DETECTION, SUSCEPTIBILITY TESTING AND WHOLE GENOME SEQUENCING OF MYCOBACTERIUM TUBERCULOSIS ISOLATED FROM CEREBROSPINAL FLUID OF PATIENTS WITH SUSPECTED TUBERCULOUS MENINGITIS

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JUDUL : Detection, Susceptibility Testing and Whole Genome Sequencing of Mycobacterium Tuberculosis Isolated from Cerebrospinal Fluid of Patients with Suspected **Tuberculous Meningitis**

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4 September 2016



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ABSTRACT

Tuberculous meningitis (TBM) represents roughly 1% of all cases of tuberculosis (TB) and it is the most severe form. Early diagnosis is difficult as current laboratory tests which are the smear and culture lack sensitivity to detect the few tubercle bacilli in cerebrospinal fluid (CSF). Diagnosis based on molecular methods have been shown to have a higher sensitivity compared to smear and culture. Treatment of TBM requires several months of treatment with anti-TB drugs that cause many side effects. Alternative drug from medicinal plants can be an important source of new antimicrobial agents with little toxicity. Mycobacterium tuberculosis (M. tuberculosis) is the etiologic agent of TB and is shown to have genetic variation between each strain that may contribute to the varied outcome in *M. tuberculosis* infection. In this study, we evaluated the diagnostic value of smear, culture, multiplex PCR and GeneXpert MTB/RIF to detect *M. tuberculosis* in CSF samples from patients with suspected TBM registered in Queen Elizabeth Hospital (QEH). We also evaluated the anti-mycobacterial activity of Aloe vera (A. vera) and Allium sativum (A. sativum) against the *M. tuberculosis* isolate. Whole genome sequencing (WGS) was done to the *M. tuberculosis* isolate in order to understand the genome feature and genetic variation. CSF samples were centrifuged and the deposits were used for conventional and modified Ziehl-Neelsen (ZN) stain, microscopic observation drug susceptibility (MODS) assay and multiplex PCR targeting MPB64 and IS6110 genes. GeneXpert MTB/RIF was performed according to the manufacturer's instruction. Drug-susceptibility testing (DST) was done to determine the susceptibility of M. tuberculosis clinical isolate to isoniazid (INH) and rifampicin (RIF) by using the MODS assay. The M. tuberculosis isolate was further tested with water extract of A. vera and A. sativum at concentration ranged between 0.05 mg/ml - 3.0 mg/ml using the MODS assay. WGS was done using the Pacbio platform and annotation and comparative analysis were done using GeneMarks, RAST and BLAST. Of the 55 CSF samples collected, 12 (21.8%) samples were positive by multiplex PCR, 3 (5.4%) samples were positive by GeneXpert MTB/RIF and only 1 (1.8%) sample was positive by smear and culture. The positive culture was susceptible to both INH and RIF at concentration of 0.4 μ g/ml and 1 μ g/ml respectively. Anti-mycobacterial activity of A. vera and A. sativum extracts showed minimum inhibitory concentration (MIC) of 1.5 mg/ml and 0.15 mg/ml respectively. Comparative genome analysis showed that the genome of *M. tuberculosis* isolate has variation compared to the reference genomes. In this study, multiplex PCR showed higher sensitivity for detecting *M. tuberculosis* in unconfirmed TBM patients and has the potential to be used as a diagnostic method. A. vera and A. sativum can be a new source of anti-mycobacterial agents for the development of drugs in treatment of TB. The genome feature of the *M. tuberculosis* isolate has variation with the reference genomes.

ABSTRAK

PENGESANAN, UJIAN KECENDERUNGAN DAN PENJUJUKAN GENOM ISOLAT MYCOBACTERIUM TUBERCULOSIS DARI SEREBROSPINA PESAKIT YANG DISYAKI MENGHIDAP MENINGITIS TUBERKULOSIS

Meningitis tuberkulosis (meningitis TB) mewakili kira-kira 1% daripada semua kes batuk kering (TB) dan merupakan yang paling teruk. Diagnosis awal adalah sukar kerana ujian makmal iaitu smear dan kultur kurang sensitif untuk mengesan bakteria yang sangat sedikit dalam bendalir serebrospina (CSF). Diagnosis berdasarkan kaedah molekular dilaporkan mempunyai sensitiviti yang lebih tinggi berbanding dengan smear dan kultur. Rawatan meningitis TB mengambil masa beberapa bulan kering menagunakan ubat batuk yang mempunyai banvak kesan sampingan.Ubat-ubatan alternatif dari tumbuh-tumbuhan boleh menjadi sumber anti-mikrob yang baru dengan tahap keracunan yang sedikit. Mycobacterium tuberculosis (M. tuberculosis) adalah agen yang menyebabkan penyakit TB dan setiap strain mempunyai variasi genetik yang mungkin menyumbang kepada hasil atau level penyakit yang berbeza-beza dalam jangkitan M. tuberculosis. Dalam kajian ini, kami menaksir nilai diagnostik smear, kultur, multiplek PCR dan GeneXpert MTB/RIF untuk mengesan M, tuberculosis dalam sampel CSF daripada pesakit yang disyaki menghidap meningitis TB yang telah didaftarkan di Hospital Queen Elizabeth (HQE), Kami juga menilai aktiviti anti-tuberkulosis lidah buaya dan bawang putih terhadap isolat M. tuberculosis. Penjujukan genom telah dilakukan untuk memahami gambaran genom and variasi genetik dalam isolat M. tuberculosis. Sampel CSF telah disentrifugasi dan deposit digunakan untuk perwarnaan konvensional dan modifikasi Ziehl-Neelsen (ZN), Microscopic observation drug susceptibility (MODS) dan multipleks PCR mensasarkan MPB64 dan IS6110 gen. GeneXpert MTB/RIF dilakukan mengikut arahan manual. Drug susceptibility testing (DST) telah dilakukan untuk menentukan kecenderungan isolat M. tuberculosis terhadap isoniazid (INH) dan rifampicin (RIF). Seterusnya, M. tuberculosis diuji dengan ekstrak air lidah buaya dan bawang putih pada kepekatan di antara 0.05 mg/mL - 3.0 mg/mL menggunakan MODS assay. Penjujukan genom untuk isolate M. tuberculosis dilakukan dengan menggunakan platform PacBio dan anotasi dan perbandingan genom dilakukan menggunakan GeneMarks, RAST dan BLAST. Daripada 55 sampel CSF, 12 (21.8%) sampel positif untuk multipleks PCR, 3 (5.4%) sampel positif untuk GeneXpert MTB/RIF dan hanya 1 (1.8%) sampel positif untuk smear dan kultur. Isolat M. tuberculosis cenderung kepada ubat INH dan RIF pada kepekatan 0.4 µg/mL dan 1 µg/mL. Aktiviti anti-tuberkulosis exstrak lidah buaya dan bawang putih menunjukkan kepekatan perencatan minimum (MIC) sebanyak 1.5 mg/mL dan 0.15 mg/mL. Perbandingan genom menunjukkan variasi genetik dalam isolat M. tuberculosis dan rujukan genom. Dalam kajian ini, multipleks PCR mempunyai sensitiviti yang lebih tinggi untuk mengesan M. tuberculosis pada pesakit yang disyaki menghidap meningitis TB dan mempunyai potensi untuk digunakan sebagai kaedah diagnostik. Lidah buaya dan bawang putih boleh menjadi sumber baru agen anti-mikrob untuk penghasilan ubat-ubatan dalam rawatan TB. Isolat M. tuberculosis mempunyai yariasi genom dengan rujukan genom.

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LIST OF ABBREVIATION

CSF		Cerebrospinal Fluid
DW	.	Distilled Water
INH	-	Isoniazid
MDR	-	Multi-Drug Resistant
NC	-	Negative Control
PCR	-	Polymerase Chain Reaction
QEH	·	Queen Elizabeth Hospital
RIF		Rifampicin
тв	1 43	Tuberculosis
твм	 • • • • 	Tuberculous Meningitis
who		World Health Organization
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LIST OF SYMBOLS

bp	-	Base pair
cfu	1. C. F. F. S.	Colony forming units
Cm	-	Centimeter
9		Gravitational force
9		gram
mg	÷	Milligram
ml	8 S. 19	Milliliter
pmole		Picomole
hà	STT Mer	Microgram
μì		microliter
UV	J-A-V	Ultraviolet
v	A B A S UN	Voltage MALAYSIA SABAH
°C		Degree Celsius
%		Percentage

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CHAPTER 1

INTRODUCTION

1.1 Background

Tuberculosis (TB) is a public health problem worldwide that had infected and killed millions of people throughout the ages. Despite being preventable and treatable, TB continues to scourge human lives. Between 2000 and 2013, it had been estimated that 37 million lives were saved and it's also been declining slowly from year to year (WHO, 2014). Nevertheless, from the annual report of global TB cases worldwide by World Health Organization (WHO) published since 1997, millions of new TB cases had been reported every year and death tolls still reached approximately a million. In Malaysia, TB remains a major health threat with over 24 000 cases reported annually since 2013 (Sman, 2016). An increase of 14% and 1.7% of TB cases and deaths were reported from 2011 to 2015 (Hamid *et al.*, 2016). According to states, Sabah recorded the highest cases of TB with 4,464 cases followed by Selangor (4,429) and Sarawak (2,575) in 2015 (Sman, 2016).

Controlling TB is the key factor for reducing mortality and morbidity cases. A strengthened control measure involves few factors such as rapid diagnosis, effective treatment as well as the improved understanding of the nature of the etiologic agent involved. However, the current diagnosis and treatment of TB are somewhat effective. The knowledge about the nature of the pathogen involved is also limited. Hence, many studies are still needed to increase and deepen the knowledge of TB in these areas.

1.1.1 Challenge in Diagnosis of Tuberculous Meningitis

Pulmonary TB (PTB), which is the infection of Mycobacterium tuberculosis (M. *tuberculosis*) in lungs is the most common form of TB. However, TB also affects other parts of body such as brain, liver, kidney and spine. Among all types of TB, tuberculous meningitis (TBM) is the severest form as it causes death or severe neurological defects in more than half of those affected although it represents only about 1% of all TB cases (Rock et al., 2008; Takahashi et al., 2012). Early diagnosis is important as delay in starting the treatment regimen will lead to death or severe neurological defects. However, diagnosis is always difficult as early stage of TBM shows non-specific symptoms and the load of bacteria in cerebrospinal fluid (CSF) is very low. Smear and culture have been the gold standard for detection of M. tuberculosis. However, they lack sensitivity to detect the *M. tuberculosis* mainly in CSF which only have few bacilli. Nucleic acid amplification technique (NAAT) such as polymerase chain reaction (PCR) is a rapid and sensitive method for detection of bacteria in clinical samples. Previous studies reported that PCR was more sensitive and rapid to detect *M. tuberculosis* in CSF compared to conventional smear and culture. Despite of the better performance, a wide variability of sensitivity was obtained from different studies mainly due to different protocol assays as well as the type and number of target genes being used (Takahashi et al., 2012). Multiplex PCR that targets few genes was reported to have a higher sensitivity compared to monoplex PCR to detect *M. tuberculosis* in different biological samples as it had been reported that some *M. tuberculosis* strains lack certain genes (Kusum *et al.*, 2011; Singh et al., 2013). GeneXpert MTB/RIF system is the newest automated PCR-based technology that can detect the presence of *M. tuberculosis* and determines the susceptibility of the detected *M. tuberculosis* to rifampicin (RIF) simultaneously. Compared to other diagnosis methods either by conventional or PCR-based assays, GeneXpert MTB/RIF is advantageous in term of rapidity as it can give result within 2 hours. Furthermore, the closed-cartridge system enables it to be used outside the laboratory environment and studies had reported that GeneXpert MTB/RIF carries smaller biohazard risk than the smear microscopy (Lawn and Nicol, 2011). However, only few studies had evaluated the diagnostic value of both multiplex PCR and GeneXpert MTB/RIF in diagnosis of TBM and hence, more studies are still needed to evaluate the utility of both PCR-based assays for diagnosing extra-pulmonary TB.

1.1.2 Need of New Drug for the Treatment of Tuberculosis

The current drugs for TB treatment consisted of few drugs that had been developed more than five decades ago. These drugs are proven highly effective for treatment of TB, however, adverse side effects due to reactions resulted from the drug interactions and long term administration were frequently reported. Furthermore, the emergence of drug-resistant *M. tuberculosis* strains mainly due to the failure in treatment management has led to the use of more potent yet harmful drugs. Both adverse side effects and emergence of drug-resistant *M. tuberculosis* strains have resulted for the need to search and develop new drugs that are effective in killing the *M. tuberculosis*, can be administered for short period of time as well as have little to no side effects. Medicinal plants such as Aloe vera (A. vera) and Allium sativum (A. sativum) can meet this need as both had been proven to have medicinal properties. Both A. vera and A. sativum have been used as drugs for treatment of many ailments since long time ago and also been frequently reported to have anti-bacterial, anti-viral and anti-fungal activities. Both plants have also been reported to have activity against M. tuberculosis although studies on anti-mycobacterial effect are limited. Due to the various levels of active constituents of A. vera and A. sativum planted at different geographical areas, there is a need to evaluate the anti-mycobacterial activity of both plants to further validate their efficacy as potential candidates for development of anti-mycobacterial drugs.

1.1.3 Genetic Variability of *Mycobacterium tuberculosis* Isolates

Evidences showed that exposure of an individual to *M. tuberculosis* resulted in varied outcomes of disease presentations. Prior to the development of molecular biology tools, the level of disease severity in individuals exposed to that pathogen was exclusively determined by the host genetics and environmental factors as *M. tuberculosis* was considered clonal or monomorphic (Achtman, 2012). Advancement in molecular biology tools and the availability of whole genome sequencing (WGS) technologies that enables the scanning of the entire *M. tuberculosis* genome, revealed that mycobacterial genetics were actually diverse among the *M. tuberculosis* strains (Gagneux, 2013). Due to the small genetic variation within the hosts and communities (Walker *et al.*, 2013; Casali *et al.*, 2014), the heterogeneous outcomes

of *M. tuberculosis* infection are considered due to the vast variation in mycobacterial genetics (Warner *et al.*, 2014). Studies on genetic variation of *M. tuberculosis* isolates have been done in many geographical locations, however, it is still limited in Malaysia, especially in the state of Sabah which has the highest cases of TB in the country. It is important to know the genomic features of the isolated *M. tuberculosis* in order to gain an idea or information on the characteristics or the strains of *M. tuberculosis* that are dominant in this region.

1.2 Nature of Research

In this study, we have collected CSF samples from patients with suspected TBM that were registered in Queen Elizabeth Hospital (QEH) and confirmed the status of the patients either have TBM or not by using smear, culture and PCR-based assays. Next, we did a preliminary study whether *A. vera* and *A. sativum* have anti-mycobacterial activity against the isolated *M. tuberculosis*. Lastly, we did the whole genome sequencing for the isolated *M. tuberculosis* in order to understand the genome features of the strain in detail and did a general comparison of the genome with the reference genomes. We expect that PCR-based assays would have higher sensitivity to detect the few *M. tuberculosis* in CSF samples. *A. vera* and *A. sativum* collected locally are expected to have activity against the *M. tuberculosis* isolate. The isolated *M. tuberculosis* isolate.

1.3 Objectives:

- 1. To evaluate the diagnostic value of conventional and modified Ziehl-Neelsen (ZN) stains, Microscopic Observation Drug Susceptibility (MODS) assay, multiplex PCR and GeneXpert MTB/RIF to detect *M. tuberculosis* in CSF samples from patients with suspected TBM registered in QEH.
- 2. To evaluate the *in-vitro* anti-mycobacterial activity of *A. vera* and *A. sativum* collected from Ranau and local market against *M. tuberculosis* isolate.
- 3. To understand the genome feature of *M. tuberculosis* isolated from Sabah and compare it with the reference genomes.

