, especially among the young children and the elderly. It has been reported that CAP has an increasing trend towards hospitalization, especially in the elderly population (Fry *et al.*, 2005; van Gageldonk-Lafeber *et al.,* 2009; Woodhead *et al.*, 2011).

In addition, it is still the leading cause of death in relation to infectious diseases in high-income countries (Mandell *et al.*, 2007). Severe CAP is common during Hajj and has been reported as the leading cause of critical illness in both hospital and intensive

care unit (ICU) admissions, particularly during the second week of every Hajj season. The mortality rates for CAP during Hajj ranged between 17.0% to 36.8% among the hospitalized patients and 21.5% to 46.6 % for the patients requiring ICU admission (Memish *et al.*, 2014).

Meanwhile, hospital-acquired pneumonia (HAP) or also termed as nosocomial pneumonia, is acquired in a hospital after 48 hours or more of an admission. It is not associated with any intubation at the time of admission. Another type of HAP is ventilator-associated pneumonia (VAP), which is developed in more than 48 hours after endotracheal intubation and is usually related with a higher risk of death. The Infectious Diseases Society of America/American Thoracic Society included healthcare-associated pneumonia (HCAP) in 2005 HAP guidelines to define pneumonia that is related to healthcare facilities such as nursing homes, hemodialysis centers, and outpatient clinics (Mandell *et al.*, 2007).

1.3.3 Pulmonary tuberculosis

Tuberculosis is one of the top leading cause of death around the world. The World Health Organization (WHO) has reported that tuberculosis caused global morbidity on 10 million people with the mortality rate of 1.6 million in the year 2017 (WHO, 2018a). The incidence rate of tuberculosis varies among countries, ranging from less than 25 cases per 100,000 populations in North America, to above 300 cases per 100,000 populations in Africa and South-East Asia (Figure 1.4). Majority of new tuberculosis cases (62.0%) in 2017 came from high burden countries, including South Africa, South-East Asia and Western Pacific Regions (WHO, 2018a).

It has been reported that 50.0% of the Hajj pilgrims are from the high burden countries (Al-Orainey, 2013). In such massive gathering during Hajj, the risk of tuberculosis transmission is very high. This was proven by the high frequency of tuberculosis among the hospitalized pilgrims with pneumonia (Mandourah *et al.*, 2012). In addition, the Saudi Ministry of Health in their 2010 annual report claimed that three out of 30 respiratory disease mortality among pilgrims were actually due to tuberculosis (Al-Orainey, 2013). The over-crowd during Hajj and the presence of undiagnosed active tuberculosis pilgrims possess a high risk to other pilgrims for being infected (Yezli *et al.*, 2017).

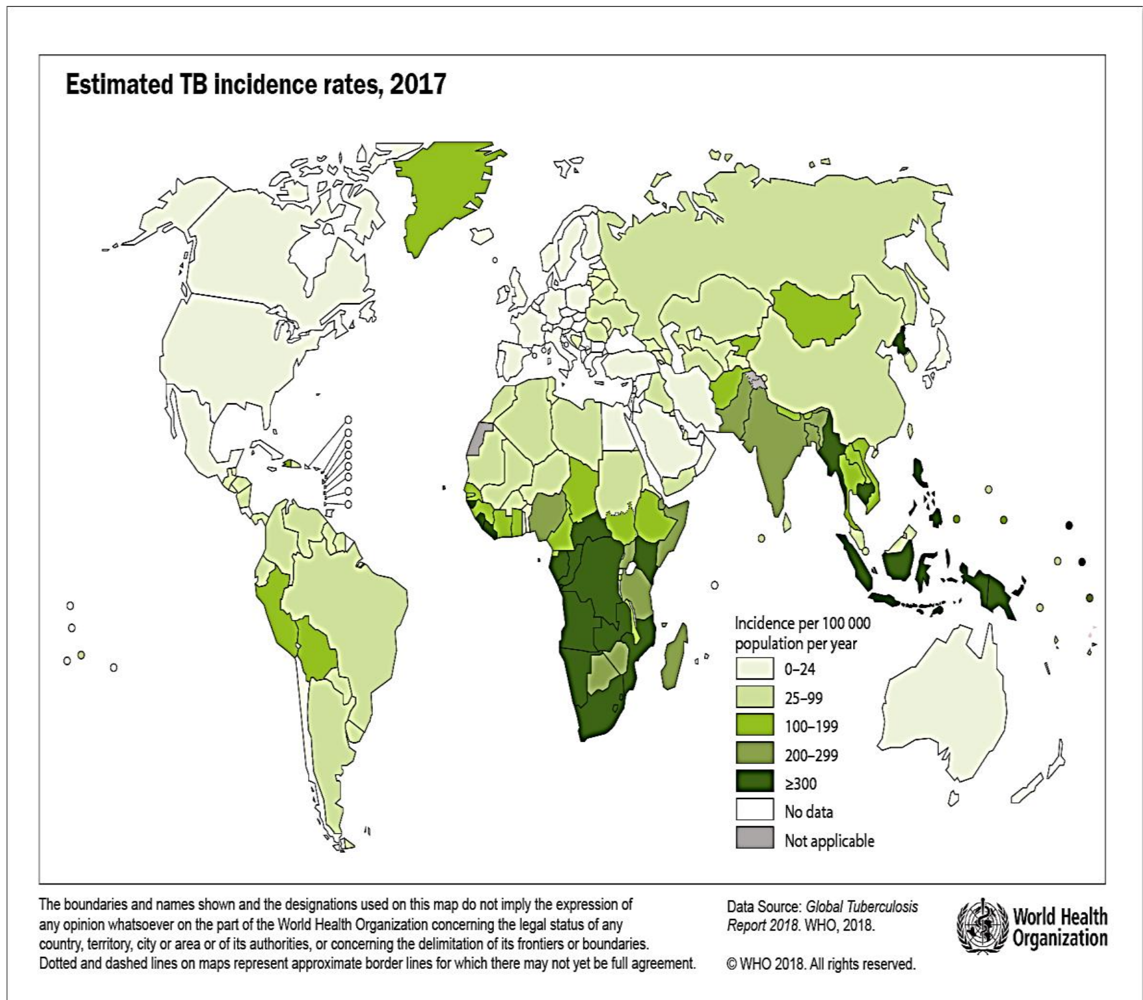


Figure 1.4: Global incidence rate of tuberculosis in 2017 (adopted from WHO, 2018a).

1.4 Etiologic bacteria for RTIs

Respiratory tract infections (RTIs) are common among Hajj pilgrims, yet the acquisition of respiratory pathogen during Hajj is not well identified due to the limitation of diagnostic coverage. Based on previous studies, most frequent bacteria acquired by Hajj pilgrims were *Haemophilus influenzae, Klebsiella pneumoniae*, *Streptococcus pneumoniae* and *Staphylococcus aureus* (Alzeer *et al.*, 2009; Memish *et al.*, 2015; Al-Tawfiq *et al.*, 2016). Meanwhile, *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* as the most common causative organisms have been reported among patients with pneumonia who failed the first line of therapy and required hospital admission during Hajj (Asghar *et al.*, 2011; Mandourah *et al.*, 2012; Shirah *et al.*, 2017). Other atypical bacteria for pneumonia are *Mycoplasma pneumoniae, Chlamydophila pneumoniae* and *Legionella pneumophila*, in which sputum is usually absent (Memish *et al.*, 2014).

The overall acquisition rate for at least one bacteria was reported as 28.3% during the annual Hajj seasons, which is about two times higher than the normal rate before attending for Hajj pilgrimage (15.4%) (Memish *et al.*, 2015). Since early 1990s, previous studies have reported that *H. inflenzaae* and *S. pneumoniae* have been the two predominant bacteria involved in the aetiology of RTIs during Hajj (El-Sheikh *et al.*, 1998; Al-Tawfiq *et al.*, 2016). Besides, these organisms are the two commonest bacteria in adult CAP (Macfarlane *et al.*, 1993; Bosch *et al.*, 2013). *K. pneumoniae, S. aureus* and *P. aeruginosa* are frequently isolated from the pilgrims in around 3.1% to 7.5% (Memish *et al.*, 2015). Meanwhile, although the overall prevalence of *M. tuberculosis* during Hajj is considered low (1%), infection by this organism could lead to severe conditions, which results in first line antibiotic failure and prolonged hospitalization (Alzeer *et al.*, 2009; Mandourah *et al.*, 2012).

1.5 Risk factors of RTIs

Constant exposure of the respiratory tract to the gaseous environment provides the chance of microorganisms including viruses, bacteria and spores to cause infections. Although most of the particles are eliminated by the respiratory tract defenses, certain pathogens may have their specific means to penetrate the host. For example, influenza viruses use their surface antigens to adhere to mucosal epithelial cells, while some bacteria are resistant to antimicrobial factors and/or phagocytosis (Inglis, 2007). These show that the respiratory tract immune systems can still be bypassed by pathogens and may be impaired by endogenous factors such as genetic defects and iatrogenic disorders; or exogenous factors such as chemical pollutants and respiratory viruses, which thus, making the host susceptible to occasional pathogens, including commensal organisms (Alonso, 2008).

RTIs are among the most frequent communicable diseases recorded during mass gatherings. The causative pathogens can be easily transmitted in crowded environment by the air, droplet, or direct hand-to-hand contact with infected secretions. The pathogens subsequently pass to the respiratory tract and produce symptoms corresponding to the area being infected (Mossad, 2013). It is also well known that elderly community, infants and children, pregnant women, and those with chronic illnesses like cardiac, diabetic or immune system deficiencies, are at-risk groups who are more vulnerable to RTIs. With the weakened immune system, their chance of being infected from other people is high even though they are not directly involved in any mass gathering.

1.6 Clinical signs of bacterial RTIs

Bacterial infections are usually predominant in the lower part of respiratory tract, in which the vast majority are due to pneumonia. Among the common signs and symptoms for bacterial RTIs include the presence of cough, sputum production, fever, and dyspnea (shortness of breath). Some patients might present with non-respiratory symptoms such as headache, confusion, gastrointestinal discomfort and myalgia.

Bronchitis and bronchiolitis are commonly preceded by upper RTIs and present with cough. Bronchiolitis is usually present with coryza and fever, and prominent with airway obstruction. Although most cases of bronchitis and bronchiolitis are caused by viruses, some bacteria such as *H. influenzae* and *S. pneumoniae* can be associated with chronic bronchitis, while *M. pneumoniae* occasionally causes bronchiolitis. Patients with chronic bronchitis have typical symptoms of incessant cough with large amount of sputum, especially in the morning (Eccles *et al.*, 2014).

Clinical sign of pneumonia can be distinguished from bronchitis and bronchiolitis by acute inflammation of the lung parenchyma (air sacs) caused by various pathogens. A clinical diagnosis of pneumonia, mainly CAP, is based on the presence of lower RTI symptoms, which include new focal chest signs (chest discomfort or pain, high pulse rate of above 100/minute at rest and shortness of breath) and at least one systemic feature (fever, sweating or rigors). In the hospital settings, additional diagnosis is noteworthy by the presence of new pulmonary infiltrate on a chest X-ray (Eccles *et al.*, 2014).

Pneumonia due to some bacteria might present with specific or uncommon clinical presentations such as gradual onset of dry cough and extra-pulmonary manifestations (Prina *et al.*, 2015). For example, patients with CAP caused by *P. aeruginosa* has been

reported to have acute symptoms, including chest pain, dry cough, or hemoptysis (bloody sputum). Hemoptysis is resulted from the necrotizing vasculitis and parenchymal necrosis. These symptoms are followed by fever and in some cases, are further developed to hypotension with a rapid progression to septic shock (de'Campos *et al.*, 2014).

Pulmonary tuberculosis is another common type of bacterial respiratory infections in humans, mainly caused by *M. tuberculosis*. The symptoms of active pulmonary tuberculosis include chronic cough of more than three weeks, chest pain, night sweat and fever, followed by fatigue and anorexia. Human immunodeficiency virus (HIV)-infected individual is usually absence with all the classical symptoms of tuberculosis, but is having positive culture for *M. tuberculosis.* Clinical diagnosis of active pulmonary tuberculosis involves microscopic examination and culture plus subsequent drug-susceptibility testing. Latent infection can be diagnosed with either a tuberculin skin test or an interferon-gamma release assay (Zumla *et al.*, 2013).

1.7 Management of RTIs during Hajj

The massive gathering during Hajj can potentially increase huge challenges to the public health authorities in the management of RTIs, especially in the aspect of therapy and prevention strategies. Thus, understanding the specific diseases, etiologic microorganisms and pathophysiology are important for effective treatment and management.

URTIs, LRTIs and tuberculosis are classified as aerosol transmission of infectious diseases (Jones and Brosseau, 2015). Hence, the application of preventive measures as outlined by CDC in the transmission-based precautions is potentially helpful to prevent and reduce these respiratory infections (CDC, 2017). Among the preventive approaches are hygiene practices (including hand hygiene and cough etiquette), personal protective equipment and immunization. The Saudi Ministry of Health also collaborates with multi-nationalities of pilgrims' origin to provide guidelines, educational materials and information before attending for Hajj (Al-Tawfiq and Memish, 2012).

Besides, KSA also emphasizes for global collaborations with the international public health agencies, including WHO and CDC in providing updated health regulations. For every Hajj season, KSA provides an approximate of 25,000 additional health workers and 8 hospitals equipped with high quality facilities in both Mecca and Mina (Shafi *et al.*, 2016). These are to ensure that all the ill-health Hajj pilgrims receive appropriate therapy, thereby reducing any transmission of infections to other pilgrims or to the KSA residents.

1.7.1 Treatment and prevention of URTIs

Most of the URTIs are self-limiting illness and are due to viral infections. The treatments for viral respiratory infections are often limited. Therefore, the management of URTIs during hajj is focusing towards surveillance and prevention strategies. For example, the global prevalence of 2009 H1N1 and the concern of potential 2012 MERS-CoV outbreak have led to frequent screening of these viral pathogens to increase awareness and keep the surveillance record updated (Al-Tawfiq *et al.*, 2016). Besides the preventive approaches, the Saudi Ministry of Health also recommends seasonal influenza vaccination for all the pilgrims.

1.7.2 Treatment and prevention of LRTIs

Patients diagnosed with bacterial infections, particularly pneumonia need to have rapid and empirical treatments. However, the coverage of causative microorganisms is relatively low, of which about 40.0% to 56.0% from the total positive cases (Memish *et al.*, 2014). Therefore, attributable to the failure of pathogen detection, antibiotic therapy is given based on the patient's condition, history, age, and scoring. The KSA Ministry of Health has provided guidelines for the clinicians towards an effective management of common infectious diseases during Hajj (Alghamdi *et al.*, 2016).

According to the KSA guidelines, patients with low-severity CAP, for instance the formerly healthy outpatients with no record of antibiotic used for the past three months, are given a standard five- or seven-day course of single antibiotic with azithromycin or clarithromycin (Alghamdi *et al.*, 2016). Outpatients with comorbidities and/or antibiotic consumption within the past 3 months and inpatients (non-ICU) are recommended for a combination of clarithromycin with either cefuroxime or amoxicillin-clavulanate. The hospitalized patients in ICU should receive intravenous

antibiotic in a combination of ceftriaxone and vancomycin with either clarithromycin or azithromycin. For the ICU patients with suspected *Pseudomonas* spp., piperacillin-tazobactam in combination with gentamycin and clarithromycin or azithromycin are recommended.

S. pneumoniae and *H. influenzae* infections are considered as vaccine-preventable diseases. However, the Saudi Ministry of Health has not recommended a mandatory use of the current available pneumococcal vaccines or Hib vaccine due to dissimilarities of the causal strains with the vaccine coverage strains (Al-Tawfiq and Memish, 2016; Alfelali *et al.*, 2016). Nevertheless, those who are at increased risk of invasive pneumococcal disease are recommended to take pneumococcal vaccination (Rashid *et al.*, 2013).

1.7.3 Treatment and prevention of tuberculosis

WHO has highlighted that the patient with active tuberculosis should receive a six-month course of treatment with a combination of four anti-microbial drugs: rifampicin, isoniazid, pyrazinamide and ethambutol. All these four first-line medications should be administered for the first two months. In the following four months, the patient should continue with a combination of rifampicin and isoniazid. In cases of multiple drug resistant (MDR) strains, second-line medications, which include fluoroquinolones (levofloxacin, moxiloxacin) and aminoglycoside or polypeptide injections are the options for treatment (Jilani and Siddiqui, 2018). MDR strains for tuberculosis demonstrate resistance to rifampicin and isoniazid, which have emerged from inappropriate used of tuberculosis medication. Currently, the emergence of extensively drug resistant (XDR) tuberculosis increases the burden of health security

threat. The choice of treatment is limited to the third-line medications, such as clarithromycin, bedaquiline and delamanid (Glaziou *et al.*, 2018).

WHO pursues End TB Strategy with the aim to reduce global tuberculosis morbidity, mortality and catastrophic costs by 2030 (Floyd *et al.*, 2018). For this purpose, WHO emphasizes the engagement of all health-care providers to ensure that tuberculosis diagnoses and treatment meet the international standards. Among the strategies are to improve the efficacy of detection methods, such as the recommendation of molecular-based tests (DR-MTB and Xpert MTB/RIF); to introduce new vaccine or prophylactic treatment for latent infections; and to improve anti-tuberculosis medications especially for MDR- and XDR-tuberculosis (Glaziou *et al.*, 2018).

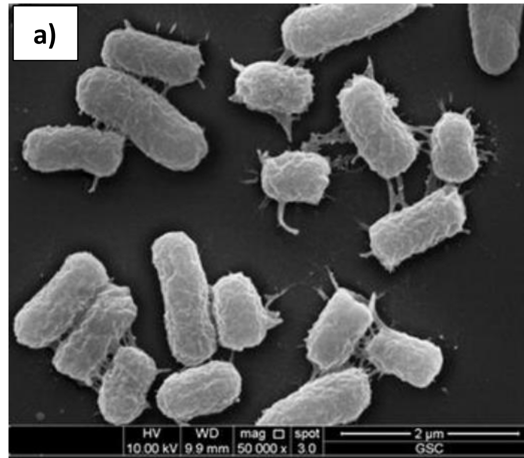
1.8 *Klebsiella pneumoniae*

K. pneumoniae is a human pathobiont that has been associated with a number of serious health problems, including pneumonia and bacteremia. This organism has gained global attention due to the increasing severity of infections and the scarcity of effective treatments (Paczosa and Mecsas, 2016). Historically, *K. pneumoniae* was first isolated in the late 19th century as Friedlander's bacterium. Although it is well known to cause severe infections among immunocompromised patients, the pathogen has recently been found as emerging hyper-virulent strains (Shon *et al.*, 2013) or become resistant to antibiotics (Boucher *et al.*, 2009). The spread of these hyper-virulent and antibiotic resistant strains may expand the infections to a healthy and immunocompromised individual (Paczosa and Mecsas, 2016).

1.8.1 Bacteriology

K. pneumoniae is a Gram-negative, rod-shaped bacterium with the size of about 0.3 to 1.0 µm in width and 0.6 to 6.0 µm in length (Figure 1.5(a)). This encapsulated bacterium naturally resides in the environments, such as soils and water. *K. pneumoniae* also colonizes the mucosal surfaces of gastrointestinal tract and oropharynx in humans. In difference to other enterobacteria, *K. pneumoniae* is unique for its thick polysaccharide capsule, which is significantly important for the pathogenesis. It is a lactose fermenter bacterium and appears as mucoid colonies on the agar media (Figure 1.5(b)).

The genome of *K. pneumoniae* consists of one circular chromosome of sized around 5.3 million base pair (Mb) and encoding for about 5,000 to 6,000 genes. The average guanine-cytosine (G+C) content is 57.0%. Different *K. pneumoniae* strains have different number of plasmids, while some strains (e.g. 1082 and ED2) lack the virulence plasmids (Ogawa *et al.*, 2005; Liu *et al.*, 2012). A pan-genome analysis has identified a total of 4,170 core genes and 5,493 accessory genes (Lam *et al.*, 2018). The accessory genes breach down *K. pneumoniae* into opportunistic, hyper-virulent and MDR strains. Comparative analyses have found that this species has a highly conservative of virulence genes in both core and accessory genome (Wu *et al.*, 2009; Lam *et al.*, 2018). The accessory genome is also important for the bacterial adaptation and response to environmental stress. Among the unique genes of *K. pneumoniae* is the phosphohydrolase (*php*) that has been used to discriminate this organism against its closely related species (Garza-Ramos *et al.*, 2015). This gene is responsible for hydrolysis reaction and lipid uptake from extracellular space.



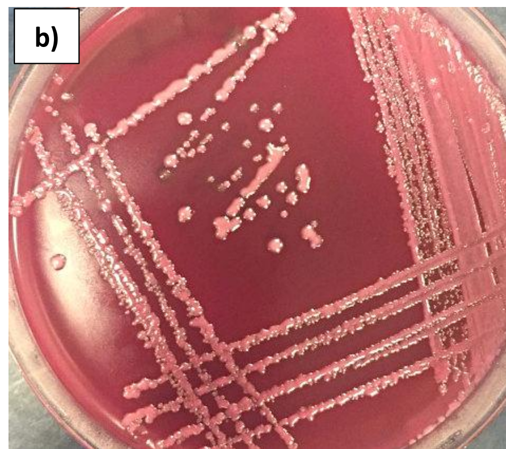


Figure 1.5: The morphology of *K. pneumoniae;* a) micrograph of *K. pneumoniae* cells under scanning electron microscope (SEM); b) characteristics of mucoid, pinkish lactose-fermenting colonies of *K. pneumoniae* on MacConkey agar (adopted from Cho *et al.*, 2012; Aryal and Gonzalez, 2018).

1.8.2 Epidemiology

K. pneumoniae is a part of human microbiota that colonizes in the gastrointestinal tract of 5.0% to 38.0% adult populations (Ashurst and Dawson, 2018). A small proportion of around 1.0% to 6.0% populations carry this bacterium in the upper respiratory tract. *K. pneumoniae* has been found as the causative pathogen in 3.0% to 5.0% of overall CAP patients from the Western countries. The proportion is expected to be higher in other developing regions.

The commensal *K. pneumoniae* can emerge into few groups based on their accessory genes (Martin and Bachman, 2018). The first group can potentially develop into an opportunistic pathogen and causes infections in those who are critically ill and having deficient immune system. Common infections caused by this group include healthcare associated of pneumonia, urinary tract infections (UTIs), and septicemia. Another group is the hyper-virulent strains of *K. pneumoniae*, which has been associated with severe infections such as pyogenic liver abscess and meningitis in the community settings. A third group is the carbapenemase-encoding *K. pneumoniae* strains, which is highly resistant to many antibiotics, causing them difficult to treat.

1.8.3 Pathogenic factors

Various physiological factors and genetic traits contribute to the pathogenesis of *K. pneumoniae*. Currently, four major virulence factors: polysaccharide capsule, lipopolysaccharides (LPS), fimbriae and siderophores, have been well recognized for *K. pneumoniae* to invade the host at different tissue sites. These factors provide protection and defense strategies for the bacterium to colonize, replicate and consequently cause infections in the host. Polysaccharide capsule plays important roles for coating the cell and protects this bacterium against phagocytosis and bactericidal