EPIDEMIOLOGY AND PREVALENCE ANALYSIS OF NTM USING 16S rRNA IN JOHOR

MISBAHA BINTI JILANI

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DEDICATION

This thesis is dedicated to my family and many friends. A special feeling of gratitude to my father Jilani Mahmod, my loving husband Mohd Fairuz bin Zakariah and my kids, whose words of encouragement and push for tenacity ring in my ears.

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ABSTRACT

The geographical distribution of nontuberculous mycobacteria (NTM) in Malaysia is unknown, however the number of cases reported is markedly increasing. Furthermore, the current Standard Operation Procedure for diagnose is time consuming and not up to the species level. This may lead to prolonged treatment, mistreatment with current Tuberculosis regime and perhaps may create multidrug resistant strains. Therefore, a new method is urgently needed to replace current identification analysis. The goal of this study was to determine the prevalence of clinical NTM cases in Johor, Malaysia using the current procedure and Runyon classification, investigate potential sources of NTM from a specific geographic area using both 16S rRNA sequencing and Runyon classification and construct a phylogenetic tree of Malaysia NTM based on species. The data was analyzed from mycobacterial cultures of sputum specimens collected from Johor Public Health Laboratory from 2011 to 2014. Water samples were collected from sputum collection center in selected Health Clinics in Johor and subjected to NTM identification analysis to investigate the potential origins of NTM. The phylogenetic tree was constructed using molecular sequencing by using MEGA-X software. In this present study, analysis of the NTM cases in Johor from year 2011 to 2014 increased with age and gender-related where male and individual of 61 years old and above are more likely to be infected respectively. Furthermore, Johor Bahru was more likely to record higher cases. There were differences in the findings from the same sources and sample collected geographically when grouped by Runyon and 16S rRNA. The Runyon classification method produced a false positive result on pathogenicity on rapidly growing NTM when compared to 16S rRNA. The precision of Runyon classification method on pathogenicity of slow grower was less. In conclusion the prevalence of NTM data for Johor were compiled and 16S rRNA sequencing was more precise in diagnosing the NTM up to the species level.

ABSTRAK

Geografi penyebaran kes jangkitan Mikobakterium Bukan Tuberkulosis (MBT) di Malaysia tidak diketahui, namun bilangannya dilaporkan meningkat. Tambahan pula, Prosedur Operasi Standard yang sedia ada untuk diagnosa memakan masa dan tidak sehingga ke tahap spesies. Ini mengakibatkan kesilapan rawatan, masa yang panjang atau kesilapan rawatan dengan rejim Tuberkulosis sedia ada dan berisiko menghasilkan strain yang rintang terhadap pelbagai ubat. Oleh itu, kaedah baru sangat diperlukan untuk menggantikan analisis identifikasi sedia ada. Kajian ini direkabentuk untuk menentukan prevalensi kes MBT klinikal di Negeri Johor berdasarkan prosedur semasa iaitu klasifikasi Runyon, menyiasat sumber MBT yang berpotensi dari kawasan tertentu menggunakan penjujukan 16S rRNA dan klasifikasi Runyon dan bagi pembinaan pokok filogenetik. Data telah dianalisis dari kultur mikobakteria spesimen kahak dari Makmal Kesihatan Awam Johor dari 2011 hingga 2014. Untuk menyiasat sumber asal MBT, sampel air telah dikumpulkan dari pusat pengumpulan kahak di Klinik Kesihatan terpilih di Johor. Pokok filogenetik telah dibina menggunakan penjujukan molekul spesies MBT menggunakan perisian MEGA-X. Kajian ini mendapati kes MBT di Johor dari tahun 2011 hingga 2014 berkait rapat dengan jantina dan meningkat mengikut umur yang mana lelaki dan individu berumur 61 tahun ke atas lebih cenderung dijangkiti. Tambahan pula, Johor Bahru lebih cenderung mencatat kes yang lebih tinggi. Terdapat perbezaan dapatan dari sumber yang sama dan sampel yang dikumpulkan secara geografi apabila dikelaskan dengan Runyon dan 16S rRNA. Kaedah klasifikasi Runyon menghasilkan keputusan positif palsu ke atas MBT patogenik bagi kumpulan yang tumbuh secara cepat berbanding kaedah 16S rRNA. Kaedah klasifikasi Runyon terhadap MBT patogenik bagi kumpulan yang tumbuh secara lambat adalah kurang tepat. Kesimpulannya, data prevalensi MBT untuk Negeri Johor telah dikompilasi dan penjujukan 16S rRNA adalah lebih tepat dalam mendiagnosis MBT sehingga ke tahap spesies.

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LIST OF ABBREVIATIONS

AFB	-	Acid Fast Bacilli
C	-	Celcius
CDC	-	Communicable Disease Control
DNA	-	Deoxyribonucleic Acid
dATP	-	Deoxyadenosine triphosphate
dCTP	-	Deoxycytidine triphosphate
dTTP	-	Deoxythymidine triphosphate
EDTA	-	Ethylenediamine tetracyclic acid
g	-	Gravitational acceleration
LAM	-	Lipoarabinomannan
LJ	-	Lowensteins Jensen
MAI	-	*Mycobacterium avium intracellulare*
MgCI2	-	Magnesium chloride
mL	-	Mililitre
mM	-	Milimolar
MTBC	-	*Mycobacterium tuberculosis complex*
NaOH	-	Natrium hydroxide
Ng	-	Nanogram
NTM	-	Non tuberculous mycobacteria
PCR	-	Polymerase chain reaction
Pmol	-	Picomole
RGM	-	Rapidly growing mycobacteria
SGM	-	Slowly growing mycobacteria
Rpm	-	Revolution per minute
Taq	-	*Thermus aquaticus*
TB	-	Tuberculosis
TE	-	Tris-EDTA
TEM	-	Transmission electron microscopy
U	-	Unit
µm	-	Micromilimetre

µl	-	Microlitre
vol/vol	-	Volume per volume
wt/vol	-	Weight per volume
ZN	-	Ziehl Neelson

LIST OF SYMBOLS

^{o}C	-	Degree celcius
%	-	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background

In recent years, tuberculosis remains among the high burden communicable diseases in developing world. Mycobacteria are the causative organisms for diseases such as tuberculosis (TB) and pulmonary non tuberculous mycobacterial disease, to name the most important ones. In 2015, globally, almost 10 million people developed TB, and almost half a million patients suffered from its multidrug-resistant form. The U.S. National Institutes of Health reported an increase from 20 to 47 cases/100,000 persons (or 8.2% per year) of pulmonary non tuberculous mycobacterial disease among adults aged 65 years or older throughout the United States, with 181,037 national annual cases estimated in 2014. Pulmonary non tuberculous mycobacterial disease is an emerging public health challenge (Betty A. Forbes, 2018). Pulmonary NTM disease epidemiology in Malaysia particularly in Johor is not as well described as for pulmonary tuberculosis. With the increasing incidence of case reports and series from diverse countries and regions of the world, the distribution of NTM species isolated from pulmonary samples appear to vary significantly by region. However, very little is known about the contribution(s) of NTM to tuberculosis-like disease, and significant knowledge gaps exist regarding their geographical distribution, clinical and molecular epidemiology in low and middle-income countries where there is a high burden of disease caused by MTBC.

Ministry of Health Malaysia has been publishing a guideline on managing tuberculosis among pulmonary TB (PTB) patients aiming to assist clinicians and other healthcare providers in making evidence-based decisions about appropriate management and treatment of tuberculosis (TB) specifically in screening and

diagnosis, treatment, and further follow-up, prevention and patients‘ referral. According to this Clinical Practice Guidelines, our current diagnostic methods were only focusing on tuberculosis caused by *Mycobacteria tuberculosis* (MTB) complex.

All members belonging to *M. tuberculosis* complex and certain species in non tuberculous mycobacteria cause tuberculosis infections. While *M. tuberculosis* is the cause of most cases for human tuberculosis particularly in developed countries, isolating non tuberculous is of clinical significance since these organisms may cause similar symptom and disease as *M. tuberculous* complex (Radha Gopalaswamy, Sivakumar Shanmugam, Rajesh Mondal and Selvakumar Subbian, 2020).

Conventional method Runyon classification being applies for the identification of non tuberculous mycobacteria causing pulmonary disease (Igor Porvaznik, Ivan Solovicˇ, and Juraj Mokry, 2016). Speciation on NTM were made on this conventional method using phenotypic classification approach developed seventy years back by Ernest H Runyon for non tuberculous mycobacteria (NTM), primarily as a starting point in trying to understand their clinical relevance. These techniques while remaining an important baseline modality of investigations currently, they lack the desired sensitivity or are time consuming (Christine Y. Turenne, 2019).

Based on Runyon classification, most NTM correspond to one of two main groups, slowly growing mycobacteria (SGM) and rapidly growing mycobacteria (RGM). This phenotype is defined on the basis of growth (from a diluted inoculum) occurring either before or after 7 days. The SGM are further categorized as photochromogenic (Group I: pigmentation upon exposure to light), scoto-chromogenic (Group II: always pigmented) or non-photochromogenic (Group III: no, weak or late-pigmenting, irrespective of light); RGM belong to Group IV. Though tremendously useful, the Runyon classification was not intended as absolute, but served as a starting point to help assess clinical significance of NTM since Groups I and III (e.g. *M. kansasii* and *M. avium* complex) contained the more pathogenic groups, while Groups II (e.g. *M. gordonae*) and IV (RGM) were deemed *usually* not clinically relevant (Runyon, 1959).

In view of the above setbacks, there is an urgent need for a readily available, simple, reliable and cost-effective rapid diagnostic test for TB which can be applied to a clinically diverse patient population for PTB cause by both MTB or NTM. The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. 16S rRNA gene sequence analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains, can be routinely used for identification of mycobacteria, and can lead to the recognition of novel pathogens and noncultures bacteria (Franco-Duarte *et. al*, 2019)

1.2 Problem Statement

Data on the contribution of NTM to pulmonary disease in Johor is uncertain. Malaysia was poorly represented in the 'global' NTM collection that led to a description of the global epidemiology of NTM in colonisation and disease. The scarcity of data on the contribution of NTM to pulmonary mycobacterial infections in Malaysia is most likely due to the following reasons:

(1) Poor awareness among treating physicians and microbiologists on the clinical relevance of NTM.
(2) Lack of laboratory infrastructure for culture and identification of mycobacterial isolates.
(3) The high burden of TB and HIV in this region of the world attracts the bulk of the attention of the health care system. Therefore, the fiscal inputs required for diagnosis and treatment NTM disease are lacking.
(4) Misdiagnosis - since sputum smear microscopy is the mainstay of TB diagnosis in most high burden TB settings, the risk of acid-fast NTM in pulmonary samples, especially in previously treated patients with lung damage, poses a diagnostic challenge.

Given the paucity of data on NTM and the little understanding of the epidemiology of pulmonary NTM in disease and colonisation in the Johor, I conducted a comprehensive, NTM disease in Johor and investigated the epidemiology of NTM found in pulmonary samples in Johor.

1.3 Research Objectives

The objectives of the research are:

i. To describe the prevalence of NTM species among pulmonary NTM patients in Johor.
ii. Since proven scientifically from other study, to collect environmental samples from sputum collecting center in selected Health Clinic in Johor.
iii. By using data from the environmental analysis, to compare the evolutionary of NTM.

1.4 Scope of Research

The scopes of research are:

i. Development of NTM prevalence data in Johor from Johor Public Health Laboratory.
ii. Collect samples from Health Clinic with the highest recovery of NTM from the prevalence data.
iii. Run analysis of sample using 16r RNA.

1.5 Hypothesis

Non-tuberculous mycobacteria are prevalent in sputum samples of TB suspects in Johor and can cause pulmonary TB-like disease. Patients may get NTM from environmental sources as NTM infections are acquired from environmental (water, soil) reservoirs.

1.6 Significant of Study

Pulmonary NTM disease is a neglected and emerging public health disease in Johor that requires enhanced surveillance to fully quantify the diversity of and contribution of NTM to pulmonary disease and to reduce the risk of misdiagnosis of tuberculosis.

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