RESPIRATORY MICROBIOME AND ITS SUSCEPTIBILITY TO *Mycobacterium tuberculosis* INFECTION

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RESPIRATORY MICROBIOME AND ITS SUSCEPTIBILITY TO *Mycobacterium tuberculosis* INFECTION

by

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LISTS OF ABBREVIATIONS AND SYMBOLS

<	Less than
>	More than
%	Percentage
≤	Less than equal to
≥	More than equal to
µL	Microliter
ºC	Degree Celsius
ACE	Abundance coverage estimator
AFB	Acid fast bacillus
AI2	Auto inducer 2
ANOSIM	Analysis of similarity
BCG	Bacillus Calmette -Guerin
CF	Cystic Fibrosis
COPD	Chronic Obstructive Pulmonary Disease
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Treatment Short Course
DST	Drug susceptibility test
DTH	Delayed-typed hypersensitivity
EMB	Ethambutol
EPTB	Extra pulmonary tuberculosis

HMP	Human Microbiome Project
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disease
IFN-γ	Interferon gamma
IGRAs	Interferon-gamma release assays
INH	Isoniazid
LDA	Linear discriminant analysis
LEfSe	Linear effect size
LTBI	Latent tuberculosis infection
MDR-TB	Multi-drug resistant TB
MTB	*Mycobacterium tuberculosis*
MetaHIT	Metagenomics of the Human Intestinal Tract
NGS	Next generation sequencing
NTM	Non-tuberculous mycobacteria
OTU	Operational taxonomic units
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PTB	Pulmonary tuberculosis
PPD	Purified protein derivative
PZA	Pyrazinamide
QIIME	Quantitative Insights into Microbial Ecology
SCFA	Short-chain fatty acids

STR	Streptomycin
RIF	Rifampicin
rRNA	Ribosomal ribonucleic acid
TB	Tuberculosis
TST	Tuberculin skin test
Unifrac	Unique Fraction
UPGMA	Unweighted pair-group method with arithmetic means
WGNCA	Weighted correlation network analysis
WHO	World Health Organization

MICROBIOME PERNAFASAN DAN KEPEKAAN TERHADAP JANGKITAN *Mycobacterium tuberculosis.*

ABSTRAK

Saluran pernafasan bertindak sebagai ceruk ekologi bagi pelbagai mikroorganisma. Komuniti mikrob ini memainkan peranan penting dalam memastikan tahap kesihatan manusia. Walaupun kecenderungan untuk mengkaji tentang komposisi mikrobiota di dalam saluran pernafasan baru-baru ini telah meningkat, namun kemungkinan kaitan antara komposisi dan kelimpahan mikrobiota dalam niche ekologi tekak manusia yang dikaitkan dengan jangkitan *Mycobacterium tuberculosis* masih lagi kurang difahami. Kajian ini bertujuan untuk mengkaji komposisi mikrobiota tekak dalam pesakit tuberkulosis pulmonari berbanding dengan subjek sihat yang mempunyai ujian positif TST (Ujian Kulit Tuberkulin) dan subjek kajian negatif TST. Mikrobiota tekak itu sepenuhnya dicirikan oleh jujukan 16s rRNA metagenomik. Kajian ini mendedahkan bahawa terdapat 7 fyla dan 35 genera dalam subjek kajian. Fyla utama adalah *Firmicutes, Bacteroidetes, Proteobacteria* dan *Actinobacteria*. Komuniti utama genera adalah *Streptococcus, Staphylococcus, Capnocytophaga, Rothia* dan *Enterococcus.* Komposisi mikrobiota tekak pesakit TB dan TST positif subjek tanpa gejala lebih heterogenus berbanding dengan mikrobiota tekak subjek TST negatif yang sihat. *Streptococcus* adalah genera yang paling dominan dalam tekak subjek yang sihat berbanding dengan subjek TST positif tanpa gejala dan pesakit TB. Bagi pesakit TB, *Prevotella, Neiserria, Leuconostoc, Fusobacterium, Campylobacter,* dan *Enterococcus* adalah antara genera yang banyak terdapat di tekak mereka. Komposisi mikrobiota tekak pesakit TB dan subjek TST positif tanpa simptom lebih heterogenus berbanding dengan

mikrobiota tekak subjek TST negatif yang sihat. *Streptococcus* didapati sangat banyak dalam tekak subjek yang sihat berbanding subjek TST positif dan pesakit TB. Dalam pesakit TB, *Bifidobacterium*, *Bulleidia*, *Butyrivibrio*, *Chryseobacterium* dan *Pediococcus* adalah antara genera yang hadir dengan banyaknya di tekak mereka berbanding dengan subjek TST negatif. Pada peringkat genus, *Lactobacillus salivarius* adalah jauh lebih banyak dalam tekak pesakit TB berbanding dengan individu positif TST. *Streptococcus sobrinus* dan *Bulleidia moorei* juga jauh lebih banyak pada pesakit TB apabila dibandingkan dengan individu TST negatif. Kajian kepelbagaian alfa dan beta juga dijalankan dalam kajian ini. Tidak terdapat perbezaan yang ketara antara ketiga-tiga kumpulan dalam indeks alfa, menunjukkan kepelbagaian mikrobiota yang sama dari segi kekayaan dan kesamarataan antara spesies. Dalam analisis kepelbagaian beta, unifrac tak berwajaran kumpulan pesakit TB dan TST negatif adalah lebih tinggi secara statistik berbanding dengan TST positif, menunjukkan kehadiran spesies atau kluster yang lebih tinggi dalam tekak mereka. Unifrac berwajaran untuk TST positif adalah lebih tinggi secara statistik berbanding kumpulan pesakit TB dan TST negative, menunjukkan perkaitan organisma yang jauh dalam komuniti. Kajian ini konsisten dengan kajian terdahulu dan hasil kajian ini menunjukkan bahawa mikrobiota tekak mungkin memainkan peranan dalam kerentanan atau kerintangan terhadap jangkitan *Mycobacterium tuberculosis*. Kaitan mikrobiota ini mungkin perlu diambil kira untuk rawatan dan pengawalan penyakit TB yang lebih baik pada masa akan datang.

RESPIRATORY MICROBIOME AND ITS SUSCEPTIBILITY TO *Mycobacterium tuberculosis* INFECTION.

ABSTRACT

Respiratory throat acts as an ecological niche for various microorganisms. These microbial communities play important roles in the maintenance of human health. Although the interest in microbial composition of microbiota in respiratory tracts has recently been increased, the possible relationship between the composition and abundance of microbiota in the ecological niche of human throat with *Mycobacterium tuberculosis* infection are poorly understood. This study was aimed to investigate the composition of throat microbiota in pulmonary tuberculosis patients in comparison to healthy TST (Tuberculin Skin Test) positive and TST negative subjects. The microbiota of the throat was fully characterized by 16s rRNA Metagenomics sequencing. The study revealed that altogether, there were 7 phyla and 35 genera in the throat of enrolled subjects. The main phyla were *Firmicutes*, *Bacteriodetes*, *Proteobacteria* and *Actinobacteria.* The predominant communities of genera were *Streptococcus*, *Staphylococcus*, *Capnocytophaga*, *Rothia* and *Enterococcus*. The microbiota composition of TB patients and asymptomatic TST positive subjects were more heterogeneous as compared to the throat microbiota of healthy TST negative subjects. *Streptococcus* was found to be significantly abundant in throat of healthy compared to asymptomatic TST positive subjects and TB patients. In TB patients, *Bifidobacterium, Bulleidia, Butyrivibrio, Chryseobacterium* and *Pediococcus* were among the genera present abundantly in their throats compared to TST negative. At genus level, *Lactobacillus salivarius* was significantly higher in the throat of TB patients compared to TST positive individuals.

Streptococcus sobrinus and *Bulleidia moorei* were also significantly higher in TB patients when compared with those in the throats of TST negative individuals. Alpha diversity and beta diversity analysis were also performed in this study. No significant difference was observed between the three groups in alpha indices indicating the equally diverse microbiota in terms of richness and evenness of the species. In beta diversity analysis, the unweighted unifrac of TB patients and TST negative groups were statistically higher compared to TST positive group, indicating the higher presence of particular cluster or species in their throats. Weighted unifrac of TST positive was statistically higher than that of TB patients and TST negative groups which indicates more distantly related organisms inhabiting the communities. This investigation was consistent with earlier studies and our findings indicate that throat microbiota may play a role in the susceptibility or resistance to *Mycobacterium tuberculosis* infection. The relevance of these microbiota may need to be taken into consideration to improve methods of control of TB disease in the future.

CHAPTER 1

INTRODUCTION

1.0 Respiratory Microbiota

The populations of microbial species such as bacteria, viruses, fungus, and microbial eukaryotes that colonized within human body are known as human microbiota (Weinstock, 2012). The human microbiota is relatively diverse and colonizes in human body internally and externally such as skin, intestinal epithelial mucosal, lung, vagina and respiratory tracts (Clemente *et. al* 2012).The diversity of microbiota in a given habitat can be defined as the number and abundance distribution of different type of organisms which has a high impact on human health and diseases (Consortium, 2012).

Consequently, human host is believed to harbor a large number of bacterial cells which are more numerous than the human cells. For instance, the bacterial cell in human intestine is estimated to harbor up to 10^4 microbes cell by a ratio of ten to one, outnumbered the human cell (Belkaid & Hand, 2014). It plays an important role in regulation of the immune system, metabolisms of drugs, provides function such as digestion of nutrient and contributes to metabolic pathway and resistance to pathogenic infection (Dethlefsen *et al*., 2008; Li *et al*., 2008; Weinstock, 2012).

Human respiratory tract acts as a major portal of entry for various airborne microorganisms. Several studies have reported the correlation between the respiratory tract-associated microbiota with the development of airway diseases such as asthma,

chronic obstructive pulmonary diseases, pulmonary cystic fibrosis, and nosocomial pneumonia (Zhou *et al*., 2010; Cabrera-Rubio *et al*., 2012; Marri *et al*., 2013).

Microorganisms vary in their growth requirements such as physical conditions and nutrient resources and the growth of certain bacteria species are said to select for their preferred environmental niches. It is thus interesting to consider that the differential composition or microbial infections in particular niches are the consequences of these occurrence. Therefore, assessing microbiota in the throat could provide insight into the physical characteristics, possibility of colonization and the degree of disease progression caused by a particular pathogen (Rogers *et al*., 2015).

The need to describe the genomic of the communities of non-pathogenic microbes that inhabit the human body and to understand their significance has been identified as the microbiome research has become a growing interest (Weinstock, 2012). However, it is not yet known which of the many hundreds of species are fundamental in host health, and little is understood about the molecular host–microbiome interactions that influence host metabolic pathways (Li *et al*., 2008) .

1.2 Throat Microbiota Composition

Throat is a part of the respiratory tracts that acts as a niche for various microorganisms. This upper respiratory tract is colonized by pathogenic microorganisms and serve as a site for local respiratory tract infection and subsequently may cause invasive diseases (Charlson *et al*., 2010). Besides that, throat microbiota is known as a high stable microbial

community as it displayed the lowest phylotype richness as compared to that in stomach and lower intestine (Andersson *et al*., 2008).

Until currently, it has been generally proposed that the upper respiratory tract of a healthy individual is colonized by abundant of bacteria when compared to lower respiratory tract that is sterile during healthy state (Hilty *et al*., 2010; Dickson *et. al*., 2014). However, Bassis et al. (2015) proposed that the entire respiratory tract should be taken as a single ecosystem extending from the oral cavities and nasal to the alveoli which includes niches that characterized microbiome structure. Subsequent studies also demonstrated that lower respiratory tract is colonized by varies bacteria communities in both disease and healthy state (Mizgerd, 2006).

The actual characteristics, composition and abundance of the throat microbiota is important to define between healthy and unhealthy microbiota. Generally, hundreds of samples have been profiled using various technologies which led researchers to finally agreed that the human throat microbiome is mostly composed of five phyla; *Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, Actinobacteria.* They dominated most of the respiratory tracts in most studies that compared between control populations and those with diseases (Andersson *et al*., 2008; Botero *et al*., 2014).

Apparently, when analyzed for relative abundance of the bacteria in the human throat at phylum level, the most common phyla constantly observed has been *Firmicutes* (Gong *et al*., 2013). The name *Firmicutes* are taken from Latin words, 'Firmus' which means strong

and ‘Cutis’ means skin that referred to cell wall. Most of the organisms in this phylum are characterized by Gram-positive cell wall, many are endospore forming and mostly anaerobic bacteria. This phylum constitutes more than 250 genera, including *Lactobacillus* and *Streptococcus* (Dos Santos, 2013).

This information of microbiota composition has been determined predominantly by the application of Next Generation Sequencing (NGS) technologies that include shotgun metagenomic sequencing and 16S rRNA-based taxonomic profiling. Researchers have been using various sequencing technologies, molecular techniques and methodologies such as Sanger, 454 Pyrosequencing and Illumina platform (Kuczynski *et al.*, 2012).

1.3 Factors Shaping the Throat Microbiota Composition

The composition of the throat microbiota has been discussed by many researchers since the formation of the Human Microbiome Project (HMP) (Turnbaugh *et al*., 2007). They suggested that microbiota composition can be influenced by multiple factors including age, sex, host genotype, diet, lifestyle, diseases, ethnicity, socio-economic status, environment, and geographical region. These factors serve as the main influences in the variation of human microbiome as every individual was believed to originally exhibit a common or core microbiome (Conlon & Bird, 2014).

A core microbiome is constituted of the common members of two or more microbial communities that colonized within a habitat (Turnbaugh *et al*., 2007). Shade and Handelsman (2012) suggested that it is important to identify the core species or

operational taxonomic units (OTUs) to define 'healthy' community of microbial assemblages in a given habitat. The core microbiome theory has proposed that the variation of human microbiome in given habitat could be the consequences of the combination of few factors such as genotype, disease status (host pathobiology), diet (host lifestyle), immune systems (host physiology status) and host environment as shown in Figure 1.1 (Turnbaugh *et al*., 2007).

Subsequently, it also appears that drugs (especially antibiotics) and organic diseases can modulate microbiome composition and activities (Nicholson, 2006). Antibiotics treatment disturbs the microbiome by altering its composition and reducing its size which then can lead to infection and presence of antibiotic-resistance organisms such as *Clostridium difficile* that normally controlled by the microbiome to be overgrown in the host (Miller et al., 1957; Sekirov *et al*., 2008).

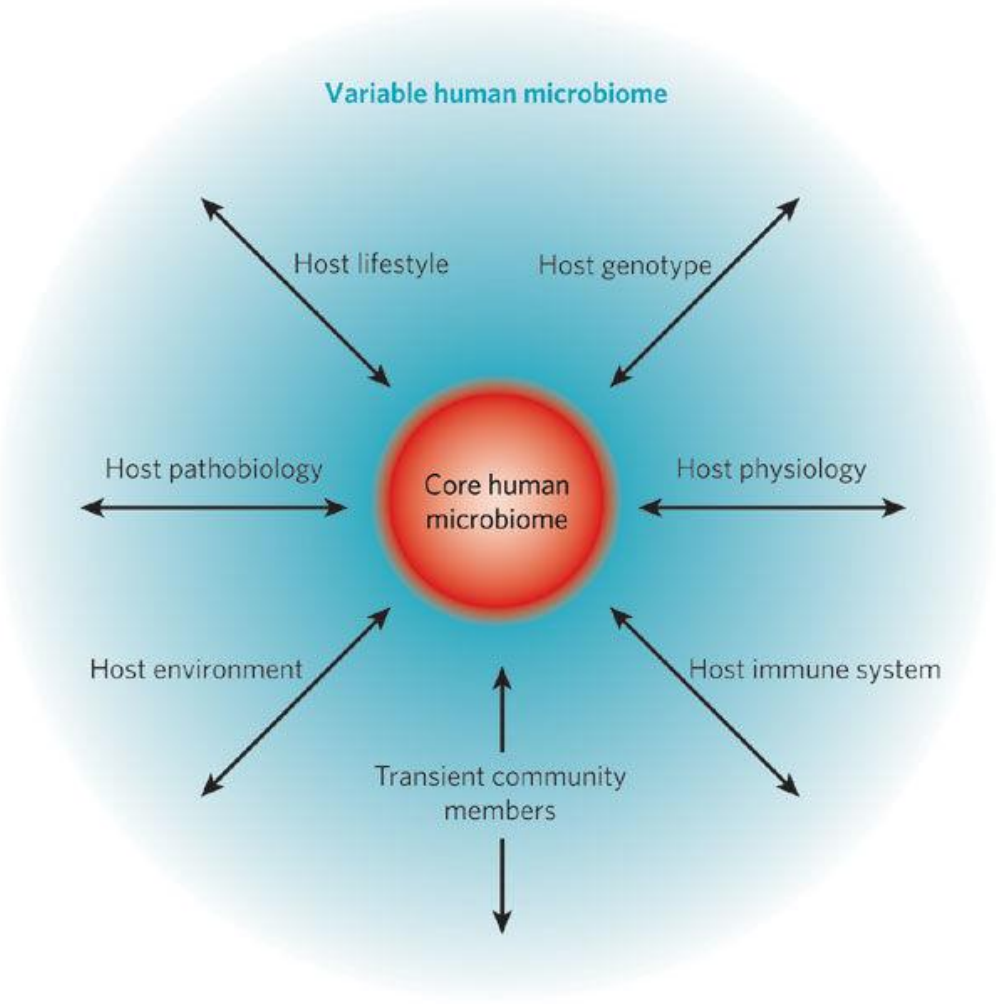

Figure 1.1: The concept of core microbiome (Adopted from Turnbaugh *et al*. (2007))

1.4 Applying Ecology to Explain Throat Microbiota

Many studies were aiming on looking for individual microbial species that are responsible for the development of diseases by comparing between healthy (control) and unhealthy (disease) groups. The studies have also identified some differences in diversity between these two groups by defining the richness and evenness of the species. Low-diversity microbiome has been consistently reported in those with disease (Arumugam *et al*., 2011).

To understand the relationship between human microbiome and disease, the use of ecological perspective has been discussed (Dos Santos, 2013). Rogers *et al*., (2013) stated that the correlation of human microbiota and disease can be understood best in such that certain disease may be associated with the presence of certain microbiota and the structure of microbial niche can influence its microbial compositions.

A model of the relationship between the microbial composition due to selective properties of airway environment and the ability of the infection to promote disease has been proposed (Rogers *et al*., 2013). As shown in Figure 1.2, Rogers *et al*., (2013) stated that there were two possible processes that involve in disease progression. It can be either the severity of the disease is contributed by the member of the local microbial communities, or the disease progression which influences the microbial shift in the host (Rogers *et al*., 2013). Microbiota can be the causative for disease progression in such that they exhibit pathogenic behavior themselves or by indirectly make use of other species' pathogenic traits. As for disease driven microbial shift, it can be the consequence of selective pressure

due to the use of antibiotics treatments or the increasing levels of host immune system (Daniels *et al*., 2013; Rogers *et al*., 2013).

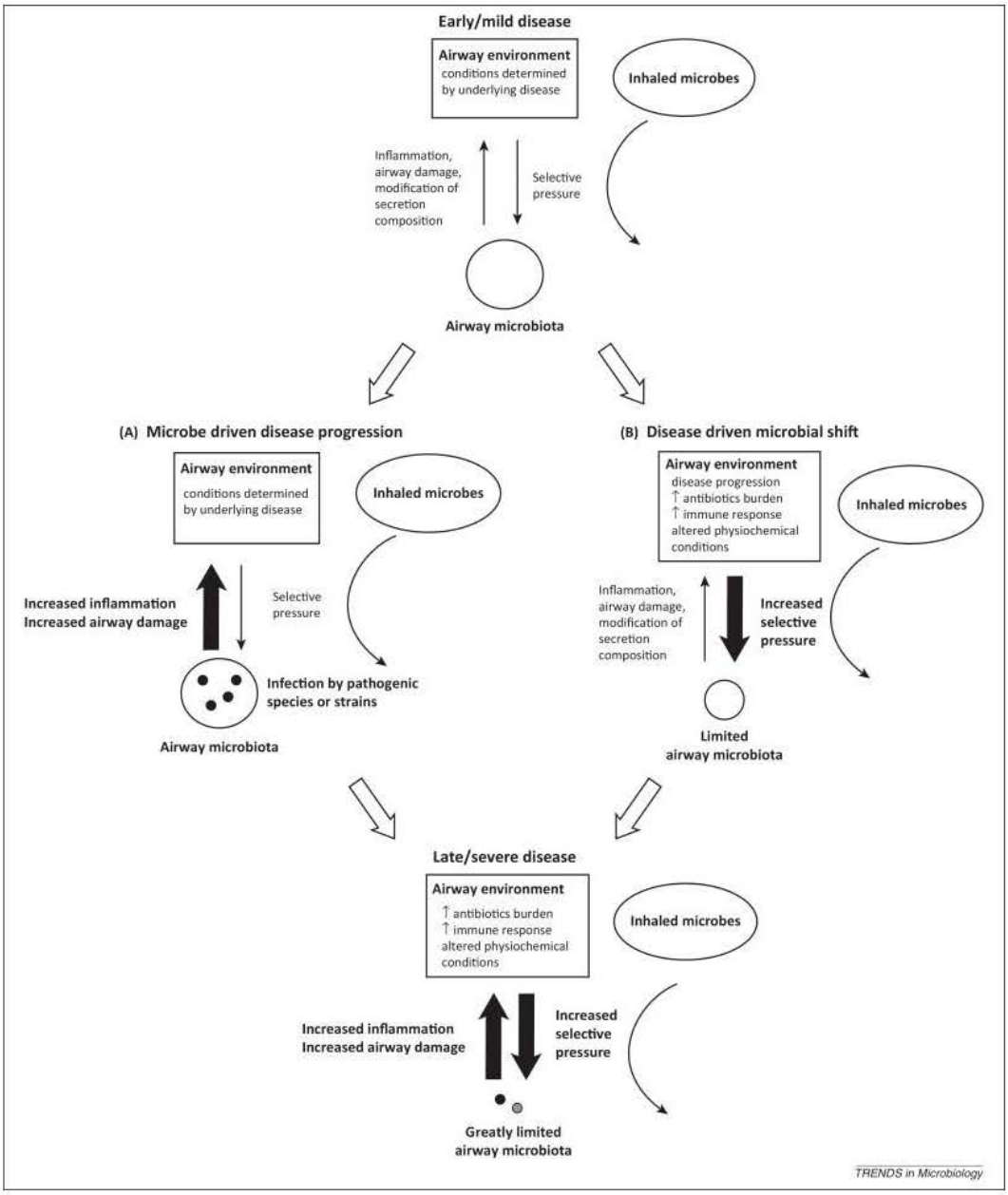

Figure 1.2: A model of the relationship between the selective properties of airway environment on microbiota and the ability to promote damage. Adopted from Rogers *et al*., (2013).

1.5 Mycobacterium tuberculosis infection

The causative agent that causing Tuberculosis (TB) in human being is known as *Mycobacterium tuberculosis*, a pathogenic mycobacterium that is able to infect animals and human hosts since domestication of animals about 10 000 years ago (Van Ingen et al., 2012). A single *M. tuberculosis* organism has a generation time of 18-24 hours at 37ºC with optimal source of oxygen and nutrients. It forms a white to light-yellow colonies on egg-based medium within 3-4 weeks of incubation. The acid-fast organism does not form spores and can become dormant within human body. Besides that, the aerobic to facultative anaerobe bacteria is also surrounded by a thick and impermeable cell wall which consist of polysaccharides, peptidoglycans, unusual glycolipids and lipids (Gengenbacher & Kaufmann, 2012).

TB is an ancient infectious disease that remains as one of the major health problems worldwide for many years. For the past 5 years, it has been ranking above Human Immunodeficiency Virus (HIV) for the disease from single infectious agent and has become the ninth leading cause of death worldwide. Pulmonary tuberculosis (PTB) is the most common TB occurred due to the infection of *M. tuberculosis* in the lung. However, it also can affect other parts of the body, causing extra pulmonary tuberculosis (EPTB). TB is well known as an airborne disease in which it can be spread easily by breathing in air-droplets with *M. tuberculosis* bacteria from coughing or sneezing of the people infected with active pulmonary (WHO, 2016).

1.6 History of Tuberculosis (TB)

Tuberculosis (TB) is an ancient disease that has infected humankind and was acknowledged in Egypt, China and India since a long time ago. It may have killed more people than any other infectious diseases did (Daniel, 2006). The oldest skeletal evidence of its infection in early human was discovered from the Neolithic era in eastern Mediterranean since 9000 years ago (Hershkovitz *et al.*, 2008). Gutierrez et al. (2005) suggested that TB is much older than other diseases including malaria, typhoid fever, or plague. Besides that, they also concluded that the causative agent for TB, *M. tuberculosis* has been present as early as 3 million years ago in East Africa and may have infected early hominids during that time (Gutierrez *et al*., 2005).

In 6th century, TB was considered as unpreventable and became the major cause of mortality in Europe. The epidemic of TB then spread slowly to other countries due to exploration and colonization. In 1880, the native people of North African begun to experience major TB outbreak. The people in Africa was infected with this disease and experience high mortality rate when they met Europeans, whereby the existence of TB disease in Asia has only begun towards the end of 19th century. India and China were the countries with the peaks TB incidence observed in Asia (Barberis *et al*., 2017).

A Prussian physician, Robert Koch has announced the discovery of *M. tuberculosis* as the causative agent of TB for the first time in 24th March 1882. This discovery was the most important step in controlling and eliminating this disease which aggressively killed one out of every seven people living in the Europe and United States during that time. In 1890,

Koch has developed a purified derivative of the bacteria, known as tuberculin for immunization purpose. However, it has been proven as inefficient for immunization. Later in 1908, Charles Mantouq has found the tuberculin as an effective tool for diagnosis of TB (Centers for Diseases Control and Prevention, CPC 2016; Barberis *et al.*, 2017).

1.7 Transmission of TB

M. tuberculosis which also known as tubercle bacilli is carried via airborne particles called droplet nuclei with diameter of 1-5 microns, expelled by people infected with pulmonary TB through sneezing, coughing, singing or shouting. These tiny particles, depending on the environment can remain suspended in the air for several hours. The infectiousness is depending on the number of tubercle bacilli that the infected TB persons expel to the air. Basically, the infectious dose is said to be between 1-200 bacilli, and each droplet nuclei contain of at least 1-400 bacilli. Therefore, it is nearly impossible for a healthy person to be uninfected when having contact with TB patient (Centers for Diseases Control and Prevention, 2016).

Transmission happens when a person inhaled the air droplets containing tubercle bacilli and infection occurred when the bacilli reach the alveoli of the lungs by passing through the nasal passage, upper respiratory tract and bronchi. The probability of *M. tuberculosis* infection is determined by the susceptibility of the exposed person, infectiousness, environmental factors that affect the tubercle bacilli concentration and the duration or the frequency of exposure (Centers for Diseases Control and Prevention, 2016).

Bovine TB that usually infected infants or children is caused by another species of mycobacteria called *Mycobacterium bovis*. It is transmitted to the host when the infants or children drink milk that has been contaminated with the bacteria. However, that type of TB infection is rarely happened now since pasteurization of milk has been performed nowadays (Centers for Disease Control and Prevention, 2011).

1.8 Pathogenesis of TB

TB infection occurs when the tubercle bacilli contain in the droplet's nuclei inhaled by a person reached the alveoli of the lungs. A competent immune of the host will recognize these tubercle bacilli as foreign, thus the tubercle will be attacked by host's macrophages. The alveolar macrophages will destroy or inhibit these tubercle bacilli by engulfing and dissembling them. However, some of these tubercle bacilli can multiply in the alveoli and will be released throughout the body once the macrophages burst. They will then enter the bloodstream and spread to more distant organs or tissues including the larynx, brain, lung, lymph node, bone, spine, or kidney in which TB diseases is more likely to develop (Heemskerk *et al*., 2015).

Regularly, the infected area will develop into granuloma, a barrier cell that is formed when the macrophages engulf and surround the tubercle bacilli. This allows the bacilli or the *M. tuberculosis* to be in latent or dormant state which can remain for years or even decades in the human body. However, if the immune system fails to keep the bacteria under control, the tubercle bacilli will begin to multiply rapidly and causing disease

development or primary TB disease. It normally takes about three to four weeks for the newly infected individuals to become contagious to transmit the tubercle bacilli to others.

The mechanisms of the infection are depending on the immune system or the defense mechanisms of the host. Most healthy people with activated macrophages is more likely to defeat the potential infection. Patients with non-resistant active infection will slowly switch to non-infectious state after two weeks of effective treatment (Centers for Diseases Control and Prevention, 2016).

1.9 Types of TB Infection

TB infections are divided into 2 types; Latent Tuberculosis infection (LTBI) and active TB disease.

1.9.1 Latent Tuberculosis Infection (LTBI)

Individuals with LTBI are those who do not fall sick with TB infection and cannot transmit the tubercle bacilli to others even though they have the *M. tuberculosis* bacteria in their body. LTBI is established in the body once the granuloma is formed right after the initiation of white blood cells by host immune response to engulf the tubercle bacilli. They can become dormant in the host for few years or even decades. However, the person with LTBI can fall sick with active TB disease when the tubercle bacilli become active and start multiplying in the body. This usually happened when the immune response is compromised because of co-infection with other diseases such as HIV or diabetes or might

due to aging or malnutrition. Besides, it is said that people with LTBI might have approximately 5 to 10 % risk of transforming into active TB disease (WHO, 2016).

1.9.2 Active TB disease

Active multiplication of tubercle bacilli in the body can cause active TB disease. Usually, this happen when the bacteria overcome the host's immune system and the compromised immune system fail to prevent them from growing and causing the person to fall sick with the disease. The person with active TB disease is symptomatic and with symptoms which include coughing that last for two weeks or longer, fever, night sweats, chills, loss of appetite, unintentional weight loss, fatigue, short of breath and chest pain. They are infectious to others and can spread the tubercle bacilli easily just by coughing, sneezing or singing (Centers for Diseases Control and Prevention, 2016).

A person with TB symptoms will be tested for TB by two test options, Tuberculin Skin Test (TST) or TB blood test which is also known as Interferon-gamma Release Assays (IGRAs). Positive result of TST or IGRAs's indicates that the person has been infected with TB bacteria, however it does not necessarily tell if the person have LTBI or has progressed to active TB disease. Therefore, further tests such as chest x-ray and examination on sputum sample are needed to determine if the person has been infected with active TB disease. The sputum sample will undergo few procedures such as Acid-Fast Bacillus (AFB) staining and culture method to confirm the *M. tuberculosis* bacteria. Treatments for TB patients will only begin once the TB culture is bacteriologically

confirmed as positive, where the culture normally takes about 2 to 8 weeks for confirmation of result (Heemskerk *et al*., 2015).

1.10 Epidemiology of TB

TB is a communicable and prevalent infectious disease that remains as one of the major health problems throughout the world. Despite the existence of numerous effective anti-tuberculosis drugs, it continues to be a major public health challenge. As reported by World Health Organization (WHO) in 2015, approximately 10.4 million incident of TB cases worldwide has been estimated as shown in Figure 1.3, where 1.8 million people died with TB including 0.4 million deaths resulting from co infection with HIV (WHO, 2016). On the other hand, within the year of 2000 to 2015, it was estimated that 49 million lives were saved as TB cases declining slowly each year due to effective diagnosis and treatment. However, the mortality rate of this disease is still unacceptably high and TB still remain as one of the top 10 causes of death worldwide in 2015 (WHO, 2016).

Malaysia has been categorized by WHO as an intermediate TB burden country. Even though Malaysia has succeeded in reducing the burden of other infectious disease including HIV and Malaria, TB remains as stubbornly persistent disease and continue to rise. In Malaysia, the notification rate of TB increased gradually from 61 cases per 100 000 populations to 79 cases per 100 000 population in 1990 and 2015, respectively (Kadir, 2017). This increasing TB incidence occurred due to several factors such as diabetic mellitus, HIV infection, increased in urban migration, influx of migrants from endemic

neighboring countries and also drug abuse that led to emergence of drug resistance (Ismail & Bulgiba, 2013).

Nantha (2014) has reported that there was a general focus for diabetes mellitus and HIV as the risk factors for TB infection in her review of TB research in Malaysia. A greater percentage (91.4%) of pulmonary TB patients were found among people with diabetes mellitus (Nissapatorn *et al*., 2005). This study indicated diabetes as a strong risk factor for developing pulmonary TB disease compared to non-diabetic individuals (Nissapatorn *et al*., 2005; Nantha 2014).

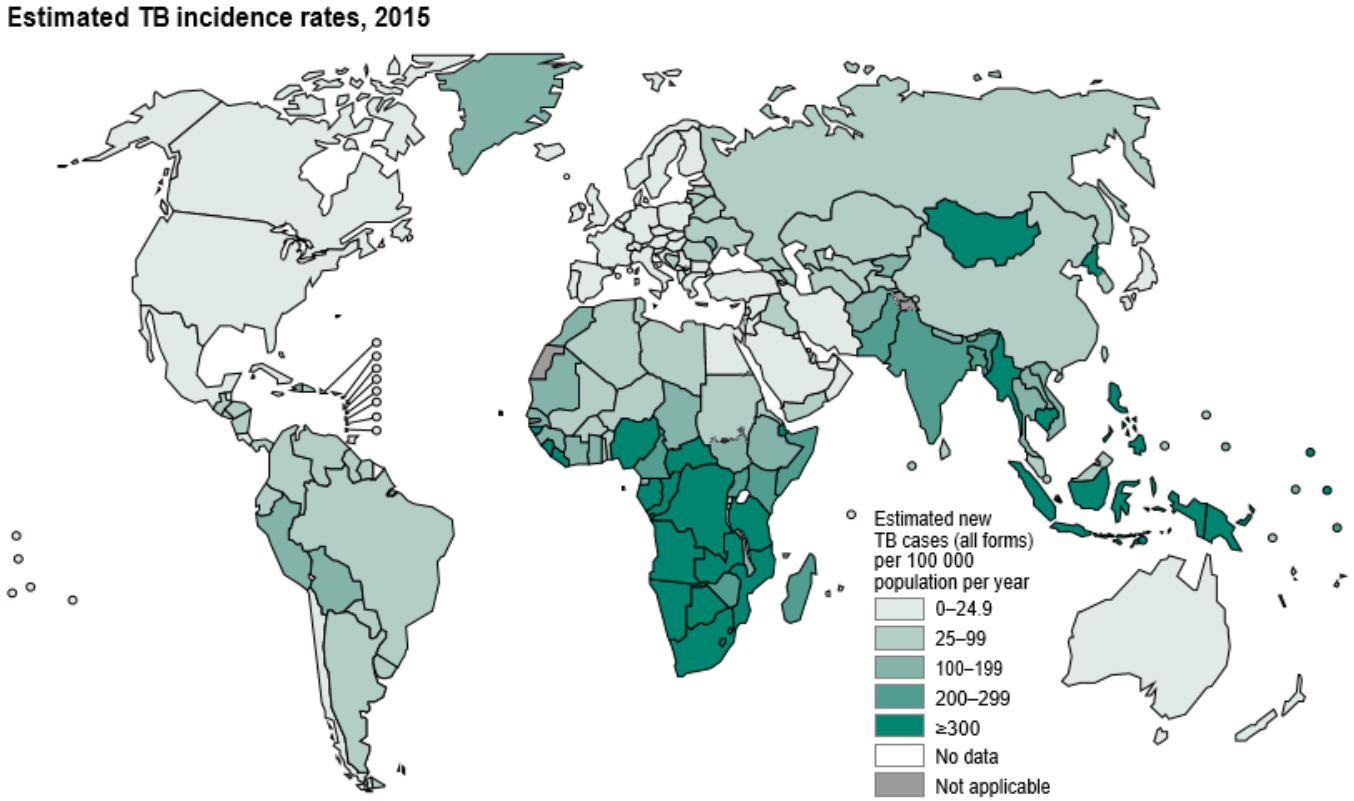

Figure 1.3: The 2015 estimated incidence rate (Adopted from (WHO, 2016)).

1.11 Multi Drug Resistant TB

Mycobacterium tuberculosis can develop resistance to antimicrobial drugs prescribed for the disease caused by the bacteria which then lead to development of multidrug-resistance TB (MDR-TB). MDR-TB is the TB that does not respond to at least 2 most powerful anti-TB drug, which are Isoniazid and Rifampicin. Resistant arises when the tubercle bacilli which capable of surviving in the presence of these drugs at optimum concentration that usually inhibits the growth or kill the bacteria. Several factors that led to the drug resistant includes improper use of antibiotics, inappropriate prescription of drugs or treatment, and failure to complete the lengthy treatment course which took at least 6 months (WHO, 2016).

The latest MDR TB surveillance according to WHO report suggested that there were 4.1% and 19% of new and previously treated TB cases, respectively to be associated with MDR-TB or Rifampicin Resistant. In 2016, about 600 000 new cases of MDR TB has been estimated by WHO with globally estimation of 240 000 deaths (WHO, 2017). In Malaysia, MDR-TB cases has not been an urgent problem and the prevalence of the cases keep declining from year to year with 124 cases in 2013 to 56 cases in 2017 (WHO, 2017).

1.12 Diagnosis of TB

A person should be suspected with TB once the person acquired some symptoms such as coughing longer than 3 weeks, chest pain, fever, loss of appetite and unexplained weight loss. Two types of tests that are used for diagnosis of TB, to determine if the person is infected with the bacteria are Tuberculin Skin Test (TST) and TB blood test (Cattamanchi *et al*., 2011).

TST or Mantoux skin test is the only method used for screening of *M. tuberculosis* infection among apparently healthy individual since early 1930s (Huebner *et al*., 1993). The current in use diagnostic method which is an intradermal technique was first described by Charles Mantoux in 1907. It was first developed by Koch, who discovered the tubercle bacillus in 1890. Nowadays, it is used to measure the prevalence of tuberculosis infection in population for epidemiological study besides of it's purposed in diagnosing TB in individual patient worldwide (Nayak & Acharjya, 2012).

The Mantoux skin test involves the intracutaneous injection of tuberculin into volar surface of the left forearm. Purified protein derivative (PPD), a derived protein from the culture of *M. tuberculosis* is the most widely used tuberculin. PPD-RT 23 which is the universally used PPD is injected into the skin with a standard dose of 5 tuberculin unit (0.1 ml) and should be read within 48 to 72 hours later. Upon administration of tuberculin into the skin, a delayed-type hypersensitivity (DTH) response will occur due to stimulation of T-lymphocytes that has been proliferated and sensitized prior to mycobacteria infection. The lymphocytes release lymphokines that will recruit other

inflammatory cells (basophils, monocytes, and neutrophils) and induce induration through local vasodilatation, fibrin deposition and edema at the area of injection (Reichman, 2008).

The interpretation of the Mantoux test is done by observing and measuring the diameter of induration size. Induration greater or equal to 15 mm is considered as positive result in healthy individuals with normal immune system and no exposure to Mtb infection. However, induration of 10 mm is considered as positive result in high risk population groups in infants, children less than 4 years old, health care workers and individuals with clinical conditions. Induration of 5 mm is positive in HIV-infected individuals, immune-suppressed individuals and recent contacts with TB patients. This test measure the degree of sensitivity to Tuberculin, but not the immune response to TB (Nayak & Acharjya, 2012).

In addition, the blood test which also known as interferon-gamma release assays (IGRAs) is another test used for the screening of TB. It measures interferon-gamma release after exposure of whole blood (QuantiFERON-TB Gold In-Tube® [QFT-GIT], Cellestis, Carnegie, Australia) or peripheral blood mononuclear cells (T-SPOT. TB® [TSPOT], Oxford immunotec, Abingdon, UK) to antigens encoded within the region of difference-1, a region of the MTB genome absents in all BCG strains and in most nontuberculous mycobacteria (Pai *et al.,* 2006). IGRAs use antigens that are more specific to *M. tuberculosis* than PPD that is used in TST and thus it has high specificity and may be

useful in low-endemic, high-income settings where cross-reactivity due to BCG might adversely impact the efficacy of TST (Cattamanchi *et al*., 2011).

1.13 Factors causing TB

TB has existed for millennia and remains as one of the major health problems throughout the world. Generally, there are several factors contributing to the increasing burden of TB cases especially in endemic countries. The established risk factors for acquiring TB include close-contact with infectious TB patients, co-infection with other diseases and malnutrition. Besides that, other factors including poverty, infants, smoking, alcohol and drug addiction, health care worker and immigration have also played an important role in the increasing susceptibility to the disease, whether at individual or community level (Narasimhan *et al*., 2013).

Co-infection with other diseases such as Human Immunodeficiency Virus (HIV) or diabetes mellitus can alter the immune system causing the person to be immunocompromised and thus increased the risk of disease progression into active TB disease. In 2015, a total number of 500 564 HIV patients has been reported by WHO to be infected with TB disease, despite the decreased percentage of TB patients with positive HIV reported globally since 2008 (WHO, 2016). Besides, consistent evidence for association of diabetes mellitus with TB disease have also been reported (Jeon & Murray, 2008).

Malnutrition is said to be one of the significant risk factors causing active TB in primary infection or LTBI due to the impaired immune functions and cell-mediated immunity which are the important host-defense mechanism against TB infection in malnourished person. This is usually caused by the lack of certain nutrients and proteins needed in the body (Cegielski & McMurray, 2004). TB infection will also lead to further malnutrition and metabolic dysfunction due to unintentional loss of appetite and weight lost (Grobler *et al*., 2016).

Health care workers including those who work in hospitals, homeless shelters, nursing or residential homes and also prisons are at high risk exposure to TB infection. This is due to the nature of their work that require close-contact with patients with active TB disease. According to WHO, the number of TB cases per 100 000 population of health care worker in 2015 was more than double the notification rate of adult population in 16 countries (WHO, 2016).

In addition, tobacco smoking, drug and alcohol addiction also increase the risk of acquiring active TB disease threefold in human population. Therefore, prevention effort must be taken to reduce the prevalence of both smoking and harmful use of alcohol and drugs in order to reduce the TB incidence in the population (WHO, 2016).

1.14 Treatment and Prevention of TB

Transmission of TB disease from one person to another can be prevented by several methods. According to WHO, the spread of TB in the high incidence communities can be most effectively stopped by curing the disease (WHO, 2016). TB is treatable and completely curable with proper treatment such as short-course chemotherapy. The most effective ways of eliminating TB from a population is by treating the disease at the source. Directly Observed Treatment Short Course (DOTS) has been proposed as the most efficient and economical strategy in controlling TB. Few strategies in DOTS include withstand political and financial commitment, diagnosis by quality ensured sputum-smear microscopy, standardized short-course anti-TB treatment given under direct and supportive observation, regular supply of high-quality anti-TB drugs, and systematic recording and reporting of TB cases (Heemskerk *et al*., 2015).

There are several anti-TB drugs used for killing the tubercle bacilli in the host. The first-line anti-TB drugs consists of Isoniazid (INH), Rifampicin (RIF), Streptomycin (STR), Ethambutol (EMB), and Pyrazinamide (PZA). These drugs are the standard short-course treatment recommended for new TB cases and have a good efficacy as well as known side effects as they have been extensively used for the treatment. Prior to Drug Susceptibility Test (DST) results, patients diagnosed with smear positive TB culture should be treated with combination of these first-line drugs. Once the result is revealed, treatment will only involve drugs that are susceptible against the patient's isolates (Heemskerk D, 2015).

However, the combination of the drugs must be done carefully in treating TB patients to prevent the emergence of drug resistant TB. Usually, the duration of treatments for active TB patients is between 6 to 9 months, depending on individual clinical condition such as co-infection with HIV or the emergence of drug resistant TB strain. Second-line anti-TB drugs include Ethionamide, Kanamycin, Fluoroquinolones, Cycloserine and Para-aminosalicyclic acid. These drugs are reserved for special cases like the emergence of MDR-TB, thus are less preferred for the new TB cases (Ai *et al.,* 2016).

The prevention of TB transmission includes vaccination, and pasteurization of milk. Vaccination is one of the effective methods in preventing TB. Bacillus Calmette -Guerin (BCG) which was first developed by Albert and Camille Guerin in the 1920s is the only available and current vaccine given for TB worldwide (Kaufmann *et al.,* 2017). It has become part of the national immunization program and is given to the infants and young children especially in endemic countries to provide them with excellent protection against TB disease. However, BCG does not always protect adult from being infected with the disease and it is suggested that the protection of BCG against TB in high incidence countries is lower which might be due to the greater exposure of the mycobacteria in that particular area (Mangtani *et al.*, 2013).

Besides, TB prevention can also be done by preventing the development of LTBI into infectious active TB disease and by infection control which prevent the spread of TB in settings such as hospitals and prisons (WHO, 2016). Some high-risk factors that contribute to the increase of LTBI activation includes HIV-infected patients, silicosis