ORIGINAL ARTICLE

Evaluation of Anyplex™ II MTB/MDR kit's performance to rapidly detect isoniazid and rifampicin resistant *Mycobacterium tuberculosis* from various clinical specimens

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Abstract

To determine the accuracy of multiplex real-time PCR (Anyplex™ II MTB/MDR kit) in detecting Isoniazid (INH)- and Rifampin (RIF)-resistant *Mycobacterium tuberculosis* strains from various clinical specimens. The performance of Anyplex™ II MTB/MDR kit in detecting INH- and RIF-resistant *M. tuberculosis* compared to the conventional drug susceptibility tests by Mycobacterial Growth Indicator Tube (MGIT). A total of 430 clinical samples had positive results for *M. tuberculosis* from both Anyplex™ II MTB/MDR kit assay and mycobacterial cultures by MGIT method. When compared to MGITs, the sensitivity and specificity of Anyplex™ II MTB/MDR kit in detecting INH-resistant TB were 85.71% and 99.75%, respectively. For the detection of MDR-TB, the sensitivity and specificity of the test were 82.35% and 99.76%, respectively. The positive predictive values and negative predictive values to detect INH-resistant TB were 96.77% and 98.75%, respectively. Anyplex™ II MTB/MDR kit can be used to rapidly detect isoniazid and rifampicin resistances. It has a high sensitivity, specificity and PPV in detecting INH-resistant TB and MDR-TB. This test can be used as an alternative test to Xpert MTB/RIF because it can rapidly detect both INH-resistant TB and RIF-resistant TB.

Keywords *Mycobacterium tuberculosis* · Real-time PCR · Isoniazid resistant · Positive predictive values · Sensitivity · Specificity

Introduction

Isoniazid (INH) is the most widely used anti-tuberculosis drug. It is an active first-line drug for the treatment of active tuberculosis (TB) disease [1]. Patients susceptible to rifampicin (RIF) but resistant to isoniazid (Hr-TB) had a higher risk of treatment failure and relapse when treated with the standard regimen 2HREZ/4HR (H: isoniazid, R: rifampicin, E: ethambutol, Z: pyrazinamide) [2]. In addition, TB can quickly develop resistance to the TB drugs. As

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a result of this, there is an increase in number of patients with RIF-resistant TB and multi-drug resistant tuberculosis (MDR-TB) due to inadequate and improper treatment of the disease [3]. A previous study conducted in Thailand showed that the prevalence of new cases of Hr-TB and MDR-TB were 11.8% and 2.5%, respectively [4].

In 2017, World Health Organization (WHO) examined the treatment outcomes from each country and developed the WHO treatment guidelines for Hr-TB [3]. In the past, the standard treatment for TB was 2HRZE/4HR which was not effective for Hr-TB and had a lot of negative treatment outcomes. Because of this, WHO has updated its treatment recommendation for treating confirmed Hr-TB to 6(H) REZ-Lfx (Lfx: levofloxacin) for six months [3]. Thus, early detection of Hr-TB and MDR-TB are crucial because many systematic reviews found that new isoniazid-resistant TB cases on standardized empirical treatment may contribute to higher rates of acquired drug resistance (8%) compared with drug susceptible TB (0.3%); and cases with MDR-TB treated with the standardized regimen had higher treatment failure rate [2, 5, 6]. Hence, prompt diagnosis of Hr-TB and MDR-TB can help physicians select the most appropriate regimen. Currently, Line probe assays (Hain Life science, Germany) can be used to detect Hr-TB, however, the diagnostic performance of this molecular assay performed on samples from negative smear was low and showed a high level of invalid results in detecting *M. tuberculosis* and its resistance to RIF and/or INH [7, 8]. Hence, WHO has not approved this assay to be used to diagnose Hr-TB in smear-negative specimens [9]. As a result of this, the multiplex real-time PCR technique (Anyplex™ II MTB/MDR Detection, Seegene, Korea) was introduced to detect *Mycobacteria tuberculosis* complex, INH- and RIF-resistant TB directly from the clinical samples such as sputum, cerebrospinal fluid, blood and tissue from any part of the body [10]. This assay is a semi-automated system and provides rapid results, within 3.5–4.5 h. Aside from that, there is less possibility of error and contamination [11] compared to the Line probe assay. Yet, WHO has not endorsed the use of multiplex real-time PCR technique to be used to detect drug resistant MTB strains [12]. However, many countries including Thailand, have already used this technique to identify resistant TB in clinical practice [13].

Anyplex™ II MTB/MDR Detection is the detection kit designed to detect MTB and drug-resistant TB by using Dual Priming Oligonucleotide (DPO) technology. It includes two sections of separating priming which are 5′-end stabilizer and 3′-end determiner, and are linked together by polydeoxyinosine to create a "bubble-like structure". This structure does not participate in the priming process but helps stabilize the 5′-end and 3′-end to have a clearer boundary. This method can increase the susceptibility and specificity of the reaction. By using the Innovative Technology for High Multiplex Real-time PCR (TOCE), it can confirm multiple targeted genes by at least five types. In particular, this method can simultaneously detect 25 mutations in the *inhA, katG* and *rpoB* genes associated with MDR TB [14].

This study evaluated the accuracy of the real-time multiplex PCR by using Anyplex™ II MTB/MDR Detection kit to detect Hr-TB and RIF-resistant/MDR-TB from clinical specimens and compared it to the outcome of standard drug-susceptibility testing (DST) (Bactec MGIT 960 System).

Materials and methods

A total of 893 clinical specimens from sputum, bronchial alveolar lavage (BAL), aspirated abscess, cerebrospinal fluid (CSF), and fresh tissue biopsy were immediately processed and subjected to various diagnostic tests to detect TB as per routine practice of the hospital (Fig. 1). Data from these tests were entered into the hospital's database. We retrospectively reviewed the medical records of the patients from Jan 2015 until Dec 2016 and selected patients with suspected active *M. tuberculosis* infection in any organ. The specimens from these patients were subjected to both culture and real-time multiplex PCR by using Anyplex™ II MTB/MDR at the King Chulalongkorn Memorial Hospital, a teaching hospital in Bangkok, Thailand. 893 specimens were identified to have *M. tuberculosis* via real-time multiplex PCR methods. We excluded 238 specimens because many of the specimens were collected from the same patients at different time points. Because of this, we selected specimens that were collected at the first visit. 167 specimens were negative for culture and the other 57 specimens were not cultured so these specimens were also excluded from the study. After excluding all of these specimens, we were left with 430 specimens.

Mycobacterium tuberculosis culture, DNA extraction, and Anyplex™ II MTB/MDR assay

All specimens (i.e., sputum, cerebrospinal fluid, blood and tissue from any part of the body) were aliquoted and submitted for mycobacterial culture using conventional methods and multiplex real-time PCR technique. For pre-treatment procedure, the specimens were processed with *N*-acetylcysteine and sodium hydroxide, followed by centrifugation and resuspension in phosphate buffer (total volume 1.5 ml) (Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. Atlanta, GA: US Department of Health and Human Services, Public Health Service, Centers for Disease Control, 1985).

For mycobacterial culture, the prepped sample was then inoculated onto a solid medium (Ogawa medium) and into a liquid medium (BACTEC MGIT (mycobacteria growth
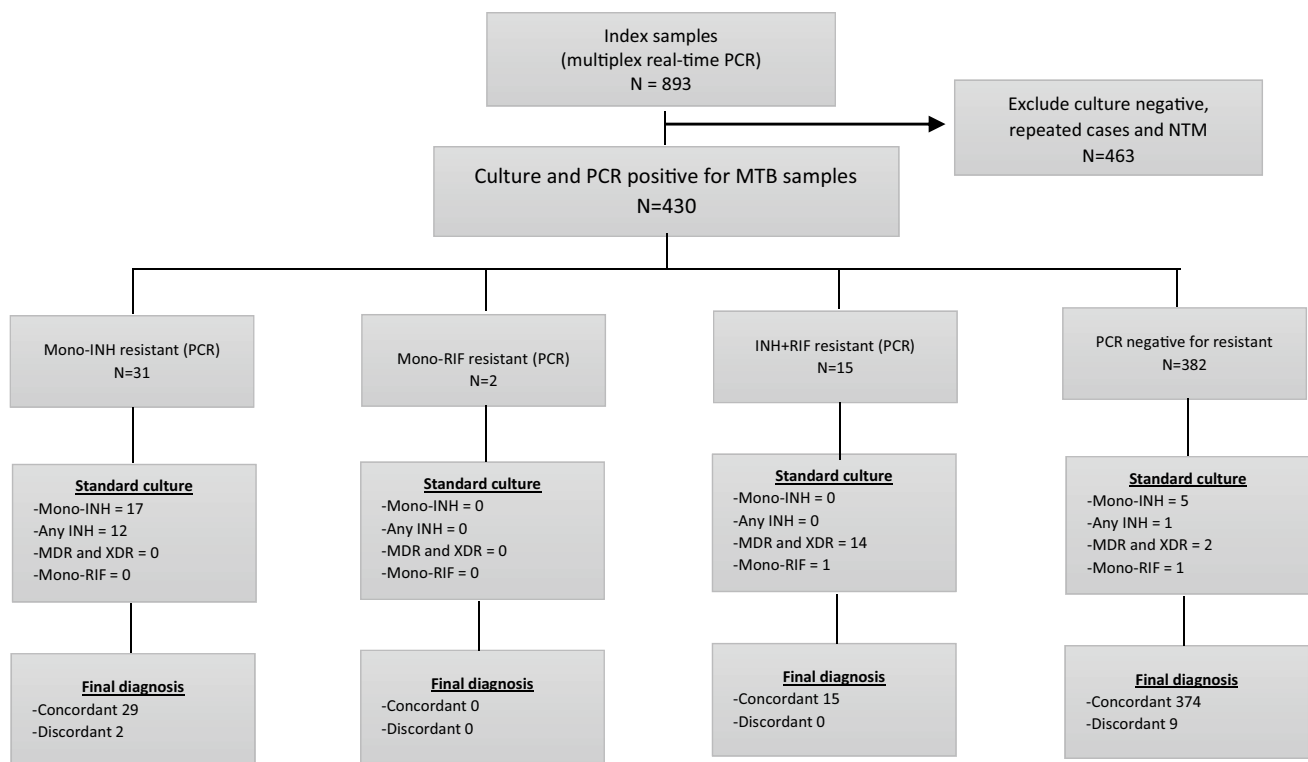
Fig. 1 Flow chart of how the clinical specimens were tested in order to identify *Mycobacterium tuberculosis* (MTB), isoniazid resistant TB (Hr-TB) and rifampicin (RIF) resistant TB by multiplex real-time PCR assay. Moreover, the same specimens were cultured by the conventional method to detect MTB and drug-susceptibility testing (DST) (Bactec MGIT 960 System) was used to identify Hr-TB and RIF resistant TB

indicator tube), using the BACTEC MGIT 960 System; BD Microbiology Systems). The identification of *M. tuberculosis* was confirmed by biochemical test (nitrate reduction) and immunochromatographic test (MPT64 antigen detection system, SD Bioline Kit; Standard Diagnostics, Inc., Korea). For drug susceptibility testing (DST), we used indirect liquid medium-based automated culture systems, the BACTEC MGIT 960 system, to detect the isoniazid and rifampicin resistance from the colonies.

DNA extraction

Before performing Anyplex™ II MTB/MDR Detection, extraction of *M. tuberculosis* DNA was done by using magLEAD® 12gC (Precision System Science, Chiba, Japan). Pretreated specimens were treated with magnetic beads-based co-extraction fully automated system. This procedure used MagDEA Dx SV, a nucleic acid extraction reagent mixed with 200 µl of decontaminated sample. The sample was then lysed, digested with proteinase K, adsorbed by magnetic bead, washed and eluted. No additional reagents were required. The 50 µl eluted nucleic acid was then obtained and subjected to Anyplex II MTB/MDR test.

DNA amplification

Anyplex II MTB/MDR was performed following the instructions of the manufacturer. Two PCR reactions were performed at the same time in pairs of tubes; the Anyplex II MTB/MDR test is designed to detect *M. tuberculosis* (*IS6110* and *MPT64*) and mutations of INH resistance (*katG, inhA* promoter region) and RIF resistance (*rpoB*) [11, 14]. The PCR solution consisted of appropriate volumes of 2×TOCE oligo mix for MDR, 2×Anyplex PCR master mix (with UDG), extracted DNA template, and RNase-free water. Amplification was performed using the CFX96TM Real-Time PCR System (Bio-Rad, USA). The turn-around time of detection is approximately 2 h and 40 min. Melting-curve data analyses and interpretation were performed automatically and interpreted via the Seegene viewer software, version 2.0 (Seegene Technologies, USA) using pre-defined threshold and cut-off values. An internal control (IC) with positive and negative amplification controls were included in the reactions.

This study was approved by the ethical committee of the Faculty of Medicine, Chulalongkorn University. Retrospective collected clinical samples (i.e., sputum, cerebrospinal fluid, and fresh tissue from any part of the body) from

patients older than 18 years old were subjected to the multiplex real-time PCR (Anyplex™ II MTB/MDR Detection Seegene, Korea) to detect MTB. Written informed consent was waived for this study. This process was performed at the Mycobacteriology Laboratory, Department of Microbiology, Faculty of Medicine, Chulalongkorn University.

Sample size calculation

We used the single sample size determination formula by Cochran (1963) to yield a representative sample for proportions. Expected sensitivity is 0.7 (70%) and substitute the acceptable error (d) with 0.15 (15%) as same as the value from the previous literature review [11, 14, 15], it provides the sensitivity results of the real-time multiplex PCR (Anyplex™ II MTB/MDR Detection Seegene, Korea) of TB strains resistant to INH about 61–93% from the clinical sampling. 36 samples that were Hr-TB were used. Based on the historical data from Rajavithi Hospital, the prevalence of new Hr-TB was 11.85% [4]. With this information, we would need 305 samples for this study.

Statistical data analysis

Data were analyzed by using SPSS version 17. After data validation, the descriptive data for the demographical variable from the patients was aggregated by using the arithmetic average and standard deviation (SD) for continuous variables, the percentage of an interesting group of clinical sampling. Comparisons of continuous data were done by using independent t test. Comparisons of categorical data were done by using the chi-square test. P-value < 0.05 with 95% confidence interval (95%CI) was considered statistically significant. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of drug-resistant TB detected by Anyplex™ II MTB/MDR Detection kit were compared to the conventional culture (Bactec MGIT 960 System). 95% CI calculation with Clopper–Pearson exact binomial, analysis and report the accuracy of the outcome by STARD 2015.

Results

Anyplex™ II MTB/MDR Detection kit detected MTB for all 893 specimens. We selected only the first specimen from each patient. Thus, 238 specimens were excluded from the study. Since 167 specimens were negative for culture and the other 57 specimens were not cultured, these specimens were also excluded from the study which left us with 430 specimens. Among the 430 samples which were culture-positive for MTB, 34 were Hr-TB, two were RIF-resistant, 16 were MDR-TB and one was extensive drug-resistance

(XDR-TB) as per the Bactec MGIT 960 System. The average age of the study population was 51 years (range 18–95), 159 samples came from female patients (36%) and 319 samples were identified as pulmonary TB infection (74.1%). The prevalence of culture-proven Hr-TB and MDR TB in this study were 7.9% and 3.7%, respectively. According to the multiplex real-time PCR, out of 430 samples, 382 samples did not have any drug-resistant gene, 31 samples were Hr-TB, 2 samples had only RIF-resistant gene and 14 samples had both isoniazid resistant and RIF-resistant genes (MDR-TB). According to the MGIT method, 53 samples out of 430 (12.3%) samples were drug-resistant TB by MGIT method. However, from the 53 samples, Anyplex™ II MTB/MDR Detection kit could not detect any drug-resistant genes from 10 samples (18%). 16 samples were culture proven to be MDR-TB. 34 samples were culture proven to be Hr-TB. On the other hand, Anyplex™ II MTB/MDR was unable to identify 12 out of 16 samples to be MDR-TB and 29 out of 34 to be Hr-TB. This indicated that multiplex real-time PCR could yield false negative results.

The diagnostic sensitivity and specificity of Anyplex™ II MTB/MDR Detection assay was analyzed by comparing it to the MGIT method. The sensitivity of the multiplex real-time PCR to detect Hr-TB was 85.3%, the specificity was 99.4%, the accuracy was 98.3%, the PPV was 93.5% and the NPV was 98.7%. For pulmonary TB specimens, the sensitivity and specificity of the multiplex real-time PCR were 95.4% and 99.3%, respectively, the PPV was 91.3%, the NPV was 99.6% and the accuracy was 99%. For extra-pulmonary specimens, the sensitivity and specificity were 76.9% and 100%, respectively. The PPV and NPV of the multiplex real-time PCR for Hr-TB in extra-pulmonary specimen were 91.3% and 99%, respectively (Table 1).

The standard culture and DST approach were able to detect multidrug-resistance (MDR-TB) and extensive drug-resistance (XDR-TB) in 17 samples of which one sample was XDR TB. Multiplex real-time PCR was unable to detect 4 MDR-TB samples. In addition, an XDR-TB sample in our study which showed that it was resistant to isoniazid, rifampicin, ethambutol, pyrazinamide, ofloxacin and streptomycin by phenotypic DST was identified as MDR-TB by multiplex real-time PCR due to its limitation. The PCR is dependent upon the primers so if the mutated genes are not within the range of the primer, then that gene will not be amplified or detected The sensitivity of the multiplex real-time PCR to detect MDR-TB was 75.4%, the specificity was 99.5%, the accuracy was 98.6%, the PPV was 85.7% and the NPV was 99.4%. For pulmonary TB specimens, multiplex real-time PCR's sensitivity for detecting MDR-TB was 83.3% and the specificity was 99.3%. Moreover, the PPV was 83.3%, the NPV was 99.3%, and the accuracy was 98.2%. As for the extra-pulmonary TB specimens, the sensitivity and the

Table 1 The sensitivity, the specificity, the positive predictive value (PPV) and the negative predictive value (NPV) of isoniazid (INH)-resistant tuberculosis (TB) detected using multiplex real-time PCR versus phenotypic drug-susceptibility testing (BACTEC MGIT 960 System)

INH-R (mono and any isoniazid resistance)	Total (N = 430) (%)	Sensitivity (%) (95%CI)	Specificity (%) (95%CI)	PPV (%)	NPV (%)	Accuracy (%)
All specimens (95% CI)	430 (100)	85.3 (29/34) (68.9–95)	99.5 (394/396) (98.2–99.4)	93.5	97.2	98.3
Pulmonary TB (95% CI)	319 (74.1)	95.4 (21/22) (77.2–98.8)	99.3 (296/297) (97.6–99.9)	91.3	99.7	99.1
Extrapulmonary TB* (95% CI)	111 (25.8)	66.7 (8/12) (46.19–94.96)	100 (99/99) (96.7–100)	100	96.1	96.4
Lymph node (95% CI)	58 (13.4)	100 (6/6) (47.8–100)	100 (52/52) (93.2–100)	100	100	100

*Other extrapulmonary specimens included cerebrospinal fluid (CSF), ascites fluid, bones, intestine tissue, pleura and pericardial fluid. The number of samples for these specimens was less than 10% so the accuracy could not be calculated

specificity of the multiplex real-time PCR were 50% and 100% in detecting MDR-TB, and the PPV and the NPV were both 100% and 98.2%, respectively (Table 2). The sensitivity of multiplex real-time PCR was high in detecting drug resistance in pulmonary specimens (95.4%) compared to extrapulmonary specimen (66.7%) for Hr-TB. As for MDR TB, the sensitivity for pulmonary specimens was 83.3% and extrapulmonary specimens was 50%. The specificities of the molecular assay were 100% for both specimen types. For the XDR TB, the multiplex real-time PCR could detect only INH with rifampicin resistance and was unable to identify XDR-TB, instead it was identified as MDR TB because this kit did not include other probes to test for other drugs.

According to the multiplex real-time PCR, the frequencies of INH resistant mutations in *inhA* and *katG* genes from all specimens were 10/47 (21.2%) and 29/47 (61.7%), respectively.

Discussion

To the best of our knowledge, this is the first study to determine the accuracy of multiplex real-time PCR (Anyplex™ II MTB/MDR Detection, Seegene, Korea) in detecting INH- and RIF-resistant strains from both pulmonary and extrapulmonary specimens in Thailand. The result from our study showed that the PPV of multiplex real-time PCR in predicting Hr-TB infection was 93.5%. Our findings indicated that multiplex real-time PCR has a high accuracy rate in detecting drug resistant TB. Since the PPV was greater than 90%, this indicated that the multiplex real-time PCR will be useful in countries with high TB burden because it will be able to detect Hr-TB. The results from this assay will definitely influence the physician's treatment regimen to include fluoroquinolone at the early stage of diagnosis as per WHO's recommendation. When we compared the performance of the multiplex real-time PCR to the gold standard culture, the sensitivity to detect Hr-TB was 85.3%. Because the PPV of this test was high, the best treatment

Table 2 The sensitivity, the specificity, the positive predictive value (PPV) and the negative predictive value (NPV) of multiplex real-time polymerase chain reaction (PCR) versus phenotypic drug-suscepti-bility testing (BACTEC MGIT 960 System) in detecting multi-drug resistant tuberculosis (MDR-TB)

MDR TB (INH-R+RIF-R)	Total (N = 430) (%)	Sensitivity (%) (95%CI)	Specificity (%) (95%CI)	PPV (%)	NPV (%)	Accuracy (%)
All specimens (95% CI)	430 (100)	75 (12/16) (47.6–92.7)	99.5 (412/414) (98.3–99.9)	85.7	99	98.6
Pulmonary TB (95% CI)	319 (74.1)	83.3 (10/12) (51.6–97.9)	99.3 (305/307) (97.7–99.9)	83.3	99.3	96.8
Extrapulmonary TB* (95% CI)	111 (25.8)	50 (2/4) (6.8–93.2)	100 (107/107) (96.6–100)	100	98.2	98.2
Lymph node (95% CI)	58 (13.4)	100 (1/1) (2.5–100)	100 (57/57) (93.7–100)	100	100	100

*Other extrapulmonary specimens included cerebrospinal fluid (CSF), ascites fluid, bones, intestine tissue, pleura and pericardial fluid. The number of samples for these specimens was less than 10% so the accuracy could not be calculated

There are some limitations in this study. Even though the multiplex real-time PCR can identify early MDR-TB and Hr-TB infections, however it was substandard to the conventional culture method because it could not differentiate between low-level INH resistance versus high-level INH resistance. This is important because if we do not know the level of INH resistance, then this can affect the dose of INH used to treat TB. High dose of INH can treat patients with low-level INH resistance whereas those with high-level resistance would require a different [25]. Second, WHO recommends the use of Xpert MTB/RIF to detect TB infection and RIF-resistant TB in pulmonary and extra-pulmonary clinical specimens [26]. Therefore, the Xpert MTB/RIF is widely accepted worldwide. Third, this study did not collect the AFB smear results of these specimens and did not compare the results to the Line probe assays (LPAs). LPA is endorsed by WHO to identify drug-resistant TB from AFB positive specimen to detect first- and second-line drug-resistant MTB in smear positive specimen and in isolated culture but not endorsed to be used in smear negative specimen. This study also did not compare the results to MGIT960 culture and Xpert MTB/RIF. Head-to-head comparison between multiplex real-time PCR and Xpert MTB/RIF should be further studied to confirm the performances between the two assays.

Multiplex real-time PCR has a high accuracy in diagnosing Hr-TB and MDR-TB infections in high TB prevalence countries. It could be another option that can be used to diagnose MDR-TB and INH mono-resistant TB before initiating TB treatment. Results from this assay can help the physician administer the proper regimen to the patient while waiting for the confirmatory results. Multiplex real-time PCR (Anyplex™ II MTB/MDR Detection) may be an alternative choice to Xpert MTB/RIF. Its ability to also detect Hr-TB strains makes this tool very attractive, especially in countries with high TB burden.

Compliance with ethical standards

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