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Running head
Performance of Xpert MTB/RIF Assay for Pulmonary Tuberculosis Detection

Keywords
Mycobacterium tuberculosis, Xpert MTB/RIF, AFB smear, mycobacterial culture, rifampin resistance

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SUMMARY

This study aimed at evaluating performance of Xpert MTB/RIF (Xpert) regarding detection of pulmonary tuberculosis compared to acid-fast bacilli (AFB) smear and culture, and concordance of rifampin resistance with drug susceptibility test. Specimens simultaneously referred for AFB smear, culture, and Xpert during April 2015 to March 2018 were retrospectively reviewed. Sensitivity, specificity, and mean cycle-threshold (Ct) values of Xpert and rifampin resistance results were analyzed. Results of Xpert for pulmonary tuberculosis were evaluated by AFB smear grade. Among the total of 3,840 specimens, 491 were positive in Xpert and 626 were positive in culture. Sensitivity and specificity of Xpert was 75.6% and 99.4%, respectively. Sensitivity of Xpert in smear-positive/culture-positive specimens was 98.6% and those of smear-negative and trace/culture-positive was 63.1%. Positivity of Xpert in culture-positive specimens were 89.9%/98.6%/95.7%/100.0%/100.0% in smear grade trace/1+/2+/3+/4+. Ct values of 491 specimens significantly lowered as AFB smear grade increased (p<0.0001). Ct of smear-positive/smear-trace/smear-negative specimens were 21.7 ± 4.2/26.5 ± 3.9/27.4 ± 3.6, respectively. Rifampin resistance tested by Xpert and culture was 98.3% concordant. Region covered by probe E was the most frequently mutated (50.0%). Xpert showed reliable performance in detecting pulmonary tuberculosis in smear-positive/culture-positive specimens and further improvements are needed for smear negative/culture positive specimens.
INTRODUCTION

Tuberculosis (TB) is one of the leading causes of death worldwide with high mortality and morbidity. In 2019, according to a report by WHO, there were 10.0 million cases of newly diagnosed TB and 1.2 million deaths among HIV-negative patients (1). In Korea, TB is endemic with 59 new cases per 100,000 people in 2019, one of the highest rates in the world, and is still a serious health problem in spite of national TB control plan (2). Acid-fast bacilli (AFB) smear-based microscopic examination of the sample has relatively short turn-around time than that of the culture. However, it does not differentiate Mycobacterium tuberculosis complex (MTBC) from nontuberculous mycobacteria (NTM), and has low sensitivity (3). For rapid detection of MTBC, several polymerase chain reaction (PCR) based molecular diagnostic methods have been developed.

The Xpert MTB/RIF assay (Cepheid, CA, USA) is a MTBC-specific qualitative real-time PCR assay, which simultaneously detects rifampin resistance (4). It is a rapid user-friendly automated system with cartridge-based nucleic acid amplification assay that requires less manual handling, and was endorsed by WHO in 2011 for the initial diagnosis of TB (5). It detects MTBC based on the cycle-threshold (Ct) of the first positive probe; therefore, it can provide semi-quantitative results (6). Recent studies on the relationship between smear grade and the PCR positivity showed good correlation between AFB smear and positivity of molecular assay, where molecular assay exhibited even higher sensitivity than smear, thereby becoming a possible substitute of smear microscopy (7, 8). There were studies regarding relationship between Ct values and AFB smear grade (9) and relationship between Ct values and AFB smear positivity (10). However, there has been no report that compares positivity and Ct values of Xpert MTB/RIF assay to mycobacterial culture and smear grade, and even
rifampin resistance for all specimens commissioned in the laboratory, not selected samples.

The aims of this study were to evaluate the performance of Xpert MTB/RIF assay for MTBC detection compared to the mycobacterial culture and AFB smear grade, and to analyze the concordance of resistance results compared with the phenotypic drug susceptibility test (pDST) using real-world results tested for 3 years in a clinical microbiology laboratory. For these purposes, \( C_t \) values of respiratory specimens were analyzed according to the AFB smear grades. In addition, upon detection of rifampin resistance, resistance-causing probes were also analyzed.

**MATERIALS AND METHODS**

**Study subjects:** This study was conducted at Asan Medical Center, a 2700-bed tertiary hospital in Seoul, Korea. All of the specimens, which were simultaneously referred for smear microscopy, culture, and Xpert MTB/RIF to the clinical microbiological laboratory during April 2015 to March 2018, were retrospectively enrolled. Drug susceptibility test for MTB was carried out for MTBC positive specimen. This work was approved by the Institutional Review Board. Informed consent was waived by the Institutional Review Board of Asan Medical Center because this study was performed retrospectively without requirement of any extra clinical specimens.

**Smear microscopy:** All the specimens were examined by auramine-rhodamine fluorescent stain, following Ziehl-Neelsen staining for confirmation of AFB on smear according to the CLSI M48-A guideline (11). The AFB smears were examined and graded according to the American Thoracic Society/Centers for Disease Control and Prevention guidelines (12). We used “trace” grade when 1-2 AFB per 300 fields are seen in the AFB smear.
**Xpert MTB/RIF assay:** Xpert MTB/RIF cartridge with GeneXpert Dx system (Cepheid, CA, USA) were used in this study. The assay was performed directly on clinical samples according to the manufacturer’s instructions and previously described method (4). Sensitivities and $C_t$ values of MTBC-positive specimens were analyzed according to the AFB smear grades. Distribution of probes responsible for rifampin resistance were analyzed. Upon detection of positive signals for the specimens that resulted in NTM in the culture, the type of probe and species information of the NTMs were identified.

**Mycobacterial culture and drug susceptibility test:** Mycobacterial culture was carried out using both solid 2% Ogawa medium (Korean Institute of Tuberculosis, South Korea) for 8 weeks and BACTEC MGIT liquid media (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD) for 6 weeks. Upon obtaining positive result in liquid media or colony growth on solid medium, Ziehl-Neelsen staining and duplex PCR using Seeplex TB detection (Seegene, Seoul, Korea) or Advansure TB/NTM real-time PCR (LG Chemistry, Seoul, Korea) were performed for differentiation between MTBC or NTM. For specimens that grown MTBC, drug susceptibility test was performed by both pDST and genotypic drug susceptibility test. Colonies of MTBC were sent to the Korean National Tuberculosis Association for pDST. The absolute concentration methods described by Canetti et al. (13) using Löwenstein-Jensen agar were used for pDST. Genotypic drug susceptibility test was performed using GenoType MTBDRplus assay (Hain Lifescience, Nehren, Germany) as described previously (14). $rpoB$ sequencing for identification of disputed $rpoB$ mutation was performed as described previously (15). The results of Xpert MTB/RIF were analyzed and compared to those of culture-based tests as gold standard. When Xpert MTB/RIF was positive whereas culture and AFB smear resulted negative, clinical information including chest-imaging study were evaluated.
**Statistical analysis:** Sensitivity and specificity of the AFB smear and Xpert MTB/RIF were calculated using Excel (Microsoft Excel 2013) based on the culture results. *t*-test using MedCalc (Medcalc, Mariakerke, Belgium) was used for comparison of *C*ₜ values of Xpert MTB/RIF-positive/smear-positive and Xpert MTB/RIF-positive/smear-negative. Trend of *C*ₜ values of Xpert MTB/RIF and smear grade was analyzed using Spearman correlation analysis using SPSS software version 19.0 (SPSS, Chigaco, IL, USA). Correlation between Xpert MTB/RIF results and AFB smear grade and significance of the mean *C*ₜ values of the Xpert MTB/RIF grouped by AFB smear were analyzed by Pearson’s Chi-square test using SPSS software version 19.0 (SPSS, Chigaco, IL, USA).

**RESULTS**

**General characteristics:** Total 3,840 specimens including 3,813 lower respiratory samples, 26 lung biopsies, and 1 gastric lavage were collected. By culture, 626 specimens (16.3%) were MTBC positive. Among 3,214 MTBC-negative specimens in cultures, 477 specimens were NTM-positive. The Xpert MTB/RIF assay was positive in 491 specimens (12.8%). AFB smear was positive in 348 (9.1%), trace in 201 (5.2%), and negative in 3,291 specimens (85.7%). Among 348 AFB smear-positive specimens, numbers of 1+, 2+, 3+, and 4+ were 114, 90, 75, and 69, respectively.

**Comparison of Xpert MTB/RIF assay and AFB smear:** Among 491 Xpert MTB/RIF assay-positive specimens, 473 were culture-positive for MTBC and 18 were culture-negative. Among all Xpert MTB/RIF assay-positive specimens, 217 (44.2%) specimens were smear-positive, while 274 (55.8%) were smear-negative. Among 626 MTBC culture-positive specimens, 220 (35.1%) were smear-positive, 89 (14.2%) were smear-trace, and the remaining
317 (50.6%) were smear-negative. Sensitivities of Xpert MTB/RIF in culture-positive specimens were 98.6%, 89.9%, and 55.5% in AFB smear-positive, AFB smear-trace, and AFB smear-negative specimens, respectively (Table 1). The positivity of the Xpert MTB/RIF was significantly correlated to the AFB smear grade \((p<0.0001)\) (Table 1).

**C\(_t\) values of Xpert MTB/RIF and AFB smear grade:** Mean probe C\(_t\) value of 491 specimens, which were shown as positive in Xpert MTB/RIF was 24.9 ± 4.76. Those of specimens which were also positive in the AFB smear were significantly lowered as AFB grade increased as follows \((p<0.0001)\): 24.6 ± 3.95 in smear 1+, 21.8 ± 3.38 in smear 2+, 21.1 ± 3.25 in smear 3+, and 18.4 ± 3.14 in smear 4+. Correlation coefficient was -0.660 between mean probe C\(_t\) value and AFB grade. There were 81 specimens which were positive in Xpert MTB/RIF and showed trace at AFB smear, and their mean C\(_t\) value was 26.5 ± 3.93 (Figure 1). Mean C\(_t\) value of Xpert MTB/RIF-positive but smear-negative specimens was 27.4 ± 3.6 \((p<0.0001)\).

**Resistance profile comparison of Xpert MTB/RIF and pDST:** Total 412 specimens that were detected MTBC-positive by Xpert MTB/RIF undergone pDST with conventional methods. Four hundred and five (98.3%) specimens showed concordant results in two test methods. Seven specimens had discordant test results, five of which were rifampin resistance by Xpert MTB/RIF but were susceptible to rifampin by pDST, while the remaining two showed resistant to rifampin by pDST but no resistance detected by Xpert MTB/RIF. Among five specimens that showed rifampin resistance only by Xpert MTB/RIF, two were wild type, the other two had \(rpoB\) disputed mutations (L511P and D516V), and the remaining one had D516Y mutation. The latter two specimens with rifampin resistance only by pDST showed wild type in one specimen, and wild type losses in the other specimen by GenoType MTBDR\textit{plus}. There
were total 553 specimens that undergone pDST, of which 39 specimens (7.1%) had rifampin resistance. Among these specimens, which showed rifampin resistance in pDST, 31 specimens were also resistant to rifampin in Xpert MTB/RIF (78.5%), two specimens showed no resistance in Xpert MTB/RIF, and the rest of six resulted in negative MTBC (Table 2). Five specimens resulted ‘indeterminate’ in rifampin resistance by Xpert MTB/RIF, and their pDST revealed all as susceptible to rifampin.

The largest number of probe associated with rifampin resistance was probe E, which comprised 19 specimens (50.0%), followed by probe B in 10 specimens (26.3%), probe D in 5 specimens (13.2%), and the rest with probe C (5.3%) and probe A (5.3%). In one specimen, which had grown MTBC in culture, positive signal was generated only in probe B with C\textsubscript{t} value of 39.7, and it showed ‘MTBC not detected’ in Xpert MTB/RIF.

**Analysis of probe signal in specimens of NTM growth:** There were 477 specimens of culture-proven NTM in this study. Among these, 24 generated one positive probe signal in their Xpert MTB/RIF tests including 22 in probe A and 2 in probe C. The former 22 were all revealed as *Mycobacterium intracellulare*, and the latter two were identified as one *Mycobacterium terrae* and one *Mycobacterium kansasii*, respectively.

**Clinical diagnosis of discordant Xpert MTB/RIF/culture specimens:** There were 18 specimens, which showed positive in Xpert MTB/RIF but no growth in mycobacterial culture and no AFB seen in AFB smear (Table 3). Ten of them were graded as ‘very low’, seven as ‘low’, and the rest one as ‘medium’ in Xpert MTB/RIF. Chest imaging study including chest radiograph and computed-tomography revealed 16 of them as ‘active TB.’ The rest two were also diagnosed with TB, one by bronchial endoscopy and diagnosed with endotracheal TB, and the other by positive result in TB-PCR with bronchoalveolar lavage sample.
DISCUSSION

This study aimed to evaluate the performance of Xpert MTB/RIF for the detection of MTBC and rifampin resistance in pulmonary TB patients. AFB smear grade and Ct values of Xpert MTB/RIF showed good correlation. Previous study, which analyzed the relationship between AFB smear and Xpert MTB/RIF, has also shown strong correlation between two methods, and even Xpert MTB/RIF provided faster and more stable results than AFB smear (6). Another study in 2011 also revealed inversely linear correlation between Ct values and AFB smear grade (9). Lange et al. conducted meta-analysis to evaluate relationship between Ct value of Xpert MTB/RIF and AFB smear positivity. In this study, Xpert MTB/RIF and AFB smear were strongly associated. However, they could not find cut-off Ct values, which could replace AFB smear microscopy (10).

In this study, smear-negative specimens and smear-trace specimens in the culture-positive specimens comprised 50.6% (317/626) and 14.2% (89/626), respectively. The rate of smear-negative but culture-positive specimens varies according to regions, from 25.6% to approximately 60% (16-18). It is well known that TB can be transmitted from patients with smear-negative results (19); therefore, the efforts to detect MTBC in those patients are needed to prevent further transmission. Molecular diagnosis of MTBC, including Xpert MTB/RIF, may be a way for early diagnosis of smear-negative TB patients. The sensitivity of smear negative and smear-trace specimens, which were culture-positive for MTBC was 63.1%, which did not match the sensitivity (72%) suggested by the guidance for industry and FDA staff (20), indicating that further improvements are needed in specimens of smear negative/MTBC culture positive. Since WHO has recommended the use of next-generation MTB/RIF assay (Cepheid,
CA, USA) as a replacement for the Xpert MTB/RIF in March 2017, improvements in detecting specimens of smear negative/MTBC culture positive are expected (21).

In our results, there were 18 discordant specimens, which showed Xpert MTB/RIF positive but no growth in mycobacterial culture. The AFB smear of those all resulted negative except one that showed trace. However, based on clinical information, they were all diagnosed as TB. Even though there were no growth in culture and almost all were negative in AFB smear, other test findings were in support of clinical TB. With these clinical evidences of TB, the results of Xpert MTB/RIF, which were classified as discordant Xpert MTB/RIF and culture, can be considered all true-positive. Previous study has shown that Xpert MTB/RIF could not be more sensitive than cultures (22), but recent study reported that it could be more sensitive for MTBC detection from saliva, sputum, and specimens from previously treated patients (23) and it will help in diagnosis of smear-negative TB. Kim et al. compared Xpert MTB/RIF and TB real-time PCR, and in the study diagnostic sensitivity of Xpert MTB/RIF is higher in smear-positive TB (93.8% vs. 87.5%) and lower in smear-negative TB (75.9% vs. 65.5%) than those of TB real-time PCR (24). In HIV prevalence setting, Xpert MTB/RIF especially showed superior performance than AFB smear, line probe assay, and real-time PCR (25).

In rifampin resistance, Xpert MTB/RIF and culture showed consistent results with concordant rate of 98.3% (405/412). Previous studies stated that Xpert MTB/RIF provides accurate results for rifampin resistance (26), with sensitivity and specificity of detecting rifampin resistance up to 94.1% and 97.0% (27), respectively. The rifampin-resistance in Xpert MTB/RIF could be resulted from disputed mutations located in the core region of the *rpoB* gene (28). In this study, there was a disputed *rpoB* mutation in two specimens with rifampin-resistance only by Xpert MTB/RIF. There could be discrepancies between genotypic
susceptibility test and pDST in isolates with disputed mutations (29).

The indeterminate results of rifampin resistance among culture-proven MTBC specimens in Xpert MTB/RIF were generated in five specimens in this study, and they all showed “very low” category in Xpert MTB/RIF. One study analyzed the indeterminate results generated by Xpert MTB/RIF (30), in which the rate of indeterminate result was 2.9% when tested with direct sputum samples, and the pDST needed to resolve the indeterminate cases (30). Another study showed indeterminate result in 3.7% of specimens, which dropped to 1.2% when one test was repeated with the same specimen of indeterminate results (31). In this study, 5 out of 473 specimens (1.1%) resulted in indeterminate, which was slightly lower than previous studies. Further tests, such as pDST or repeated Xpert MTB/RIF, could resolve indeterminate result as previous studies.

Five probes in the Xpert MTB/RIF cover the 81 nucleotide long sequence of the core region of rpoB gene. Because more than 95% of all rifampin resistant mutations occurred within the core region, it is highly predictable target to detect rifampin resistance (32). In this study, half of the rifampin resistance mutations were caused by dropout of probe E, which covers nucleotide sequence or rpoB gene from 11214 to 11230, and approximately 26% were caused by probe B. By this result, we could assume that the mutation in the region of 11214 to 11230 in rpoB gene causes approximately 50% of rifampin resistance in Korean population. Resistant causing probe distribution varies by geographical region. For example, dropout of probe E caused rifampin resistance in 64.9 % (133/205) in a Bangladeshi study (33) and 77.0 % (314/408) in a Pakistan study (34). Meanwhile, in American study, main probes for rifampin resistance were both probe E (37.0 %) and also probe D (34.1 %) (35). No previous study has been conducted regarding a specific mutation site in rpoB gene in Korean population and
further study with more data is needed for estimating the mutation hotspot within *rpoB* gene in Korean population. Unfortunately we did not sequence *rpoA* and *rpoC*, in which compensatory mutations were reported (36). For discrepant cases between Xpert MTB/RIF and pDST, whole genome sequencing including *rpoA* and *rpoC* could reveal further mechanisms other than *rpoB* mutations.

Interestingly, all 22 *M. intracellulare* showed positive only in probe A and not in other probes. Probe A covers 18-nucleotide long sequence, “CGCCGACTGTCGGCGCTG,” and 16 of these nucleotides matched to *M. intracellulare* subspecies yougonense 05-1390 chromosome. Probe A appeared to be positive in *M. intracellulare*, probably because of the shared 16 nucleotides between the two sequences. The technician who perform the test easily overlook the result when only one probe is positive because two or more probes must be positive to detect MTBC in Xpert MTB/RIF. If clinical infection from NTM were suspected, it would be helpful as a tool to predict the species of NTM before ordinary identification result carried out.

In conclusion, Xpert MTB/RIF showed reliable performance in detecting pulmonary TB in smear-positive/MTBC culture positive specimens and concordant well with clinical diagnosis of TB in MTBC culture-negative specimens. Further improvements are needed in specimens of smear-negative/MTBC-culture positive for better performance.

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**Conflict of interest** None to declare
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Figure legends

Figure 1. Box-plot of the mean cycle-threshold (Ct) values of the positive specimens using
Xpert MTB/RIF by AFB smear grade

*Analyzed using Spearman correlation analysis

Abbreviations: Ct, cycle-threshold
Table 1. Sensitivities of Xpert MTB/RIF in culture-positive specimens when AFB smear results are divided into negative, trace, and positive

| AFB smear         | Sensitivity of Xpert MTB/RIF | p value* |
|-------------------|-----------------------------|---------|
| Positive          |                             |         |
| AFB smear-1+      | 98.6 % (217/220)            |         |
| AFB smear-2+      | 98.6 % (69/70)              |         |
| AFB smear-3+      | 95.7% (45/47)               |         |
| AFB smear-4+      | 100.0 % (52/52)             |         |
| Trace             | 89.9 % (80/89)              |         |
| Negative          | 55.5 % (176/317)            |         |
| Overall           | 75.6 % (473/626)            | <0.0001 |

*Analyzed using Pearson’s Chi-square test
Table 2. Comparison of rifampin resistance tested with culture-based phenotypic drug susceptibility test and Xpert MTB/RIF

| Rifampin resistance                                    | Number |
|--------------------------------------------------------|--------|
| Phenotypic DST-resistant/Xpert MTB/RIF-resistant       | 31     |
| Phenotypic DST-resistant/Xpert MTB/RIF-susceptible     | 2      |
| Phenotypic DST-resistant/Xpert MTB/RIF-ND              | 6      |
| Phenotypic DST-susceptible/Xpert MTB/RIF-resistant     | 5      |
| Phenotypic DST-NT /Xpert MTB/RIF-resistant             | 2      |

Abbreviations: DST, drug susceptibility test; ND, *M. tuberculosis* complex not detected; and NT, not tested
**Table 3.** Analysis of discordant specimens which showed positive Xpert MTB/RIF results but no growth in *Mycobacterium* culture

| No. | Grade of Xpert | Mycobacterial culture | AFB smear | Other test results including imaging study | Final interpretation |
|-----|----------------|-----------------------|-----------|--------------------------------------------|----------------------|
| 1   | Very low       | No growth             | No AFB seen | X-ray: R/O active TB with TB pleurisy     | Pulmonary TB         |
| 2   | Very low       | No growth             | No AFB seen | CT: R/O miliary TB                        | Pulmonary TB         |
| 3   | Very low       | No growth             | No AFB seen | CT: R/O reactivation of TB                | Pulmonary TB         |
| 4   | Very low       | No growth             | No AFB seen | X-ray: R/O reactivation of TB             | Pulmonary TB         |
| 5   | Very low       | No growth             | No AFB seen | CT: R/O active tuberculoma                | Pulmonary TB         |
| 6   | Very low       | No growth             | No AFB seen | X-ray: R/O active TB                      | Pulmonary TB         |
| 7   | Very low       | No growth             | No AFB seen | X-ray: R/O active TB                      | Pulmonary TB         |
| 8   | Very low       | No growth             | No AFB seen | X-ray: R/O reactivation of TB             | Pulmonary TB         |
| 9   | Very low       | No growth             | No AFB seen | X-ray: R/O active TB                      | Pulmonary TB         |
| 10  | Very low       | No growth             | Trace      | Active R/O endotracheal TB                | Pulmonary TB         |
| 11  | Low            | No growth             | No AFB seen | X-ray: R/O cavitory lung TB               | Pulmonary TB         |
| 12  | Low            | No growth             | No AFB seen | X-ray: R/O probable active TB             | Pulmonary TB         |
| 13  | Low            | No growth             | No AFB seen | CT: R/O active TB                        | Pulmonary TB         |
| 14  | Low            | No growth             | No AFB seen | TB-PCR with BAL: positive                 | Pulmonary TB         |
| 15  | Low            | No growth             | No AFB seen | X-ray: R/O active TB                      | Pulmonary TB         |
| 16  | Low            | No growth             | No AFB seen | X-ray: R/O active TB                      | Pulmonary TB         |
| 17  | Low            | No growth             | No AFB seen | CT: R/O active TB                        | Pulmonary TB         |
| 18  | Medium         | No growth             | No AFB seen | X-ray: R/O active TB                      | Pulmonary TB         |

Abbreviations: TB, tuberculosis; CT, computed tomography; TB-PCR, polymerase chain reaction for *Mycobacterium tuberculosis*; and BAL, bronchoalveolar lavage.
