Med & Health Jun 2022; 17(1): 198-210 https://doi.org/10.17576/MH.2022.1701.15

ORIGINAL ARTICLE

Molecular Characterization of *Mycobacterium* species Isolates from Patients with Pulmonary Tuberculosis in Sabah, Malaysia

NORFAZIRAH J[1], ZAINAL ARIFIN M[1], ZAW L[1], MYO THURA Z[1], NOR AMALINA E[1], KAMRUDDIN A[1,2], JAEYRES J[1], CHERONIE SHELY S[1], CHIN KL[1]*

[1]Faculty of Medicine and Health Science, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia
[2]Borneo Medical and Health Research Centre, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia.

ABSTRAK

Tuberkulosis (TB) adalah salah satu penyakit yang paling berbahaya di seluruh dunia, disebabkan oleh kuman-kuman Mycobacterium tuberculosis complex (MTBC), kebiasaannya oleh Mycobacterium tuberculosis (Mtb) dan Mycobacterium bovis. Di Malaysia, Sabah merupakan salah satu negeri yang mendapat perhatian kesihatan awam dengan kes TB tertinggi. Ciri-ciri klinikal TB dan penyakit paru-paru non-tuberculous mycobacteria (NTM) adalah serupa dan mikrobakteria kelihatan sama ketika diagnosis piawai dengan mikroskopi menggunakan apusan kahak, menyebabkan kesukaran dalam diagnosis TB. Pengenalpastian spesies Mycobacterium adalah penting untuk pengurusan rawatan penyakit mikrobakteria yang berkesan serta strategi untuk kawalannya. Justeru, kajian ini bertujuan untuk mengenalpasti spesies Mycobacterium daripada pesakit TB yang disyaki di Sabah menggunakan kaedah molekular. Sampel kahak (n=595) telah disaring dengan GeneXpert MTB/RIF (Xpert), dan sampel TB positif (n=67) telah diproses dan dikultur dalam BACTEC MGIT. Empat puluh lima isolat berjaya dihidupkan dalam MGIT dan pencirian isolat mikrobakteria menggunakan PCR dan/atau penjujukan dengan gen rpoB, RD9, hsp65, dan 16S rRNA mengesahkan kehadiran Mtb dalam 41 sampel, dan empat sampel non-mycobacteria, iaitu Microbacterium laevaniformans, Streptomyces sp., Streptomyces misionensis, dan Gordonia sp. Isolat non-mycobacteria ini menunjukkan keputusan negatif apabila diuji terus dengan Xpert. Kesimpulannya, Mtb adalah spesies utama MTBC yang didapati di Sabah. Kehadiran isolat non-mycobacteria dalam kajian ini adalah disebabkan oleh pencemaran bakteria dalam MGIT, bukan kerana kereaktifan silang bakteria

Address for correspondence and reprint requests: Kai Ling Chin. Faculty of Medicine and Health Science, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia. Tel: +6014 6839539 Email: chinkl@ums.edu.my

dalam Xpert, membayangkan kepekaan dan kekhususan Xpert yang tinggi untuk diagnosis TB.

Kata kunci: diagnostik molekular, pembezaan spesies, tuberkulosis

ABSTRACT

Tuberculosis (TB) is one of the deadliest diseases worldwide, caused by members of *Mycobacterium tuberculosis* complex (MTBC), commonly by *Mycobacterium tuberculosis* (Mtb) and *Mycobacterium bovis.* In Malaysia, Sabah is one of the states of public health concern with the highest TB cases. Clinical presentations of TB and non-tuberculous mycobacteria (NTM) lung disease are similar, and mycobacteria appear to be identical under standard diagnosis with sputum smear microscopy, causing difficulty to diagnose TB. Identification of *Mycobacterium* species is essential for effective management of mycobacterial diseases treatment and their control strategy. Thus, this study aimed to identify the *Mycobacterium* species from suspected TB patients in Sabah using molecular methods. Sputum samples (n=595) were screened with GeneXpert MTB/RIF (Xpert), and positive TB samples (n=67) were processed and cultured in BACTEC MGIT. Forty-five isolates were successfully recovered in MGIT and characterisation of the mycobacterial isolates using PCR and/or sequencing with *rpoB,* RD9, *hsp65,* and 16S rRNA genes confirmed the presence of Mtb in 41 samples, and four non-mycobacteria, i.e. *Microbacterium laevaniformans*, *Streptomyces sp.*, *Streptomyces misionensis* and *Gordonia sp.* These non-mycobacteria isolates showed negative results when tested directly with Xpert. In conclusion, Mtb is the predominant species of MTBC circulating in Sabah. The presence of non-mycobacteria in this study was due to bacterial contamination in MGIT, not bacterial cross-reactivity in Xpert, implying the high sensitivity and specificity of Xpert for diagnosis of TB.

Keywords: molecular diagnostic, species differentiation, tuberculosis

INTRODUCTION

Tuberculosis (TB) is one of the top ten leading causes of mortality in the world and it is one of the most prevalent infections, ranked above HIV/AIDS (WHO 2020). In 2019, 10 million new TB cases, mostly in men (56%), followed by women (32%), and children (12%), with 1.4 million deaths reported (WHO 2020). Two-thirds of the new cases were reported in these eight countries, with the highest incidence in India, followed by Indonesia, China, the Philippines, Pakistan, Nigeria, Bangladesh, and South Africa (WHO 2020). Main concern in the treatment of TB is the presence of drug-resistant strains. Multidrug-resistant TB (MDR-TB) is defined as TB cases resistant to

at least rifampicin (RIF) and isoniazid (INH). These two drugs are bactericidal drugs and RIF is the most effective first-line drug. A total of 206,030 people with multidrug- and rifampicin-resistant tuberculosis (MDR/RR-TB) were detected in 2019, which was 10% higher compared to 2018 (WHO 2020).

In Malaysia, 25,173 TB cases were reported across the country with an estimated incidence rate of 92 cases per 100,000 people in 2018 (WHO 2019). A greater rate of TB has been documented in the state of Sabah, which is in Malaysia's Borneo region. Even though Sabah only accounts for 10% of the total population of the country, it has been reported that TB cases account for 20-30% of the total population in Malaysia (Rundi et al. 2011). The reasons for the high prevalence of TB in Sabah may be due to the following conditions i.e. there was high prevalence rate of TB in neighboring countries like the Philippines and Indonesia, and frequent movement of people across the international borders which might have promoted the transmission (Goroh et al. 2020). Other observations showed that TB patients in Sabah have advanced disease at diagnosis, long diagnostic delays, and high smoking rates among males (William et al. 2015).

Pulmonary TB (PTB) is caused by members of *Mycobacterium tuberculosis* complex (MTBC). Although *Mycobacterium tuberculosis* (Mtb) is the main cause of human TB, it has been estimated that 1.4% of the total 10 million new TB cases in 2018 were due to *Mycobacterium bovis* (*M. bovis)* (WHO 2019). Opportunistic pulmonary infections by non-tuberculous mycobacteria (NTM) present the same clinical presentation as PTB, causing difficulty in the diagnosis of the diseases (Gopalaswamy et al. 2020). Also, in clinical specimens, diagnosis of TB using conventional sputum smear acid-fast staining shows positivity for both MTBC and NTM, and could not differentiate the organisms (Gopalaswamy et al. 2020). Although culture method is the "gold standard" for diagnosis of TB, it is a time-consuming process as Mtb has a slow growth rate, requiring 3 to 6 weeks for its isolation on Löwenstein–Jensen medium, plus an additional 1 to 2 weeks for its speciation (Park et al. 1984).

Hence, for specific diagnosis of *Mycobacterium* infections, there is a need for molecular-based differentiation and speciation. GeneXpert MTB/RIF (Xpert) is a useful tool for simultaneous identification of MTBC and rifampicin resistance by amplifying an MTBC-specific sequence of the *rpoB* gene and subsequently probing it with five molecular beacons (Probes A to E) for detection of mutations inside the 81-bp rifampin-determining region (RRDR) (Lawn & Nicol 2011). Although Xpert is a rapid diagnostic tool for PTB, with higher sensitivity and specificity than sputum smear (Agrawal et al. 2016), a study showed that Xpert recognised a few NTM species as MTBC at a high bacterial load of 10^6, implying its non-specificity (Pang et al. 2017). Also, Xpert is unable to detect TB patients with NTM co-infections, resulting in

incorrect treatments (Sarro et al. 2018). Thus, in this study, GeneXpert-positive isolates recovered from Mycobacteria Growth Indicator Tube (MGIT) were characterised using polymerase chain reaction (PCR) with duplex PCR assay of RNA polymerase beta subunit (*rpoB*) gene to detect MTBC and NTM (Kim et al. 2004); region of difference 9 (RD9) gene to detect Mtb and *M. bovis* (Warren et al. 2006); and heat shock protein 65 (*hsp65*) gene to detect *Mycobacterium sp.* (Ringuet et al. 1999); and 16S ribosomal RNA (16S rRNA) gene sequencing for identification of bacteria (Engelbrektson et al. 2010). Differential diagnosis of MTBC and NTM is important for the appropriate treatment of individual patients and epidemiological purposes.

MATERIALS AND METHODS

Sample Collection

This study was conducted in collaboration with the TB mobile clinic of the Sabah State Health Department (JKNS). Sputum samples were collected from several districts in Sabah including Kota Kinabalu, Papar, Lahad Datu, Tawau, and Semporna following the guidelines approved by Universiti Malaysia Sabah (UMS) (Ethical clearance reference code: JKEtika 2/16(6)]. The criteria of patients for sputum collection were abnormal chest X-ray, cough, cough with blood, appetite loss, weight loss, night sweat, among others. Each sample (2 mL) was collected in a 60 mL screw-capped specimen container without preservatives and transported under cold chain in a cooler box with coolant packs at temperature below 4°C to the Tuberculosis Laboratory, Faculty of Medicine and Health Sciences, UMS. The sample was stored at 4°C and screened with Xpert within 24 hours.

Diagnosis of TB using GeneXpert MTB/RIF

Sputum samples were processed and analysed using GeneXpert MTB/RIF (Xpert) (Cepheid, Sunnyvale, CA, USA) according to the manufacturer's protocol. Briefly, sputum sample (0.5 mL) was mixed with sample reagent (1.5 mL) and incubated at room temperature for 15 minutes, with vigorous shaking at 10 and 15 minutes. The processed sample (2 mL) was transferred to an Xpert cartridge and the cartridge was loaded into the Xpert machine for analysis. Result interpretation was done with software version 4.3 provided by the company. Four semiquantitative categories of mycobacterial load based on cycle threshold (Ct) value were reported, i.e. very low for Ct >28, low for Ct 22-28, medium for Ct 16-22, or high for Ct <16. Leftover sputum samples were kept at -20°C until ready to be cultured at the Biosafety Laboratory Level-3 (BSL3), Biotechnology Research Institute, UMS.

Culturing of TB Positive Sputum Samples in MGIT

According to the manufacturer's instructions, sputum sample (0.5 mL) was decontaminated with BBL® MycoPrep™ Specimen Digestion/

Table 1: Primers and thermal cycling conditions used in this study

Target	Primers	Thermal cycling conditions	PCR products	References
rpoB	MTBC Tbc1: 5′-CGTACGGTCGGCGAGCTGATC CAA-3′ TbcR5: 5′-CCACCAGTCGGCGCTTGTGGG TCAA-3 NTM M5: 5′-GGAGCGGATGACCACCCA GGACGTC-3′, RM3: 5′-CAGCGGGTTGTTCTGGTC CATGAAC-3′	Activation (1 cycle): 95°C for 5 minutes Amplification (30 cycles): 95°C for 30 seconds, 68°C for 30 seconds, and 72°C for 1 minute Final extension (1 cycle): 72°C for 10 minutes	MTBC – 235 bp NTM – 136 bp	Kim et al. 2004
RD9	F: 5′-CAAGTTGCCGTTTCGAGCC-3′ Int: 5′- CAATGTTTGTTGCGCTGC-3′ R: 5′-GCTACCCTCGACCAAGTGTT-3′	Activation (1 cycle): 95°C for 5 minutes Amplification (35 cycles): 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute Final extension (1 cycle): 72°C for 10 minutes	Mtb – 235 bp M. bovis – 108 bp	Warren et al. 2006
hsp65	Tb11: 5′-ACCAACGATGGTGTGTCCAT-3′ Tb12: 5′-CTTGTCGAACCGCATACCCT-3′	Activation (1 cycle): 95°C for 5 minutes Amplification (35 cycles): 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute Final extension (1 cycle): 72°C for 10 minutes	Mycobacterium sp. – 441 bp	Ringuet et al. 1999
16S rRNA	27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ 1392R: 5′-GGTTACCTTGTTACGCATT-3′	Activation (1 cycle): 94°C for 3 minutes Amplification (30 cycles): 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1.5 minutes Final extension (1 cycle): 72°C for 10 minutes	~1500 bp	Engelbrektson et al. 2010

Decontamination Kits (Becton Dickinson, Franklin Lakes, NJ USA) [N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) solution] and was added in a 7H9 MGIT liquid culture with oleic acid, albumin, dextrose, and catalase (OADC) supplement; and polymyxin-B, amphotericin-B, nalidixic acid, trimethoprim, and azlocillin (PANTA) antibiotic mixture (Becton Dickinson, USA). The tube was incubated at 37°C in a BD BACTEC™ MGIT™ 320 instrument (Becton Dickinson, Franklin Lakes, NJ USA) and bacterial growth was checked with an indicator present in the instrument. Time to detection (TTD) of the bacteria in MGIT was recorded. Tube showing

no growth after 42 days was removed from the system.

DNA Extraction from Bacteria Culture

Bacterial cultures from positive tubes were collected for DNA extraction. The DNA was extracted using Masterpure™ Complete DNA and RNA Purification kit (Epicentre Biotechnologies, Madison, WL, USA). The sample (7 mL) was transferred into a falcon tube (15 mL) and boiled at 95°C for 20 minutes to deactivate the bacteria. The sample was centrifuged 3,000 x g for 15 minutes and the supernatant was discarded, leaving 25 µL of liquid. The liquid was transferred into a 2 mL microcentrifuge tube. Tissue and Cell Lysis Solution (600 µL) and Proteinase K (40 µL) were added into the microcentrifuge tube and were incubated at 65°C for 3 hours. RNase A (2 µL) was added and incubated at 37°C for 30 minutes. Then, MPC Protein Precipitation Reagent (300 µL) was added and vortexed vigorously for 10 seconds. The tube was centrifuged 10,000 x g at 4°C for 10 minutes and the supernatant was transferred into a new tube. Isopropanol (500 µL) was added into the tube and centrifuged 10,000 x g at 4°C for 20 minutes. The supernatant was discarded and washed twice with 70% ethanol (500 µL). Finally, after the final wash of ethanol, the supernatant was discarded, and the DNA was resuspended in nuclease-free water (35 µL).

Polymerase Chain Reaction (PCR) and Sequencing

The extracted DNA was used as a template for PCR with *rpoB* and RD9 genes. Each PCR reaction contained 50 ng of DNA template, 1X PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 1µM of each primer (10 pmol/L), 2.5 U of Taq DNA polymerase (Apical Scientific Sdn. Bhd, Malaysia), and was made up to 25 µl with nuclease-free water. The primers used in this study were listed in Table 1. The PCR mixture was amplified according to its optimal thermal cycling conditions (Table 1). PCR products were electrophoretically fractionated in 3.0% agarose incorporated with Fluorosafe stain

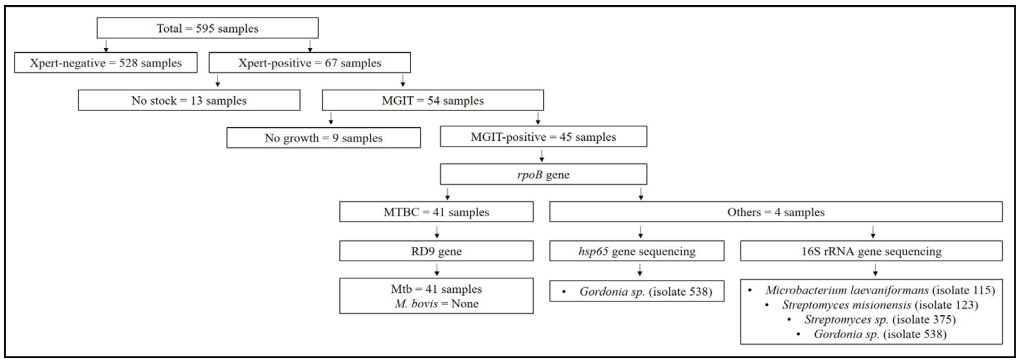

Figure 1: Overview of the study. Sputum samples screening with Xpert, culturing of bacteria in MGIT, and characterization of bacteria with PCR and sequencing

Table 2: Summary of GeneXpert MTB/RIF and BACTEC MGIT results

Patient ID	Xpert MTB/RIF			Time of storage (days)/ No stock	Time to detection MGIT (days)/ No growth
	Average Ct	Semi quantitative	RIF resistance (RR)		
Xpert- and MGIT-positive samples (n=45)	Mean = 25.89 ± 4.67 Min = 14.72 Max = 34.12	High – 1 Medium – 6 Low – 22 Very low – 16	Detected - 1 Not detected - 44	Mean = 60.51 ± 51.28 Min = 3 Max = 166	Mean = 11.58 ± 5.04 Min = 3 Max = 26
Xpert-positive and MGIT-negative samples (n=9)	Mean = 28.73 ± 5.28 Min = 15.94 Max = 34.60	High – 1 Low – 1 Very low – 7	Not detected - 9	Mean = 76.67 ± 48.20 Min = 23 Max = 144	No growth
Xpert-positive samples (n=13)	Mean = 25.29 ± 6.56 Min = 15.82 Max = 32.24	High – 1 Medium – 4 Low – 2 Very low - 6	Detected - 1 Not detected - 12	No stock	

(Apical Scientific Sdn. Bhd, Selangor, Malaysia) in 1xTAE buffer at 100 V for 30 minutes. Expected results were listed in Table 1.

Selected samples were amplified with high fidelity enzyme, PrimeSTAR GXL DNA polymerase (Japan), replacing the Taq DNA polymerase targeting *hsp65* and 16S rRNA genes (Table 1). The PCR products were purified with FavorPrep GEL/ PCR Purification Kit (Favorgen Biotech Corp, Taiwan) and sequenced by Integrated DNA Technologies (IDT) (processed by Apical Scientific Sdn. Bhd, Malaysia). The forward and reverse sequences were aligned using the BioEdit 7.0.9 program. Isolates were identified by comparing unknown sequences to reference databases by National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) search.

Statistical Analysis

The correlation between mean Ct value of Xpert, sputum storage period, and TTD in MGIT were analysed using IBM® SPSS® Statistic 27 (IBM Corp., Armonk, NY, USA) with Pearson Correlation test. A p-value ≤0.05 was statistically significant. A Pearson correlation, coefficient, r, indicates no association with a value of 0, positive association with a value greater than 0, and a negative association with a value smaller than 0. One-way ANOVA and Tukey post-hoc tests were conducted to determine the significant differences between groups, with a p-value ≤0.05 was statistically significant.

Table 3: hsp65 gene sequencing

No.	hsp65 sequencing	Length (bp)	Query cover	Percentage identity (%)
538	*Gordonia sp.*	441	99%	93.86%

RESULTS

A total of 595 sputum samples were collected from suspected TB patients and were screened with Xpert. Sixty-seven new TB cases (11.26%) and two RIF-resistant cases (2.99%) were detected using Xpert. Of the 67 Xpert-positive samples, 54 samples were cultured in MGIT, while no available sputum for the remaining 13 samples. These samples were kept for a range of 3 to 166 days due to limited access to BSL-3. The bacterial recovery rate in MGIT was 83.3%, with growth observed in 45 samples, while nine samples with no growth after 42 days were discarded (Figure 1, Table 2).

For the non-growing samples (Xpert-positive, MGIT-negative), their mean Ct value was higher than the growing samples (Xpert-positive, MGIT-positive), 28.73 vs 25.89, and their storage periods were longer 76.7 vs 60.5 days (Table 2), suggesting that bacillary counts and sputum storage periods might have affected the bacterial growth in MGIT. Pearson correlation coefficient was computed to assess the relationship between the Ct value, storage period, and TTD (days). There was a statistically significant positive correlation between the storage period and TTD, $r = 0.325$, $p = 0.029$, but no significant correlation between the Ct value and TTD, $r = 0.050$, $p = 0.744$.

Bacterial isolates recovered from MGIT were characterised using PCR with specific target genes. PCR results of the *rpoB* gene showed that 41 isolates were MTBC and four isolates (i.e. 115, 123, 375, and 538) were NTM. PCR with RD9 gene confirmed the presence of Mtb in the 41 isolates and no *M. bovis* was detected, while the other four isolates did not amplify with RD9 primers (Figure 1). The four NTM isolates were further characterised with the *hsp65* gene for mycobacteria identification. However, only isolate 538 was successfully amplified and the sequencing result showed the presence of *Gordonia sp.* (Table 3, Figure 1). Using universal 16S rRNA

Table 4: 16S rRNA gene sequencing and GeneXpert MTB/RIF results for non-mycobacteria samples

No.	16S rRNA sequencing	Length (bp)	Query cover	Percentage identity (%)	GeneXpert MTB/RIF
115	*Microbacterium laevaniformans*	1468	99%	99.73	Negative
123	*Streptomyces misionensis*	1473	99%	72.87	Negative
375	*Streptomyces sp.*	1469	98%	96.88	Negative
538	*Gordonia sp.*	1459	99%	96.12	Negative

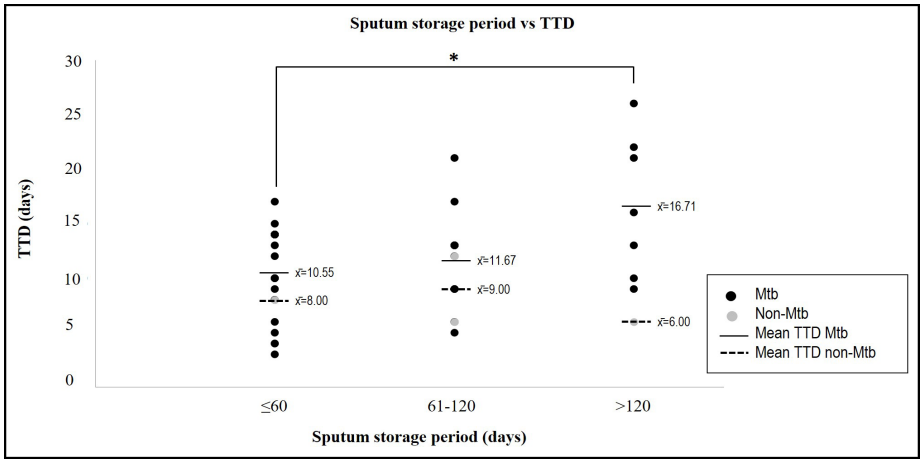

Figure 2: Scatter plot of TTD of Mtb and non-mycobacteria at different storage periods (*p-value ≤0.05)

bacterial primers, these four isolates were successfully amplified, and sequencing results of the gene showed the presence of *Microbacterium laevaniformans* (isolate 115), *Streptomyces misionensis* (isolate 123), *Streptomyces sp.* (isolate 375), and *Gordonia sp.* (isolate 538) (Table 4, Figure 1). These non-mycobacteria isolates were tested negative in Xpert (Table 4).

A scatter plot of sputum storage period vs TTD in MGIT showed that non-mycobacteria generally had a lower mean TTD compared to Mtb, regardless of the duration of the sputum storage (Figure 2). Overall mean TTD for Mtb was 11.9 days, while for non-mycobacteria was 8.0 days.

Among the Mtb strains, analysis with one-way ANOVA showed that there was a statistically significant difference of TTD at different storage periods (short, medium, and long periods) [$F(2,38) = 4.621$, $p = 0.016$]. Tukey post-hoc tests revealed TTD was significantly shorter in the sample with short storage period (≤60 days) (10.55±4.16 days, n = 22) as compared to long storage period (>120 days) (16.71±6.47 days, n = 7) ($p = 0.012$), but no significant difference between short (≤60 days) and medium storage period (61-120 days) (11.67±4.48, n = 12) ($p = 0.784$) (Figure 2).

DISCUSSION

The TB mobile clinic is one of the initiatives by the Sabah state government for TB control by screening randomly in the community of high-risk areas, particularly those that live in crowded place and are afraid or have difficulty to seek treatment. Since TB is spread via aerosol, detection of TB cases in high-risk areas should be of concern to stop the transmission of TB and provide necessary treatment to the infected people. With this strategy, we are able to identify new Mtb and RIF-resistant strains circulating in Sabah.

Xpert is a molecular method recommended by World Health

Organization (WHO) to diagnose TB due to its high sensitivity and specificity (WHO 2013). Although Xpert has significantly shortened turnaround times of diagnosis to 2 hours, the culture method still plays an important role as the "gold standard" for diagnosis of TB, particularly for negative smear/molecular specimen and for determination of drug susceptibility. However, several factors could affect the culture positivity including sputum storage time from collection, condition of the storage, sputum quantity, and bacterial loads in sputum (Tessema et al. 2011). In this study, all our samples were stored at -20°C prior to culture and 500 µL sputum samples were used as starting materials, but no growth was detected in the nine samples even after two attempts regardless of the bacilli count of high, low, and very low bacterial loads (Table 2), suggesting that sputum storage time from collection to culture might be the major factor which determines the culture positivity in this study. A statistically significant correlation between sputum storage period and TTD in MGIT was observed in this study. It was reported that bacterial lysis increases during storage and no growth can be observed after 2 months (Pathak et al. 2007). Nevertheless, our study found that there was no statistically significant growth for short (≤60 days) and medium storage periods (61 to 120 days), suggesting that bacteria growth viability would not be affected within 4 months of storage.

Members of MTBC and NTM showed similarities in terms of clinical presentation, detection by acid-fast bacillus (AFB) staining, and ability to grow in Löwenstein–Jensen (LJ) medium or MGIT, causing difficulty to differentiate between these organisms. Thus, characterisation of mycobacteria is important as an accurate diagnosis in the first step to getting effective treatment.

Utility of duplex PCR targeting *rpoB* gene of *Mycobacterium* for identification of MTBC and NTM has an advantage of detecting coexistence of MTBC and NTM in the same reaction mixture, yielding two bands (Kim et al. 2004). This method was adopted in this study and 41 isolates were identified as MTBC and four isolates (i.e. 115, 123, 375, and 538) were NTM, while no co-infections were detected. Although the TbcR5 primer of the *rpoB* gene for MTBC detection was located at the RRDR region, in which mutations are related to rifampicin resistance (Kim et al. 2004), this does not affect the sensitivity of the test. However, false-positive results were observed for M5 and RM3 primers of the *rpoB* gene which were used for detection of the NTM. A study showed that these primers could cross-react with 10 strains of *Tuskamurella, Rhodococcus,* and *Nocardia* species (Kim et al. 2004). In this study, we identified that this set of primers could also amplify other non-mycobacteria including *Microbacterium laevaniform, Streptomyces sp.,* and *Gordonia sp.* These bacteria are classified as actinomycetes which are phylogenetically closed to mycobacteria (Barka et al. 2015).

Besides Mtb, the presence of *M. bovis* has been reported in human

TB infection (Muller et al. 2013) and the use of RD9 gene has successfully differentiated the organisms (Warren et al. 2006). Species differentiation is important for the treatment of TB because *M. bovis* is intrinsically resistant to pyrazinamide (PZA), a first-line TB drug (Allix-Béguec et al. 2010). In Malaysia, *M. bovis* was found in a PTB patient in Kelantan (Suraiya & Farakhin 2018). Also, from neighboring country, *M. bovis* was detected in six patients at Surabaya, Indonesia (Yanti et al. 2020). In our study, we confirmed a total of 41 people were infected with Mtb and none were infected with *M. bovis*, suggesting that till now, no zoonotic *M. bovis*-induced TB among humans in Sabah.

The *hsp65* gene is a valuable marker for the detection of *Mycobacterium* species. It has been widely used for NTM species-level identification due of its variability (Maleki et al. 2017). However, in this study, the Tb11 and Tb12 primers of the *hsp65* gene detected *Gordonia sp.* Similarly, Busatto et al. (2016) reported that *hsp65* primers could cross-react not only with *Gordonia sp.,* but also with *Rhodococcus sp., Rothia mucilaginosa, Cryobacterium sp., Nocardia sp.,* and *Corynebacterium sp.* (Busatto et al. 2016). Thus, these primers should be used with caution for diagnostic purposes.

According to Pang et al. (2017), Xpert cross-reacts with NTM such as *M. abscessus, M. marinum, M. smegmatis, M. phlei,* and *M. aurum* at a high bacterial load (Pang et al. 2017). Also, according to the Trademark, Patents and Copyright Statements of Xpert (Cepheid, Sunnyvale, CA), *M. scrofulaceum* is detected at 10^8 CFU/mL, but not at 10^7 CFU/mL. *In silico* analysis also predicted the potential of cross-reactivity with *M. kumamontonense, M. leprae, M. mucogenicum, Tsukamurella spp.,* and *Nocardia otitidiscaviarum.* Although Xpert showed cross-reactivity with NTM and other organisms, the non-mycobacteria isolates identified in this study tested negative in Xpert, suggesting the high specificity of the Xpert.

Non-mycobacteria were recovered in this study even though standard sputum decontamination with NALC-NaOH solution and the addition of PANTA antibiotics were used to reduce the rate of contamination in the MGIT. Although MGIT culture has a shorter culture time compared to LJ medium, it has a higher rate of contamination probably due to the highly rich culture medium and overgrowth of *Rhodococcus sp.* and *Nocardia sp.* (Tortoli et al. 1999). Another study showed that *Streptomyces, Gordonia, Nocardia, Rhodococcus,* and *Actinomadura sp.* were isolated from MGIT culture. In addition, a study by Simner et al. (2016) had found that the most commonly isolated aerobic actinomycetes were *Streptomyces* species. They also stated that the average TTD of actinomycetes was 10 days, while MTBC was 14 days (Simner et al. 2016). The results were similar to our finding with shorter TTD of actinomycetes (8 days) compared to Mtb (12 days), suggesting the former had a faster growth rate.

CONCLUSION

The present study showed that Mtb was the main causative agent of TB in Sabah and *M. bovis* infection in human was not detected. Xpert is a specific tool for the detection of Mtb without cross-reactivity with NTM and other organisms. Although culture is the gold standard for diagnosis of TB, recovery of mycobacteria in MGIT from sputum specimens was affected by prolonged sputum storage period and presence of actinomycetes.

ACKNOWLEDGEMENT

This work was supported by TRGS Grant (TRG008-2016) and FRGS-RACER Grant (RACER/1/2019/SKK08/UMS//1) under the Ministry of Education Malaysia. The authors were thankful to the Sabah State Health Department for assisting in sample collection and Biotechnology Research Institute, UMS for providing necessary facilities for the study.

REFERENCES

Agrawal, M., Bajaj, A., Bhatia, V., Dutt, S. 2016. Comparative study of GeneXpert with ZN stain and culture in samples of suspected pulmonary tuberculosis. *J Clin Diagn Res* **10**(5): DC09-DC12.

Allix-Béguec, C., Fauville-Dufaux, M., Stoffels, K., Ommeslag, D., Walravens, K., Saegerman, C., Supply, P. 2010. Importance of identifying *Mycobacterium bovis* as a causative agent of human tuberculosis. *Eur Respir J* **35**(3): 692.

Barka, E.A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Meier-Kolthoff, J.P., Klenk, H.P., Clement, C., Ouhdouch, Y., van Wezel, G.P. 2015. Taxonomy, physiology, and natural products of actinobacteria. *Microbiol Mol Biol Rev* **80**(1): 1-43.

Busatto, C., Silva, A.B.S., Groll, A.V., Ramos, D., da Silva, P.E.A, Ramis, I.B. 2016. Identificação de *Mycobacterium avium* entre suspeitos de tuberculose. *Congresso Brasileiro Interdisciplinar de Promoção da Saúde.* https://online.unisc.br/acadnet/anais/index.php/CBIPS/article/view/15975 [20 October 2021].

Engelbrektson, A., Kunin, V., Wrighton, K.C., Zvenigorodsky, N., Chen, F., Ochman, H., Hugenholtz, P. 2010. Experimental factors affecting PCR-based estimates of microbial species richness and evenness. *ISME J* **4**(5): 642-7.

Gopalaswamy, R., Shanmugam, S., Mondal R., Subbian, S. 2020. Of tuberculosis and non-tuberculous mycobacterial infections – a comparative analysis of epidemiology, diagnosis and treatment. *J Biomed Sci* **27**(1): 74.

Goroh, M.M.D., Rajahram, G.S., Avoi, R., Van Den Boogaard, C.H.A., William, T., Ralph, A.P., Lowbridge, C. 2020. Epidemiology of tuberculosis in Sabah, Malaysia, 2012-2018. *Infect Dis Poverty* **9**(1): 119.

Kim, B.J., Hong, S.K., Lee, K.H., Yun, Y.J., Kim, E.C., Park, Y.G., Bai, G.H., Kook, Y.H. 2004. Differential identification of *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria by duplex PCR assay using the RNA polymerase gene (*rpoB*). *J Clin Microbiol* **42**(3): 1308-12.

Lawn, S.D., Nicol, M.P. 2011. Xpert® MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. *Future Microbiol* **6**(9): 1067-82.

Maleki, M.R., Kafil, H.S., Harzandi, N., Moaddab, S.R. 2017. Identification of nontuberculous mycobacteria isolated from hospital water by sequence analysis of the *hsp65* and 16S rRNA genes. *J Water Health* **15**(5): 766-74.

Muller, B., Durr, S., Alonso, S., Hattendorf, J., Laisse, C.J., Parsons, S.D., van Helden, P.D., Zinsstag, J. 2013. Zoonotic *Mycobacterium bovis*-induced tuberculosis in humans. *Emerg Infect Dis* **19**(6): 899-908.

Pang, Y., Lu, J., Su, B., Zheng, H., Zhao, Y. 2017. Misdiagnosis of tuberculosis associated with some species of nontuberculous mycobacteria by GeneXpert MTB/RIF assay. *Infection* **45**(5): 677-81.

Park, C.H., Hixon, D.L., Ferguson, C.B., Hall, S.L., Risheim, C.C., Cook, C.B. 1984. Rapid recovery of mycobacteria from clinical specimens using automated radiometric technic. *Am J Clin Pathol* **81**(3): 341-5.

Pathak, D., Chakravorty, S., Hanif, M., Tyagi, J.S. 2007. Lysis of tubercle bacilli in fresh and stored sputum specimens: implications for diagnosing tuberculosis in stored and paucibacillary specimens by PCR. *BMC Microbiol* **7**: 83.

Ringuet, H., Akoua-Koffi, C., Honore, S., Varnerot,

A., Vincent, V., Berche, P., Gaillard, J.L., Pierre-Audigier, C. 1999. *hsp65* sequencing for identification of rapidly growing mycobacteria. *J Clin Microbiol* **37**(3): 852-7.

Rundi, C., Fielding, K., Godfrey-Faussett, P., Rodrigues, L.C., Mangtani, P. 2011. Delays in seeking treatment for symptomatic tuberculosis in Sabah, East Malaysia: factors for patient delay. *Int J Tuberc Lung Dis* **15**(9): 1231-8.

Sarro, Y.D.,Kone, B., Diarra, B., Kumar, A., Kodio, O., Fofana, D.B., Achenbach, C.J., Beavogui, A.H., Seydi, M., Holl, J.L., Taiwo, B., Diallo, S., Doumbia, S., Murphy, R.L., McFall, S.M., Maiga, M. 2018. Simultaneous diagnosis of tuberculous and non-tuberculous mycobacterial diseases: Time for a better patient management. *Clin Microbiol Infect* **3**(3): 10.15761/CMID.1000144.

Simner, P.J., Doerr, K.A., Steinmetz, L.K., Wengenack, N.L. 2016. *Mycobacterium* and aerobic actinomycete culture: Are two medium types and extended incubation times necessary? *J Clin Microbiol* **54**(4): 1089-93.

Suraiya, S., Farakhin, N.I. 2018. *Mycobacterium bovis* infection in a human in Malaysia. *Clin Microbiol Newsl* **40**(16): 136-7.

Tessema, B., Beer, J., Emmrich, F., Sack, U., Rodloff, A.C. 2011. Rate of recovery of *Mycobacterium tuberculosis* from frozen acid-fast-bacillus smear-positive sputum samples subjected to long-term storage in Northwest Ethiopia. *J Clin Microbiol* **49**(7): 2557-61.

Tortoli, E., Cichero, P., Piersimoni, C., Simonetti, M.T., Gesu, G., Nista, D. 1999. Use of BACTEC MGIT 960 for recovery of mycobacteria from clinical specimens: multicenter study. *J Clin Microbiol* **37**(11): 3578.

Warren, R.M., Gey van Pittius, N.C., Barnard, M., Hesseling, A., Engelke, E., de Kock, M., Gutierrez, M.C., Chege, G.K., Victor, T.C., Hoal, E.G., van Helden, P.D. 2006. Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference. *Int J Tuberc Lung Dis* **10**(7): 818-22.

WHO. 2013. Policy update: Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children. *World Health Organization, Geneva.* http://apps.who.int/iris/handle/10665/112472 [20 October 2021].

WHO. 2019. Global tuberculosis report 2019. *World Health Organization, Geneva*. https://www.who.int/publications/i/item/9789241565714 [20 October 2021].

WHO. 2020. Global tuberculosis report 2020. *World Health Organization, Geneva.* https://apps.who.int/iris/bitstream/handle/10665/336069/9789240013131-eng.pdf?ua=1 [20 October 2021].

William, T., Parameswaran, U., Lee, W.K., Yeo, T.W., Anstey N.M., Ralph, A.P. 2015. Pulmonary tuberculosis in outpatients in Sabah, Malaysia: advanced disease but low incidence of HIV co-infection. *BMC Infect Dis* **15**: 32.

Yanti, B., Mulyadi, M., Amin, M., Harapan, H., Mertaniasih, N.M., Soetjipto, S. 2020. The role of *Mycobacterium tuberculosis* complex species on apoptosis and necroptosis state of macrophages derived from active pulmonary tuberculosis patients. *BMC Res Notes* **13**(1): 415.

Received: 29 Oct 2021
Accepted: 11 Jan 2022