***Mycobacterium tuberculosis* DNA Detection and Molecular Drug Susceptibility Test in AFB-stained Sputum Slides**

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Tuberculosis (TB) remains an unsolved community health problem since identification of its causing microorganism called *Mycobacterium tuberculosis* (MTB) by Robert Koch in 1882. Annually, eight million TB cases are newly reported and 2~3 million patients die from TB. Pulmonary TB is highly infectious and untreated pulmonary TB patients are believed to infect >10 people in a year. The conventional methods for diagnosis of TB are chest X-ray and isolation of the causing microorganisms from patient specimens. Screening of TB is conducted with smeared sputum in slides, and TB is confirmed by identification of MTB in cultured specimens. One of the fatal pitfalls of screening detection for smeared sputum is that it is impossible to distinguish MTB and other acid-fast bacilli (AFB) because they are stained equally with Ziehl-Neelsen (ZN) stain. Culture of MTB is the most reliable method for diagnosis of TB but it takes 4~8 weeks. In this report, we suggest a fast and highly-reliable MTB detection method that distinguishes AFB in sputum samples. Purified DNA from the AFB stained slide samples offered by The Korean Institute of Tuberculosis were used to detect infected MTB in patients. PCR, real-time PCR and reverse blot hybridization assay (REBA) methods were applied to purified DNA. Conclusively, the real-time PCR method was confirmed to produce high sensitivity and we were able to further detect drug-resistant MTB with REBA.

Key Words: MTB, Molecular DST, PCR, Real-time PCR, REBA, Molecular diagnosis

RESULTS AND DISCUSSION

Tuberculosis (TB) remains an unsolved community health problem since identification of its causing microorganism called *Mycobacterium tuberculosis* (MTB) by Robert Koch in 1882. According to the World Health Organization (WHO) report there were eight million new TB cases and deaths of

two to three million patients by TB in a year. Forty thousand new TB cases and three thousand deaths occur in the Republic of Korea every year (Dye et al., 1999; Lew et al., 1995). Microscopic detection method for smeared sputum is a conventional method that detects acid-fast bacilli (AFB) using Ziehl-Neelsen (ZN) staining. However, it has low sensitivity: at least 5,000~10,000 AFB are required per ml for detection and is impossible to distinguish MTB from

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other AFB, such as non-tuberculous mycobacteria (NTM) due to the same staining pattern (Bate, 1979; Kent and Kubica, 1985). In contrast to the screening method, culture of MTB has high sensitivity: as low as $1 \times 10^{1-2}$ bacilli in specimen can be detected but it takes 4~8 weeks (Tenover

et al., 1993; Woods, 2011). This allows opportunity for contamination by other bacterium and also renders the sample hard to detect for MTB using these time-consuming culture methods (Heo and Kim, 2013).

One of the difficulties for treating TB is drug resistance.

Table 1. MTB PCR results with AFB stained slides

Number of AFB		CDC report	Number of AFB Slide	MTB PCR results
Ziehl-Neelsen ($\times 1000$)	Fluorochrome ($\times 250$)			
0	0	Negative	24	0/24 (0%)
1~2 of 300 fields	1~2 of 30 fields	Trace	11	3/11 (27.2%)
1~9 of 100 fields	1~9 of 10 fields	1+	10	7/10 (70%)
1~9 of 10 fields	1~9 of 1 field	2+	12	11/12 (91.6%)
1~9 of 1 fields	10~90 of 1 field	3+	10	10/10 (100%)

'Number of AFB' indicates number of stained bacteria under designated microscopic fields. 'CDC report' indicates categories defined by center for disease control USA. 'Number of AFB Slide' indicates stained sputum slides numbers used for PCR analyses. 'MTB PCR results' indicates positive percentage of MTB amplification.

(A) FAM (MTB)

(B) ROX (Myc)

(C) Cy5 (IC)

Fig. 1. Real-time PCR for MTB detection in slide specimens.

For real-time PCR assays, the Real MTB-ID® kit using the multi-probe real-time PCR TaqMan® was employed and were conducted with CFX-96 real-time PCR system (Bio-Rad, USA). For amplification, 10 μ L of 2 $\times$ Thunderbird probe qPCR mix (Toyobo, Japan), 3 μ L of primer and TaqMan® probe mixture, 5 μ L of template DNA, and 2 μ L ddH₂O were added into a PCR tube. FAM (fluorescein) and ROX™ dyes were applied to detect MTB DNA and *Mycobacteria* spp. (Myc), respectively. Cy5 dye was the internal control for the real-time PCR reaction.

Fig. 2. REBA for detection of antibiotic resistant AFB in stained slides. The REBA MTB-MDR® kit is composed of 13 probes for wild-type (WT) sequences, 6 probes for the mutated (MT) sequences known for isoniazid (INH) and rifampin (RIF) resistance, one probe for MTB and one probe for mycobacterial species (Myc). Hybridization process between the PCR products that contain biotin at their 5' end and the probes immobilized on a membrane was carried out according to the manufacturer's instructions. Briefly, denatured PCR products in 2X SSPE (150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA), 0.1% SDS were incubated with REBA MTB-MDR membrane strips at 50 °C on a Mini-Incubation Tray (Bio-Rad) for 30 min. After washing twice with 2X SSPE, 0.5% SDS at 65 °C for 10 minute, the strips were incubated with streptavidin conjugated with alkaline phosphatase in 2X SSPE, 0.5% SDS at ambient temperature, after which they were washed with 50 mM Tris · HCl (pH 7.5), 150 mM NaCl. The hybridized amplicons carrying biotin tag were detected by incubating the strips with nitro blue tetrazolium (NBT) and chloride and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 mM Tris · HCl (pH 9.5), 100 mM NaCl, 50 mM MgCl₂. (U: Unknown specimens, Rv: *M. tuberculosis* H37Rv)

In Korea, it is estimated that more than half of MTB patients exhibit no clinical symptoms and TB patients who showed drug-resistance are increasing (Korean Institute of Tuberculosis, 2009). Isoniazid (INH) and rifampin (RIF) are conventional antibiotics used for combinatorial therapy, but resistance toward these antibiotics is increasing. Therefore, selection of susceptible antibiotics is becoming important for early phase treatment of latent TB patients (Moore et al.,

1997; Frieden et al., 1993). For fast and accurate detection of TB and selection of antibiotics, current drug-susceptibility test (DST) for TB may not be adequate because it generally requires more than three weeks of culture (Quy et al., 2003). Up to date, many researchers have reported DNA-based methods for TB detection and antibiotics selection, such as polymerase chain reaction (PCR) and real-time PCR (Bang et al., 2011; Cho et al., 2013; Lee et al., 2015). The DNA-based methods have produced quite comparable results with those from bacterial culture methods (Bang et al., 2011; Gous et al., 2015; Munkhdelger et al., 2013; Pai and Schito, 2015).

Here we evaluated applicability of the DNA-based detection methods toward sputum slides for detection of MTB DNA and confirmation of molecular DST. Sixty-seven slides provided by The Korean Institute of Tuberculosis were grouped based on their AFB staining results: 24 negative, 11 trace, 10 AFB 1+, 12 AFB 2+, and 10 AFB 3+ (Table 1). DNA was purified from the slides using Trizol reagent following the manufacturer's protocol. Briefly, 200 µL of Trizol was dropped on the slides and was scraped into a tube twice. The purified DNA was used as template for PCR amplification of MTB-exclusive sequence (MTB amplicon) with MTB-ID v3® (M&D, Korea). There was no amplification of the MTB sequence in all of the AFB negative slides. Among the 11 trace slides, three slides (27.2%) produced the MTB amplicon. The 32 slides of AFB 1+ and AFB 2+ produced 70% and 91.6% MTB amplicons, respectively. All of the AFB 3+ slides produced 100% MTB amplicons (Table 1). Thus, production of the MTB amplicon and AFB positive score correlated well. To compensate the known drawbacks of conventional PCR methods, real-time PCR method called Real-time MTB-ID® (M&D, Korea) was applied to the same purified DNA. Both of the PCR and the real-time PCR methods amplified the same gene of the MTB. The real-time PCR method showed higher sensitivity: there was no amplification in the AFB negative slides; all of the trace and AFB positive slides, regardless of positive intensity, produced a MTB-positive signal and the *Mycobacterium* species (Myc) signal (Fig. 1). Next, the DNA samples were used to examine molecular antibiotic susceptibility with reverse blot hybridization assay (REBA) MTB-

Table 2. The results of MTB detection and antibiotic resistance results from the AFB slide specimens using molecular diagnostic methods

Specimens	AFB stain	PCR MTB detection	Real-time PCR MTB detection	REBA Molecular DST
1	Trace	IC	TB	TB
2		TB	TB	wt
3		TB	TB	TB
4		IC	TB	TB
5		–	TB	wt
6		IC	TB	TB
7		IC	TB	wt
8		TB	TB	wt
9		IC	TB	TB
10		IC	TB	TB
11		IC	TB	TB
12	1+	TB	TB	wt
13		IC	TB	wt
14		TB	TB	wt
15		TB	TB	wt
16		TB	TB	wt
17		TB	TB	wt
18		IC	TB	wt
19		IC	TB	wt
20		TB	TB	wt
21		TB	TB	wt
22	2+	TB	TB	wt
23		TB	TB	wt
24		TB	TB	wt
25		TB	TB	wt
26		TB	TB	wt
27		TB	TB	wt
28		–	TB	wt
29		TB	TB	wt
30		TB	TB	wt
31		TB	TB	wt
32		TB	TB	wt
33		TB	TB	wt
34	3+	TB	TB	wt
35		TB	TB	wt
36		TB	TB	wt
37		TB	TB	wt
38		TB	TB	wt
39		TB	TB	wt
40		TB	TB	wt
41		TB	TB	wt
42		TB	TB	wt
43		TB	TB	wt

'IC' indicated that only amplified internal control DNA by PCR reaction. '–' indicated did not showed any amplicon by PCR reaction. 'TB' indicated that amplified and detected MTB DNA. 'wt' indicated that detected MTB DNA without any mutation related antibiotic resistance.

MDR® (M&D, Korea). The entire purified DNA from the trace and AFB positive slides were determined susceptible like MTB H37Rv, a standard MTB strain susceptible to antibiotics (Fig. 2). These results were correlated with information of the patient who are antibiotic susceptible. Results for the 42 slides were summarized in Table 2.

Our results suggest that DNA purified from stained sputum slide might be useful for MTB detection, and MTB could be detected in short time with high sensitivity without bacterial culture, even, antibiotic susceptibility can be tested with DNA. Conclusively, AFB stained slides might be useful source for molecular detection and molecular drug susceptibility test of MTB.

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Conflict of interest

The authors declare that there is no conflict of interests.

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