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Potential Use of Multiplex PCR in Diagnosis of Tuberculous Meningitis

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Abstract. Accurate and rapid diagnosis of tuberculous meningitis (TBM) is important for early administration of treatment. In this study, we have evaluated the diagnostic value of smear, culture, multiplex PCR and GeneXpert MTB/RIF to detect *M. tuberculosis* in cerebrospinal fluid (CSF) samples from patients with suspected TBM registered in Queen Elizabeth Hospital, Kota Kinabalu, Sabah, Malaysia. Of the 55 CSF samples collected, 12 (21.8%) samples were positive by multiplex PCR, 3 (5.4%) by GeneXpert MTB/RIF and only 1 (1.8%) by smear and culture. Multiplex PCR showed higher sensitivity to detect *M. tuberculosis* in patients with suspected TBM and has the potential to be used as a diagnostic method.

INTRODUCTION

Tuberculosis (TB), the leading cause of death worldwide (WHO, 2015) is a major health problem in the state of Sabah, Malaysia. The estimated incidence rate is 130 per 100 000 people (Miwil, 2016) and this surpassed the national incidence rate of 80/100 000 people (Rashid Ali *et al.*, 2015). Sabah recorded the highest cases of TB in 2015 with 4 464 cases, followed by Selangor (4 429) and Sarawak (2 575) (Sman, 2016). Tuberculous meningitis (TBM) is the severest form of TB and it constituted almost 50% of clinical diagnosis in a prospective study and the major contributor to mortality and morbidity in patients with central nervous system (CNS) infection in East Malaysia (Lee *et al.*, 2016).

TBM is devastating illness yet with no definite diagnosis. Clinical manifestations are pleomorphic and the cerebrospinal fluid (CSF) cellular contents and biochemical parameters are not consistent and often similar to other bacterial meningitis (Desphande *et al.*, 2007). Classic signs of meningitis will appear at a later stage of the disease, however, the success of recovery greatly determined by stage in which treatment is initiated (Singh *et al.*, 2015). The number of bacilli further complicate the diagnosis where conventional "gold standards" which are the smear and culture cannot detect few bacilli in CSF with sufficient sensitivity and rapidity (Singh *et al.*, 2015; Solari *et al.*, 2013).

Molecular technique such as polymerase chain reaction (PCR) is able to amplify DNA from small amounts of starting material and helpful for rapid diagnosis of TBM. Studies show good sensitivity of PCR in detecting *M. tuberculosis* in CSF specimens (Thwaites, 2013). However, most of these studies utilized only one target gene and this could lead to false negative results as some studies have reported absence of certain genes in different isolates of *M. tuberculosis* (Rafi *et al.*, 2007).

Multiplex PCR that target and amplify several genes simultaneously can be used as an alternative diagnostic method. Reports show that multiplex PCR targeting few genes have significantly increased the sensitivity of PCR (Singh *et al.*, 2015; Kusum *et al.*, 2011). GeneXpert MTB/RIF is an automated system that uses real time PCR and simultaneously detects *M. tuberculosis* and rifampicin resistance. This machine had been endorsed by the World Health Organization (WHO) for diagnosis of pulmonary TB, however, its diagnostic value for extra-pulmonary TB is still uncertain (Denkinger *et al.*, 2014; Nhu *et al.*, 2014).

This study was conducted to evaluate the diagnostic value of smear, culture, multiplex PCR and GeneXpert MTB/RIF in patients with suspected TBM. For multiplex PCR, we have used IS6110 and MPB64 genes as the target genes as IS6110 is present in multiple copies while MPB64 is a conserved sequence in the *M. tuberculosis* genome (Kusum *et al.*, 2011; Therese *et al.*, 2005).

MATERIALS AND METHODS

Clinical Samples. This study was approved by the Medical Research and Ethical Committee, Ministry of Health Malaysia and the study number is NMRR-13-1255-17830. Fifty-five CSF samples were collected from patients with suspected TBM registered in Queen Elizabeth Hospital (QEH), Kota Kinabalu, Sabah, Malaysia and were characterized based on standardized clinical case criteria (Marais *et al.*, 2010) during October 2014 to April 2015.

Processing of CSF Samples. After collection of CSF samples from the patients, each sample was distributed into 8 ml sterile universal bottles and one of the bottles was processed in Biosafety level 3 (BSL3) laboratory, Biotechnology Research Institute (BRI), Universiti Malaysia Sabah (UMS). The samples were centrifuged and the deposits were used for smear (100 µl), culture (100 µl) and multiplex PCR (100 µl).

Ziehl-Neelsen Stain. For smear, 50 µl deposit was used for conventional and modified Ziehl-Neelsen (ZN) stain. Conventional ZN stain was performed according to the World Health Organization (WHO) protocol while modified ZN stain was done according to the previously described method (Chen *et al.*, 2012).

Culture. Culture was done by Microscopic Observation Drug Susceptibility (MODS) assay as described previously (Caws *et al.*, 2007). 100 µl of CSF deposit was inoculated in a well of 24-wells plate containing 900 µl of Middlebrook 7H9 medium supplemented with OADC (oleic acid, albumin, dextrose, and catalase) growth enrichment (BBL) and PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) antibiotic mixture (BD) and the plate was kept in a ziplock bag and incubated at 37ºC. The plates were examined twice a week under the inverted light microscope at 400X magnification for evidence of growth.

Multiplex PCR. DNA was extracted by freezing of the sample at -20ºC for minimum of one day, thawing then adding 200 µl TE buffer before heating at 95ºC for 30 minutes (Awua *et al.*, 2010). The samples were then centrifuged at 6 000 x g for 10 minutes and the supernatants were stored at -20ºC until use. Detection of *M. tuberculosis* by PCR was done by using specific pairs of primers designed to amplify IS6110 and MPB64 genes in the *M. tuberculosis* genome and the expected band size was 123 bp for IS6110 and 240 bp for MPB64. The primer sequence for IS6110 was: IS6110-F: 5'-CCT GCG AGC GTA GGC GTC GG-3', IS6110-R: 5'-CTC GTC CAG CGC CGC TTC GG-3' (Deshpande *et al.*, 2007) while for MPB64 was: MPB64-F: 5'-TCC GCT GCC AGT CGT CTT CC-3' and MPB64-R: 5'-GTC CTC GCG AGT CTA GGC CA-3' (Kusum *et al.*, 2011). Positive and negative controls were included in each PCR assay for comparison of the test results. The positive control was an extracted DNA from *M. tuberculosis* culture provided by the Public Health Laboratory Kota Kinabalu (MKAKK) that contained both the target genes. The negative control contained all the PCR reagents except DNA template, which was replaced with sterile distilled water. PCR was done using the Ready-To-Go PCR tubes (GE, USA) in which 1 µl of each primer (10

pmole), 5 µl DNA template and 16 µl sterile distilled water were added and the contents were mixed well. Multiplex PCR was done in duplicate for each sample. Amplification was done according to the previously described method (Kusum *et al.*, 2011) with minor modifications: Incubation for 5 min at 94ºC for initial denaturation, followed by 45 cycles of denaturation at 94ºC for 0.50 min, annealing at 65ºC for 0.50 min, extension at 72ºC for 1 min and final extension at 72ºC for 10 min. The PCR amplified products were run on 1.5% agarose gel at 100 V. The gel was stained with ethidium bromide and visualized with UV-transilluminator. The quality of the multiplex PCR procedure was assessed by participation in an external quality control program, the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP). A total of 12 specimens consisted of *M. tuberculosis*, *M. avium* and *M. fortuitum* strains as well as negative specimens were tested in two batches.

GeneXpert MTB/RIF. One ml of CSF sample was used for GeneXpert MTB/RIF system and the assay was performed according to the manufacturer's instruction in the laboratory of Infectious Diseases department, QEH.

RESULTS

A total of 55 CSF samples were collected from patients with suspected TBM. The volumes of CSF samples used for smear, culture and multiplex PCR are shown in Table 1. Only 11 patients' case notes were able to be traced. Of the 11 patients, one each had definitive and probable TBM, 6 had possible TBM and 3 had non-TBM diseases. The non-TBM group had the diagnosis of cryptococcal meningitis and viral meningoencephalitis. Patient's details are shown in Table S1.

On microscopic examination, only one sample was positive for conventional/ modified ZN stains and MODS assay. This sample was also positive by multiplex PCR and GeneXpert MTB/RIF. The growth of *M. tuberculosis* was detected on day 8 of incubation in MODS assay and identified by the characteristic cord formation in a clear medium. One sample was contaminated and this was identified by the rapid overgrowth and clouding in the culture. The rest of the cultures were observed up to eight weeks and found negative for growth.

Twelve CSF samples were flagged positive by multiplex PCR. A sample was considered positive when bands were present for both of the target genes or if band was present for any one of the two gene targets used. Out of these 12 multiplex PCR positive samples, MPB64 band was present in 11 (20.0%) samples and IS6110 band was present in 5 (9.1%) samples. Both MPB64 and IS6110 bands were present in only 4 samples (7.3%). In quality assurance, multiplex PCR was able to correctly detect 11 out of 12 samples from RCPAQAP (Table 2).

Three samples were positive by GeneXpert MTB/RIF. As shown in Table 3, the overall sensitivity of smear microscopy, culture, multiplex PCR and GeneXpert MTB/RIF was 1.8%, 1.8%, 21.8% and 5.4% respectively.

In this study, 3 samples had less than 1 ml and were flagged negative by all the assays. The rest of the samples had more than 1 ml and the volume of the sample that was positive for smear, culture and multiplex PCR was 6 ml (Table 1).

Table 1. Volume of CSF samples obtained in this study

Volume of CSF samples (ml)	<1	1–<2	2–<3	3–<4	4-<5	5–<6	6–<7	≥7
Number of samples	3	11	11	5	9	8	6	2
Number of samples positive either by smear, culture or multiplex PCR	–	2	2	3	2	1	2	–

Table 2. Quality assessment of multiplex PCR assay

Specimen	Expected Result / Bacteria strain	Our Result	Assessment
9A1	*M. tuberculosis*	Not detected (negative)	Incorrect
9B1	*M. avium*	Not detected (negative)	Correct
9C1	*M. tuberculosis*	Detected (positive)	Correct
9D1	Negative	Not detected (negative)	Correct
9E1	*M. avium*	Not detected (negative)	Correct
9F1	*M. tuberculosis*	Detected (positive)	Correct
9A2	*M. avium*	Not detected (negative)	Correct
9B2	*M. tuberculosis*	Detected (positive)	Correct
9C2	*M. fortuitum*	Not detected (negative)	Correct
9D2	*M. tuberculosis*	Detected (positive)	Correct
9E2	Negative	Not detected (negative)	Correct
9F2	*M. tuberculosis*	Detected (positive)	Correct

Table 3. Multiplex PCR showed higher sensitivity in detecting *M. tuberculosis* in CSF samples compared to smear, culture and GeneXpert MTB/RIF

Molecular methods		Conventional methods: Smear and culture		
		Positive	Negative	
Multiplex PCR	Positive	1	11	12 (21.8%)
	Negative	0	43	43
	Total			55
GeneXpert MTB/RIF	Positive	1	2	3 (5.4%)
	Negative	0	52	52
	Total			55

DISCUSSION

Administration of early treatment for TBM depends on the rapid identification of the causative agent of the disease. In this study, we have 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. Multiplex PCR showed higher sensitivity compared to GeneXpert MTB/RIF, smear and culture which is in agreement with the previous study that showed higher percentage of MTB PCR compared to MTB-culture (Lee *et al.*, 2016). PCR is advantageous as it is able to amplify DNA from few bacterial cells. It has been reported that optimized multiplex PCR is able to detect DNA from 2-3 bacterial cells either live or dead as long as the bacteria did not lyse (Kusum *et al.*, 2011). However, at least 10-100 cfu/ml viable bacilli and approximately 10^4 organisms/ml are needed for mycobacterial culture and ZN stain respectively while the level of detection for GeneXpert MTB/RIF is between 80-131 cfu/ml (Patel *et al.*, 2013; Marlowe *et al.*, 2011; Bhigjee *et al.*, 2007). Obtaining such amount of bacilli is not practical as the CSF of patients with TBM contains only 10^0–10^2 organisms/ml (Bhigjee *et al.*, 2007).

Targeting two genes has increased the sensitivity of PCR. If only IS6110 gene was used, we could have missed the seven cases

that were positive by MPB64. Similarly, if only MPB64 gene was used, we would have missed the one sample that was positive by IS6110. Multiplex PCR was negative for RCPAQAP samples containing *M. avium* and *M. fortuitum* strains, showing that the assay is specific for the detection of *M. tuberculosis*. Furthermore, it did not show any bands in negative samples thus eliminating the possibility of cross-contamination and carry over. One RCPAQAP sample with *M. tuberculosis* was detected negative by multiplex PCR and this could be due to low number of *M. tuberculosis* in that sample. To the best of our knowledge, only two studies have previously evaluated the utility of multiplex PCR for detecting *M. tuberculosis* in CSF of TBM patients and the sensitivities reported were 58.0% and 84.8% (Singh *et al.*, 2015; Kusum *et al.*, 2011). Several factors such as volume of CSF, extraction protocol and number of target genes used could be considered as the reasons for the variation in performance of multiplex PCR in these studies.

The number of GeneXpert MTB/RIF positive samples in this study was also low compared to the previous studies where the sensitivities reported were 59.3% and 62% (Nhu *et al.*, 2014; Patel *et al.*, 2013). Total CSF samples might have influenced the percentage of positivity where the previous studies used more than 200 CSF samples while in our study, we were able to collect only 55 CSF samples during the study period. In this study, only one sample was positive by both multiplex PCR and GeneXpert MTB/RIF while two samples that were positive by GeneXpert MTB/RIF were negative by multiplex PCR. After sample collection, each sample was distributed into few universal bottles. There is a possibility that the aliquot of CSF used for multiplex PCR might not have sufficient bacilli for the assay to become positive.

Only one sample was positive by both conventional and modified ZN stains. This result is in agreement with some previous studies that showed sensitivity of smear in the range of 0–3.3% (Kusum *et al.*, 2011; Desai *et al.*, 2006). Few previous studies reported higher sensitivity for conventional ZN smear (Nhu *et al.*, 2014; Caws *et al.*, 2007). One study reported higher sensitivity for modified ZN smear since this method can reveal the intracellular *M. tuberculosis* (Chen *et al.*, 2012).

MODS assay had low sensitivity to detect the *M. tuberculosis* in CSF samples since only one sample was culture positive. Previous study conducted by Ha and colleagues that evaluated the diagnostic value of MODS assay for different types of TB specimens also showed low sensitivity where they were able to get only one culture positive for CSF samples (Ha *et al.*, 2009). Others have reported higher sensitivities of MODS assay ranging from 37.5–66.6% (Tran *et al.*, 2013; Caws *et al.*, 2007). MODS assay is advantageous in term of rapidity as it can show the growth of *M. tuberculosis* as early as 6th day of incubation compared to Lowenstein-Jensen (LJ) medium and commercial media that take more than 2 weeks. In this study, *M. tuberculosis* was detected on the 8th day of incubation which is in concordance with previous studies where growth of *M. tuberculosis* in MODS assay was detected in the time range between 6–11 days (Tran *et al.*, 2013; Ha *et al.*, 2009; Caws *et al.*, 2007).

Overall, all diagnostic methods showed low sensitivity to detect *M. tuberculosis* in patients with suspected TBM. From this study, it was shown that samples volume of more than 1 ml could increase the sensitivity of the diagnostic methods as most of the positive samples had more than 1 ml. Previous studies have reported that CSF samples with >6 ml and meticulous examination are needed for an improved detection (Nhu *et al.*, 2014; Ho *et al.*, 2013; Caws *et al.*, 2007; Thwaites *et al.*, 2004). Large samples size might be needed for an increased sensitivity as well as precise estimation of the test accuracy. In summary, we demonstrate that multiplex PCR had higher sensitivity for detecting *M. tuberculosis* in patients with suspected TBM and this assay has the potential to be used as a diagnostic method for TBM.

Supporting Information

Table S1 Laboratory and clinical information of the study patients.

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