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Comparison between the PaxView TB/NTM MPCR-ULFA Kit and current methods for *Mycobacterium tuberculosis* detection in Malang, East Java, Indonesia

Agustin Iskandar[1], Putri Wulan Akbar[1,2], Andrea Aprilia[1], Young-Kil Park[3],

[1] Faculty of Medicine, Brawijaya University, Malang, Indonesia
[2] Faculty of Medicine, UIN Maulana Malik Ibrahim, Malang, Indonesia
[3] Department of Research, PaxGenBio, Anyang, Republic of Korea
[*]Corresponding author. Email: agustin_almi@ub.ac.id

ABSTRACT

The PaxView TB/NTM MPCR-ULFA Kit, which targets the IS*6110* and *mtp40* genes for *Mycobacterium tuberculosis* (MTB) detection, is a novel tool that substitutes gel electrophoresis for universal lateral flow assays. The sensitivity and specificity of this method were compared with those of established methodologies using Indonesian clinical isolates. In this study, 148 sputum specimens isolated from suspected tuberculosis (TB) carriers were examined to evaluate the performance of the PaxView TB/NTM MPCR-ULFA Kit compared to that of smear microscopy and the Xpert MTB/RIF assay. Out of 148 cases, the rate of TB-positive samples evaluated by different methods was 18.2% (27/148; 95% CI 11.9–24.4) for smear microscopy, 20.3% (30/148; 95% CI 13.8–26.8) for the Xpert MTB/RIF, and 34.5% (51/148; 95% CI 26.8–42.1) for the PaxView TB/NTM MPCR-ULFA Kit. Twenty sputum specimens from healthy subjects were also tested, all of which rendered negative results via the three diagnostic tools examined herein. Compared to the Xpert MTB/RIF, the PaxView TB/NTM MPCR-ULFA Kit was found to possess a 96.7% sensitivity (29/30; 95% CI 90.3-100).Moreover, the PaxView TB/NTM MPCR-ULFA Kit detected 18.6% (22/118, 95% CI 11.6–25.6) of Xpert MTB/RIF MTB negative samples and 20.7% (25/121, 95% CI 13.5–27.9) of smear microscopy negative samples as MTB positive. The PaxView TB/NTM MPCR-ULFA Kit could be a useful molecular diagnostic tool to identify MTB in clinical samples in resource-limited countries, as this procedure is more cost-effective and sensitive than the Xpert MTB/RIF, and more convenient than conventional PCR gel electrophoresis approaches.

Keyword
Mycobacterium tuberculosis, multi-plex PCR, universal flow lateral assay, Xpert MTB/RIF, smear microscopy

Introduction

To this day, tuberculosis (TB) continues to be a major chronic infectious disease worldwide. According to the 2019 Global TB Report, the WHO estimated that 1.5 million people died due to TB and 10.0 million people developed TB worldwide in 2018[1].

The current estimated tuberculosis incidence in Indonesia is 845,000, which ranks third globally, only surpassed by India and China. However, only 570,289 cases are reported, and rapid molecular diagnoses account for only 12% in 2018[1]. Even though the coverage of molecular diagnosis is growing every year, a large gap remains between the notified cases and the estimated incidence. It has been estimated that 98,000 people died from tuberculosis in 2018 in Indonesia[1]. Most deaths from TB could be prevented with early diagnosis and appropriate treatment[2,3], and therefore early TB diagnosis is essential to reduce its worldwide lethality.

There are many kinds of TB diagnostic tools, from the smear microscopy (SM) approach developed by Robert Koch in 1882 to modern sophisticated approaches including Xpert MTB/RIF (Cepheid, CA, USA). Each country implements these methods alone or in combination with other approaches, depending on nation-wide socioeconomic factors. However, an optimal (i.e., effective and cost-efficient) TB diagnostic tool is yet to be developed.

Among the various existing TB diagnostic tools, molecular diagnostic tools are the fastest methods for the detection of *Mycobacterium tuberculosis* (MTB) in clinical specimens, and also possess satisfactory sensitivity and specificity[4]. For example, the Xpert MTB/RIF, a current leading molecular diagnostic tool, has been extensively used not only for the detection of rifampicin-resistant MTB but also for primary MTB case detection[5-8]. However, due to the expensive equipment, cartridges, and installation costs required by the Xpert MTB/RIF approach, this testing solution is not suitable for implementation in developing countries unless international monetary aid is provided[9,10].

In house-PCR is the cheapest molecular diagnostic method, but requires cumbersome gel electrophoresis procedures. Therefore, PaxGenBio (Korea) developed the PaxView TB/NTM MPCR-ULFA Kit, which rapidly detects MTB

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without the need for gel electrophoresis after polymerase chain reactions (PCRs). Moreover, this procedure can be used with a conventional PCR instrument without the need for a specific expensive PCR device.

Therefore, this study sought to evaluate the potential clinical applicability of the PaxView TB/NTM MPCR-ULFA Kit for the detection of MTB in clinical samples by comparing its performance with that of the Xpert MTB/RIF and SM, both of which have been extensively implemented worldwide.

Methods

Specimens

In this study, we utilized sputum specimens from suspected TB carriers, which were acquired from public TB diagnosis health centers, hospitals, or clinics from July to December of 2019 in the Malang region, Indonesia. The average age of the 148 TB suspects was 48.06 years (14<yrs<85), and the proportion of males and females among total TB suspects was 54% and 46% respectively. Additionally, 20 specimens were collected from healthy volunteers (laboratory staff and medical students) to serve as negative controls.

Ziehl-Neelsen Smear Microscopy

Ziehl-Neelsen direct AFB smear and grading was performed by the technicians from each institute according to the WHO/International Union Against Tuberculosis and Lung Disease method[11].

DNA extraction from specimens

After SM, the remaining sputum specimen was treated with Xpert MTB/RIF buffer. Afterward, a portion of the pretreated specimen was tested with the Xpert MTB/RIF procedure. Another specimen sub-sample was analyzed with the PaxView DNA Extraction Kit (PaxGenBio, Korea). Briefly, 500 µl of the specimen pretreated with the Xpert MTB/RIF buffer was transferred into 1.5 ml screw capped tubes, to which 500 µl of washing buffer were added (provided by the PaxView DNA Extraction Kit). This mixture was then centrifuged at 13,000 rpm for 3 min, after which the supernatant was discarded. After washing the specimen once more, 100 µl of elution buffer were added into the tube, which was then transferred to a 95℃ heating block for 15 min. After centrifugation, 20 µl of supernatant were transferred into a new tube and used as a template.

Xpert MTB/RIF

Pretreated specimen was amplified with the GeneXpert kit following the manufacturer's instructions.

PaxView TB/NTM MPCR-ULFA Kit PCR and interpretation of the results

The PaxView TB/NTM MPCR-ULFA Kit includes multiple primer pairs, including two for MTB-specific genes (IS*6110* and *mpt40*), as well as for the mycobacteria *rpoB* gene. After multiplex PCR, the product identities were confirmed by universal lateral flow assay (ULFA), which is based on DNA-DNA hybridization with previously immobilized complementary DNA fragments on a nitrocellulose membrane (**Figure 1**). PCRs were performed with the following protocol: 50°C for 4 min; 95 °C for 10 min; 25 cycles of denaturation (95 °C for 15 sec), annealing and extension (71 °C for 60 sec); 20 cycles of denaturation (95 °C for 15 sec), annealing (60 °C for 30 sec), and extension (72 °C for 30 sec).

After PCR amplification, 5 µl of PCR solution were added to the ULFA device inlet, after which 50 µl of running buffer (provided by the manufacturer) were added immediately. After 5 minutes, 50 µl of washing buffer were added into the inlet and the results were then read within 15 minutes.

Bands 1 and/or 2 (with or without band 3) in the detection device indicate that the sample is MTB-positive, the presence of band 3 without band 1 and 2 means that the sample is NTM-positive, bands 4 and 5 without band 1 to 3 indicate that the sample is MTB/NTM-negative, and band 5 alone indicates that the test was invalid, indicating that the amplification process did not happen due to problems with the template or reagents (**Figure 2**)

Ethical statement

This study was approved by the Institutional Review Board of Brawijaya University, under the project title "The diagnosis of pulmonary TB using MPCR-ULFA targeting IS*6110* and *mtp40*" (Approval number: 208/EC/KEPK-PPDS/07/2019).

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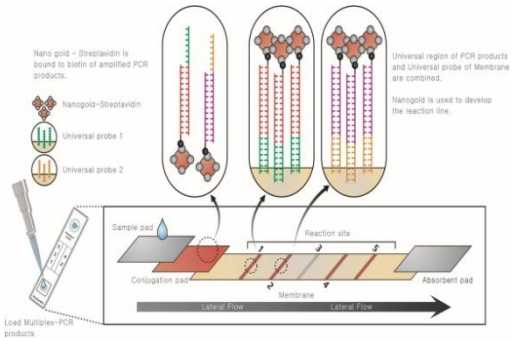

Figure 1. Principles of the PaxView TB/NTM MPCR-ULFA Kit.

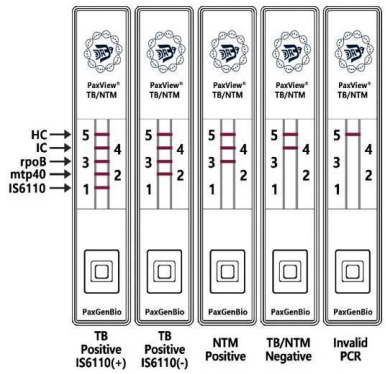

Figure 2. Interpretation of the PaxView TB/NTM MPCR-ULFA Kit results.

Results

A total of 148 clinical specimens from hospitals and clinics in the Malang area were used to compare the performances of the PaxView TB/NTM MPCR-ULFA Kit, SM, and the Xpert MTB/RIF.Out of 148 cases, 18.2% (27/148; 95% CI 11.9–24.4) were MTB positive according to SM, 20.3% (30/148; 95% CI 13.8–26.8) were positive according to the Xpert MTB/RIF, and 34.5% (51/148; 95% CI 26.8–42.1) were positive according to the PaxView TB/NTM MPCR-ULFA Kit.

Moreover, 20 specimens isolated from healthy individuals tested negative with all three methods analyzed herein. Therefore, even though the number of negative samples was very limited, the specificity of the PaxView TB/NTM MPCR-ULFA Kit was flawless.

Compared to the Xpert MTB/RIF, the PaxView TB/NTM MPCR-ULFA Kit was found to possess a 96.7% sensitivity (29/30; 95% CI 90.3-100), as the PaxView TB/NTM MPCR-ULFA missed only one case out of the 30 samples that tested positive with the Xpert MTB/RIF (**Table 1**). This may have been caused by DNA loss during the DNA extraction step, as several manual washing steps are required for DNA purification.

Furthermore, the PaxView TB/NTM MPCR-ULFA Kit detected 18.6% (22/118, 95% CI 11.6–25.6) of Xpert MTB/RIF MTB negative samples and 20.7% (25/121, 95% CI 13.5–27.9) of SM negative samples as MTB positive.

Table 1. Comparison of MTB positive samples from suspected TB carriers depending on the diagnostic tool employed

		PaxView MTB positive	PaxView MTB negative	Total
Z-N positive	Xpert MTB positive	26	1	27
	Xpert MTB negative	0	0	0
Z-N	Xpert	3	0	3

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negative	MTB positive			
	Xpert MTB negative	22	96	118
Total		51	97	148

Discussions

The PaxView TB/NTM MPCR-ULFA Kit exhibited a higher MTB detection rate than that of the Xpert MTB/RIF. This may have been due to target gene differences, as the former is IS*6110*-specific while the latter is *rpoB*-specific. Additionally, the two genes have different chromosomal copy numbers. Generally, IS*6110* has higher copy numbers than the *rpoB* gene, which only has one copy.

According to Chaidir's report, the distribution of MTB genotypes isolated in Indonesia consist of 46.9% of lineage 4, 33.7% of lineage 1, and 19.4% of lineage 2[12]. This means that at least two-thirds of Indonesian MTB isolates have high IS*6110* copy numbers, as lineages 2 and 4 are known to possess high IS*6110* copy numbers[13].

The ratio of positive cases determined by the PaxView TB/NTM MPCR-ULFA Kit to those of the Xpert MTB/RIF (34.5%/20.3%) was 1.7 (**Table 1**). This result has been confirmed in other previous reports.Reports of TB meningitis detection in Indonesia demonstrated that in-house PCR targeting IS*6110* was superior to the Xpert MTB/RIF, with sensitivities of 43% for the Xpert MTB/RIF and 64% for in-house PCR (i.e., 1.52 ratio; 64%/43%)[14]. In Sharma's report on the detection of pleural TB in India, the sensitivity of MPCR targeting for IS*6110* and *mpb64* was 2.7 times (89.6%/33.3%) superior to that of the Xpert MTB/RIF[15].

In another group's report on the detection of meningitis TB in India, the sensitivity of MPCR was 1.7 times (87.2%/50.5%) higher than that of the Xpert MTB/RIF[16] and 1.2 times (100%/82.9%) higher for the detection of pulmonary TB in Nepal[17].

In this study, the TB detection rate of the XpertMTB.RIF was only 1.12 times (20.3%/18.2%) higher than that of SM, which is only a slight difference compared the 2.16-fold (24.2%/11.2%) differences reported by Chaidir[14], 1.75-fold (84%/48%) in a study in Thailand[18], and 1.8-fold (65.5%/36.2%) in Ethiopia[19]. Generally, SM positive rates may vary depending on specimen quality, technician skill, and the number of samples treated in a day. In the latter case, having fewer samples may allow the technicians to observe the microscopy images more thoroughly.

The PaxView TB/NTM MPCR-ULFA Kit incorporates multiplex PCR and universal lateral flow assay (ULFA) and uses the IS*6110* and *mtp40* genes for TB detection. Theoretically, the occurrence of multiple copies of IS*6110* within the chromosomal DNA of MTB could increase test sensitivity when only a few of bacilli are present in a specimen compared that of single-copy genes such as *rpoB* gene or *mpt40*. However, given that strains lacking IS*6110* have been identified in some countries (e.g., India), alternative PCR targets are necessary, and *mpt40* can be a good candidate in such strains[20]. The *mpt40* gene is exclusively present in MTB, i.e., not in *M. bovis*, *M. bovis* BCG, or NTM[21]. Therefore, the *mpt40* gene is an excellent alternative PCR target to detect MTB bacilli in suspected TB-infected specimens. Unfortunately, some MTB strains do not possess an *mpt40* gene in their chromosomal DNA[22]. In these strains, the IS*6110* gene can serve as an alternative PCR target. Therefore, the use of multiple complementary PCR targets for IS*6110* and *mpt40* is critical to increase MTB detection sensitivity, as well as to accurately discriminate between MTB and NTM[23]. For this reason, the PaxView TB/NTM MPCR-ULFA kit uses these two genes as MTB detection targets.

We could not identify strains that contained exclusively *mtp40* bands without the co-occurrence of IS*6110* bands in this clinical trial, meaning that all tested strains had IS*6110* copies in a given chromosome. However, the opposite happened in some strains. For example, in **Figure 3**, lane 4 and 6 showed only band 1 without band 2, whereas lane 3 exhibited band 1 and band 2. This highlights the advantage of having multiple copies of IS*6110* in a given sample rather than only single-copy genes.

We found 2 invalid results (1.3%) out of 150 TB suspected samples, both of which were SM negative and Xpert MTB/RIF negative. This may be caused by incomplete removal of PCR inhibitors in the sputum samples during DNA extraction. Therefore, these two samples were excluded from the comparative analyses.

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Figure 3. Results of PaxView TB/NTM MPCR-ULFA Kit.

Some cases of NTM were detected with the PaxView TB/NTM MPCR-ULFA Kit; however, NTM samples were excluded in comparative analyses, as SM and the Xpert MTB/RIF cannot detect NTM.The newly-developed PaxView TB/NTM MPCR ULFA Kit incorporates multiple polymerase chain reactions and simplifies the result-reading process by implementing ULFA instead of a cumbersome electrophoresis procedure. Another advantage of the PaxView MPCR-ULFA Kit is that it only requires a standard PCR device. In contrast, the implementation of XpertMTB/RIF requires specialized and expensive equipment (**Table 2**).

ULFA was designed as follows. One strand of the primer set was designed to link a universal probe that can hybridize to an immobilized oligomer at lines 1~ 4 on the membrane. This universal probe cannot be made into a double-strand during the PCR process due to interruption by a spacer. The other primer strand set was linked with biotin, which can bind with nano gold-streptavidin when the PCR product comes in contact with the sample pad. The PCR product then flows upward through the absorbent pad through the force of running buffer. During its flow, a universal probe binds with its specific complementary oligomers, which were previously immobilized on the membrane (**Fig. 1**). Usually, within 5 minutes of adding the PCR product to the pad, a gold color is revealed on the line. However, PaxGenBio recommends waiting 15 minutes for the final results. This procedure markedly shortens PCR product reading time and, compared to gel electrophoresis, does not require preparing a gel and reading it on a UV transilluminator.

Although in-house PCR or MPCR targeting IS*6110* has satisfactory sensitivity and cost-effectiveness compared to the Xpert MTB/RIF targeting the *rpoB*gene, in-house PCR has the disadvantages of being time-consuming and requiring a cumbersome gel electrophoresis step. Therefore, the PaxView TB/NTM MPCR-ULFA Kit substitutes the gel electrophoresis step with ULFA for rapid PCR result detection.

The Xpert MTB/RIF has many advantages such as its simplicity and automatic procedures without requiring manual DNA extraction and reading PCR results. This considerably reduces the risk of sample contamination, as well as operation times. However, the reagent cost of the Xpert MTB/RIF amounts to approximately 10 USD per test in developing countries and up to 40 USD in other countries. Therefore, many developing countries implement this procedure only if financial support (e.g., Global Fund) is provided. The sensitivity of this assay is also lower than that of in-house MPCR using IS*6110* as a target (**Table 2**). Therefore, a cost-effective, user-friendly, and highly sensitive and specific TB diagnostic tool continues to be critically necessary.

Table 2. Pros and cons of various molecular MTB detection tools

	In-house PCR	PaxView MPCR-ULFA	Xpert MTB/RIF
Sensitivity compare to Xpert	Higher	Higher	-
Reagent Cost (US$)	1	3	10~40
Conveniencecompared to Xpert	10%	50%	100%
Equipment	General PCR	General PCR	Specialized
Equipment cost	Low	Low	Very high
Ventilation system	Very necessary	Very necessary	Necessary
Skillful technician	Very necessary	Very necessary	Necessary

Conclusion

In conclusion, the PaxView MPCR ULFA Kit has an excellent capacity for the detection of MTB in clinical specimens, and this kit could be implemented in laboratories with standard PCR equipment, bypassing the need to purchase expensive equipment. Moreover, the results of this kit can be obtained easily and quickly, without the need

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for unpractical electrophoresis procedures. Therefore, this approach could be very useful to detect MTB in clinical samples in conventional molecular laboratories in resource-limited countries.

Limitation and Future Studies

There are some limitation of the study.First, this PCR method doesnot detect any drug resistance genes. Second, mycobacterial culture was not done, so perhaps its performance will improve when compared with culture as standard reference.

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