INTRODUCTION

The mortality and incidence of tuberculosis (TB) in worldwide is 3% and 2% per year, respectively. 6.3 million new cases have been reported in 2016. The proportion of TB patients with multidrug-resistant tuberculosis (MDR-TB) among the new TB patients has increased, although the rate of TB deaths and incidence have decreased by the WHO-led global "The END TB strategy". In addition, 47% of all new cases were reported as MDR-TB or rifampicin.
resistant TB (RR-TB) in China, India, and Russia [1].

Successfully decreased mortality of TB patients is due to early diagnosis and appropriate treatment of the TB. The standard diagnostic method for tuberculosis is the drug susceptibility test through culture test. However, the incubation period of TB is more than 4 weeks. The molecular test method is most frequently used for early diagnosis for successful treatment up to date. These molecular tests are represented by nucleic acid based tests and development and evaluation of molecular tests such as PCR, real-time PCR, and line probe assays are underway [1,2].

Despite many molecular diagnostic methods for TB diagnosis have been developed, the molecular diagnostic methods approved by the US FDA to date has been limited. Largely, it is due to the requirement for Premarket Approval (PMA) as Class III for tuberculosis diagnosis reagents. In order to lower the entry barriers to reduce the time and cost for the approval process, it was re-graded to class II (special control) in 2013 and changed to pre-market notification (510(k)) [3].

The FDA has published a Class II special controls guideline on in vitro diagnostic reagents for detecting nucleic acid-based mycobacteria and tuberculosis antibiotic resistance-related gene mutations in respiratory specimens from respiratory specimens [4,5]. The guideline recommends confirmation of the detection of the MTB complex (M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, M. microti, and M. caprae) with 99% genetic homology. The guideline also recommend that cross reactivity is achieved by using $>10^6$ CFU/mL for mycobacteria, bacteria, fungi, $>10^5$ PFU/mL for virus, and $>10^6$ inclusion forming unit (IFU)/mL. When there is cross reactivity, it is required to describe the minimum concentration. Positive cut-off is based on receiver operating curve (ROC) analysis in a pilot study using clinical samples [4,5]. In addition, the collection of specimens, the storage of specimens, the transportation of specimens, the storage of reagents and transportation of reagents are required to report.

Pulmonary disease caused by Non-tuberculous mycobacteria (NTM) is caused by opportunistic infection. It is susceptible to NTM infection when there are problems with immunity such as bronchiectasis, cystic fibrosis (CFD), chronic obstructive pulmonary disease (COPD) and HIV infection. Recently, there has been an increasing trend of pulmonary disease due to NTM. According to the American Lung Association, 50,000 to 90,000 lung infections by NTM have been reported in the United States [6]. There are approximately 150 non-tuberculous mycobacterial species known, including M. abscessus, M. kansasii, M. abscessus complex, and Mycobacterium avium complex (MAC) [6-8]. Treatment of NTM pulmonary disease depends on the species of bacteria. Therefore, the bacteria must be identified [8].

To ensure the safety and efficacy of in vitro diagnostic devices (IVDs) before they are commercialized and marketed, the regulatory requirements for products such as reagents and systems from the Food and Drug Administration (FDA) (https://www.fda.gov/medicaldevices/deviceregulationandguidance/) should be considered. In this review, we compared the molecular tests of tuberculosis and NTM approved by the US FDA and compare the main methods currently under development.

MATERIALS AND METHODS

1. US FDA guidelines

FDA documents related to TB in vitro molecular assay approval such as reclassification of TB molecular assay, controls of Class II to molecular assay samples and mutation, and molecular assay for non-tuberculosis were summarized and compared the Korean approval for TB molecular assay.

2. Data collection

National Library of Medicine (Pubmed) database using key word 'Tuberculosis' and 'in vitro molecular diagnostic assay' was used. For the literature analysis, papers concerning US FDA approved TB and NTM molecular assays were selected. The sample characteristics and size, sensitivity and specificity of each TB and NTM molecular
3. Statistical analysis

The average of sensitivities and specificities of TB and NTM molecular assays were analyzed by GraphPad Prism 6 software (La Jolla, CA, USA).

RESULTS

1. Current in vitro diagnostic (IVD) medical device of TB

Nucleic acid-based in vitro diagnostic reagents for diagnosing tuberculosis from respiratory specimens classified as Class II have been the Amplified Mycobacterium tuberculosis Direct (MTD) test (Gen-Probe Inc.), Amplicor Mycobacterium tuberculosis (MTB) test (Roche Inc.), and Xper MTB/RIF assay (Cepheid) to date. It described in Table 1.

Amplified MTD test is a transcription mediated amplification (TMA) method for measuring fluorescence through Hybridization protection assay (HPA) to detect Mycobacterium tuberculosis ribonucleic acid (rRNA). The analytical sensitivity was presented as 1 CFU/test. Cross reactivity of Mycobacterium celatum and Mycobacterium terrae species was reported in a specificity test of 30 NTMs and 129 microbial species.

Amplicor MTB test is a test for measuring fluorescence after DNA amplification of 16S rRNA by polymerase chain reaction (PCR) and hybridization with DNA probe. The detection limit of Amplicor MTB test is ≥10 CFU/test (≥ 450 CFU/mL). The cross reactivity was not reported in the specificity test for 41 NTMs, 96 bacteria and 9 viruses. False negative was reported in presence of a small amount of MTB (2 X LoD) at high concentrations of M. avium, M. intracellulare, M. kansasi, M. gordonae, Corynebacterium spp., Gordona spuri and Rhodococcus bronchialis (＞10^5 ∼ 10^7/mL).

The Xpert MTB / RIF assay is based on a real-time PCR-based method for detecting MTB complex and the presence or absence of mutations in the core region of the rpoB gene associated with rifampin resistance using a molecular beacon probe. The detection limit of the Xpert MTB / RIF assay reported in the literature was 5×10^2 to 4×10^3 CFU/mL and the cross-reactivity was reported over 10^7 CFU/mL of M. scrofulaceum in the specificity test for

Table 1. Nucleic acid based MTB complex tests

| Trade Name | FDA No. | Class  | Method                                      | Target  | Sensitivity | Specificity |
|------------|---------|--------|---------------------------------------------|---------|-------------|-------------|
| Mycobacterium tuberculosis Xpert MTB/RIF Assay | K143302 | Class II | Real-time PCR                               | rpoB    | 93.8% (439/468), 98.7% (620/628) | 94.7% (18/19), 99.0% (404/408) |
| Amplicor Mycobacterium tuberculosis Direct Test | P940034 | Class II | Transcription mediated amplification (TMA) and Hybridization protection assay (HPA) | rRNA    | 93.2% (109/114) | 98.8% (414/419) |
| SNAP M. tuberculosis complex | K900292 | Class I | PCR, Hybridization                           | 16S rRNA | 95% (134/141) | 100% (48/48) |
| BD Proctec ET Mycobacterium tuberculosis complex culture identification kit | K000884 | Class I | NAAT, DNA probe                             | NR      | NR          | NR          |
| Accuprobe Mycobacterium tuberculosis complex Test | K896493 | Class I | Line probe Assay                            | NR      | 99.2%       | 99.9%       |
| Rapid Diagnostic System for Mycobacterium tuberculosis | K871795 | Class I | Line probe Assay                            | NR      | NR          | NR          |
| Rapid Identification Test for Mycobacterium tuberculosis complex | K862614 | Class I | Line probe Assay                            | NR      | NR          | NR          |

a, b, and c are for Class II documents from FDA. The source of documents were provided by FDA [9], [10], and [11], respectively. Abbreviations: NR, Not reported in document; d, a sensitivity for MTB complex; e, a sensitivity for Rifampin assay.
24 NTM and 87 bacteria, 7 fungi and 14 viruses. In silico tests of 18 other organism genomic databases, cross-reactivity was predicted in *M. kumamontonense*, *M. leprae*, *M. mucogenicum*, *Tsukamurellar* spp., and *Nocardia ootidiscaviarum*. The positive cut-off probes for rifampin resistance were cycle threshold (Ct) 36 for probe A, B, and C and Ct 39 for probe D and E.

The overall sensitivity and specificity of the amplified MTD test were 93.2% (109/114) and 98.8% (414/419), respectively. The sensitivity for smear positive samples and smear negative samples were 97.4% (76/78), 84.6% (33/39), respectively. When the test was repeated twice, the sensitivity increased from 87.5% to 96.9% for smear positive samples and from 64% to 72% for smear negative samples. The specificity was changed from 100% to 100% for smear positive samples and 100% to 99.1% for smear negative samples. Positive predictive value (PPV) was changed from 100% (28/28) to 100% (31/31) for smear positive samples and from 100% (16/16) to 94.7% (18/19) for smear negative samples. Negative predictive value (NPV) was changed from 63.6% (7/11) to 100% (31/31) for smear positive samples and from 87.5% (7/8) to 95.3% (141/148).

The clinical study of the Amplicor MTB test was designed for 1,833 pre-treatment patients from multiple institutions and the prevalence of tuberculosis was 5.3%. The clinical sensitivity of the Amplicor MTB test was 95% (134/141) and the specificity was 100% (48/48) in 189 specimens from 95 patients with double smear positive. Positive predictive value (PPV) was 100% (134/134) and negative predictive value (NPV) was 87.3% (48/55).

The sensitivity and specificity of the Xpert MTB / RIF assay were 93.8% (439/468) and 98.7% (620/628) in 1,096 specimens. Of these, both sensitivity and specificity for smear positive samples was 99.7% (350/351). The sensitivity and specificity for smear negative samples were 76.1% (89/117), 98.8% (555/562), respectively. The sensitivity and specificity of the rifampin test was 94.7% (18/19) and 99.0% (404/408) compared to the rifampin susceptibility test (DST).

In another clinical study performed in multicenter, 980 samples were analyzed except for culture failure, culture contamination and non-determinate results for the Xpert MTB / RIF assay. The sensitivity increased from 81.4% (175/215) to 88.1% (192/218) when the Xpert MTB / RIF assay was performed duplicate. 14 negative results and 3 non-determinate results were further derived as positive results. The specificity was slightly reduced from 98.7% (735/745) to 97.9% (746/762) as 17 non-determinate results were obtained. Positive predictive value (PPV) was changed from 94.9% to 93.3% and negative predictive value (NPV) was changed from 97.6% to 98.5%.

In addition, the nucleic acid-based tuberculosis diagnostic kit includes the AccuProbe *Mycobacterium tuberculosis* complex test (Gen-Probe Inc.), the rapid Diagnostic System for *Mycobacterium tuberculosis* (Gen-Probe Inc.), the Rapid Identification Test for *Mycobacterium tuberculosis* complex (Gen-Probe Inc.), SNAP *M. tuberculosis* complex (Syngene Inc.) and BDProbeTec ET *Mycobacterium tuberculosis* complex culture identification kit (BD & Co.) using nucleic acid amplification and DNA probes were reported to Class I before 1990, and are currently rarely used (https://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics).

2. Current IVD of NTM

NTM test kits based on nucleic acid are reported only in the Class I. The line probe assay method reported for detecting non-tuberculous mycobacterial species in the 1990s (Table 2). Recently, INNO-LiPA Mycobacteria v.2 (Innogenetics), Genotype *Mycobacterium* CM, and Genotype *Mycobacterium* AS (Hain lifesience Inc.) have been developed for the screening of major non-tuberculous mycobacterial species, but has not been reported to the FDA. Especially, INNO-LiPA Mycobacteria v.2 showed 98.8% specificity and 97.6% accuracy in 73 NTM and 21 microbial species tests. Previous studies on evaluation of the in vitro diagnostic reagents of NTB was performed using culture sample. FDA-approved Accuprobe avium complex showed 87.4% for overall specificity. For INNO-LiPA Mycobacteria v.2 non-FDA-approved commercial reagent, the specificity was 96.3%. For Genotype
Table 2. Nucleic acid based Mycobacterium species identification tests

| Trade Name | FDA No. | Class | Method |
|------------|---------|-------|--------|
| Mycobacterium species | | | |
| Accuprobe Mycobacterium avium complex culture | K921435, K896494, K897078 | Class I | Line probe Assay |
| Accuprobe Mycobacterium kansasii Identification Test | K904463 | Class I | Line probe Assay |
| SNAP Mycobacterium avium complex | K900202 | Class I | Line probe Assay |
| Accuprobe Mycobacterium intracellulare Culture Identification Test | K897077 | Class I | Line probe Assay |
| Accuprobe Mycobacterium gordonae culture identification Test | K896492 | Class I | Line probe Assay |
| Rapid Diagnostic System for Mycobacterium gordonae | K890089 | Class I | Line probe Assay |
| Rapid Diagnostic System for Mycobacteria | K864597 | Class I | Line probe Assay |
| Rapid Identification Test for Mycobacterium avium | K862613 | Class I | Line probe Assay |
| Gen-Probe Mycobacterium Rapid Confirmation System | K860782 | Class I | Line probe Assay |

Table 3. Performance evaluation of FDA approved or not approved TB IVDs in references

| Author          | Year | Sample size        | Sensitivity (%) | Specificity (%) | Reference |
|-----------------|------|--------------------|-----------------|-----------------|-----------|
| FDA approved    |      |                    |                 |                 |           |
| Xpert® MTB/RIF assay | Hai H et al. | 2017 | 2,910 sputum specimens | 96.7 | 98.3 | [12] |
| Xpert® MTB/RIF assay | Kampen SC et al. | 2015 | 5,611 sp, utum specimens | 93.1 | 96.4 | [13] |
| Xpert® MTB/RIF assay | Geleta DA et al. | 2015 | 227 sputum specimens | 65.5 | 96.3 | [14] |
| Xpert® MTB/RIF assay | Detjen AK et al. | 2015 | 4,768 respiratory specimens | 62.0 | 98.0 | [15] |
| Xpert® MTB/RIF assay | Antonenka U et al. | 2013 | 121 respiratory specimens | 74.6 | 96.2 | [16] |
| Xpert® MTB/RIF assay | Chen X et al. | 2012 | 178 sputum specimens | 95.2 | 97.9 | [17] |
| Amplified Mycobacterium tuberculosis Direct test | Papaventsis D et al. | 2012 | 152 clinical specimens | 100.0 | 85.0 | [18] |
| Amplified Mycobacterium tuberculosis Direct test | Guerra RL et al. | 2007 | 1,151 respiratory specimens | 91.7 | 98.7 | [19] |
| Amplified Mycobacterium tuberculosis Direct test | David WD et al. | 2003 | 499 respiratory specimens | 99.6 | 99.7 | [20] |
| Amplified Mycobacterium tuberculosis Direct test | Fegou E et al. | 2005 | Sputum (684) BAL (1473) SAB (625) TA (296) Pleural (189) Gastric (23) fluids(124) | 77.5, 45.6 | 88.1, 88.0 | [21] |
| Amplified Mycobacterium tuberculosis Direct test | Mitrarai S et al. | 2001 | Sputum (1088) | 61.8 | 97.4 | [22] |
| Amplified Mycobacterium tuberculosis Direct test | Choi WS et al. | 2006 | 807 respiratory specimens | 93.3, 83.3, 89.0, and 95.7 | 89.0, and 95.7 | [23] |
| Amplified Mycobacterium tuberculosis Direct test | Cho WH et al. | 2015 | 9,728 respiratory specimens | 67.2 | 98.4 | [24] |
| Amplified Mycobacterium tuberculosis Direct test | Huh HJ et al. | 2015 | 2,401 non-respiratory specimens | 67.2 | 98.4 | [25] |
| Amplified Mycobacterium tuberculosis Direct test | Lee M et al. | 2015 | 629 respiratory specimens | 78.8 | 99.5 | [26] |
| Amplified Mycobacterium tuberculosis Direct test | Moon JW et al. | 2005 | 586 respiratory specimens | 82.7 | 96.5 | [27] |
| Amplified Mycobacterium tuberculosis Direct test | Lim TK et al. | 2003 | 111 pleural effusion specimens | 17.5 | 98.1 | [28] |
| Amplified Mycobacterium tuberculosis Direct test | | | 168 respiratory specimens | 88.0 | 97.0 | [29] |

a, sensitivity based on smear positive result; b, a sensitivity based on smear negative results; c, sensitivity from bronchial washing fluid; d, sensitivity from sputum; e, sensitivity from body fluid. Abbreviation: BAL, bronchoalveolar lavage; SAB, sputa expectorated after bronchoscopy; TA, tracheal aspirate.

Mycobacterium CM/AS, the specificity was 95.6% (Table 2).

3. Sensitivity and specificity of FDA approved or not approved TB IVDs

We compared the FDA-approved in vitro diagnostic reagents for detecting TB and those that were not approved by the FDA were evaluated for their performance using commercially available reagents. For Xpert MTB/RIF diagnostic reagent, respiratory specimens were used mainly and samples were analyzed using a minimum of 121 samples and a maximum of 2910 samples. The mean sensitivity was 79.1% and the mean specificity was 97.2% (Table 3).

For the Amplified Mycobacterium tuberculosis Direct test, a minimum of 118 samples and a maximum of 1538 samples were analyzed. Non-respiratory samples and urine samples were used as well as respiratory specimens. The mean sensitivity was 93.8% and the mean specificity was 93.9%. In the case of the Amplicore MTB test, no results were tested within the last 5 years, but more than
Table 4. Performance evaluation of FDA approved or not approved NTM IVDs in references

| FDA approved | Author Year | Sample | Sample size | Specificity | Reference |
|--------------|-------------|--------|-------------|-------------|-----------|
| AccuProbe Mycobacterium avium complex identification test | Tran AC et al. 2014 | Culture | 37 | 72.9% | [29] |
| | Louro AP et al. 2001 | Culture (broth) | 34 | 82.3%; 94.1% | [30] |
| | Lebrun L et al. 1992 | Culture | 134 | 82.3% | [31] |
| FDA not-approved | Makinen J et al. 2006 | Culture | 219 | 94.4~100% | [32] |
| GenoType Mycobacterium CM/AS | Richter E et al. 2006 | Culture | 148 | 92.6%; 89.9% | [33] |
| | Lee AS et al. 2009 | Culture (solid) | 131 | 90.8% | [34] |
| | Singh AK et al. 2013 | Culture | 219 | 98.3% | [35] |
| | García-Agudo L et al. 2011 | Culture (broth) | 197 | 82.0% | [36] |
| | Padilla E et al. 2004 | Culture | 110 | 92.7% | [37] |
| | Trueba F et al. 2004 | Culture | 54 | 94.4% | [38] |

a, specificity of M. gordonae from culture bottle; b, specificity of M. avium complex; c, specificity of GenoType Mycobacterium CM; d, specificity of GenoType Mycobacterium AS.

1,000 samples were tested, with a sensitivity of 75% and a specificity of 94.5%.

The COBAS TaqMan MTB test is mainly used as a reagent which is not reported to the FDA but has been commercialized and used for research purposes. The samples are mainly used in respiratory samples, and the number of specimens is 111 and 9728. The mean sensitivity and specificity were 72.3% and 98.1%, respectively.

4. Sensitivity and specificity of FDA approved or not approved NTM IVDs

The results of the present study were as follows: 1) In vitro evaluation of non-tuberculous antibiotics was performed on cultured specimens and the average value of FDA - approved Accuprobe avium complex diagnostic reagents was 87.4%. In the case of INNO-LiPA Mycobacteria v.2, a non-FDA-approved commercialization reagent capable of simultaneous diagnosis of major NTM, the mean number of positive isolates of at least 54 and up to 197 isolated Mycobacteria isolates was 96.3%. Genotype Mycobacterium CM/AS, another commercial reagent, showed a mean of 95.6% specificity in a minimum of 131 and a maximum of 219 tests (Table 4).

DISCUSSION

In this study, we discussed the nucleic acid-based molecular assay in vitro diagnostic reagent which has been notified to FDA and the reagents that have not yet been notified to FDA but are commercialized and used for research purposes.

Currently FDA-approved in vitro diagnostic reagents are made up of a method of amplifying nucleic acid and then measuring it again using tuberculosis specific DNA probe. Recently, in the case of Xpert MTB/RIF, which is a diagnostic reagent using real-time PCR method, an optimal positive cut-off for MTB detection probe and rifampin resistance detection probe were proposed. The cut-off are important for preventing false positives and false negatives. Therefore, the cut-off should be carefully determined. The COBAS® TaqMan® MTB test (Roche), a real-time PCR-based diagnostic reagent, was recalled by the FDA due to the possibility of false negatives at the proposed cut-off criteria (https://www.accessdata.fda.gov). Nucleic acid-based tuberculosis diagnostic tests showed increased sensitivities and specificities when repeated two or more times. Therefore, it is recommended to repeat the test more than 2 times and guidelines should notice interpretation of the data with ambiguous cut off for very low signal and absence of internal control, and invalid sample.

The final clinical evaluation of the FDA’s PMA and 510 (k) was based on the culture results of the tuberculosis standard diagnostic method. The sensitivity and specificity according to the smear results were separately presented. Recently, there have been developed methods for detecting
mutations in genes associated with resistance to isoniazid, quinolone antibiotics, and aminoglycoside antibiotics for the diagnosis of multidrug-resistant tuberculosis and broad-spectrum tuberculosis. There is no approved product other than the rifampin resistance detection kit. Since the mutation detection of the relevant gene does not necessarily imply susceptibility to the drug, analysis of the phenotype DST or nucleotide sequence should be allowed in the future for approval of in vitro diagnostic reagents.

For non-tuberculous antibiotics, there is not much evaluation of direct samples yet, which should be further studied. In recent year, it should be considered in conjunction with the clinical evaluation of NTB using direct samples, because there have been various developed methods for simultaneous diagnosis of TB and NTB.

In order to confirm inclusivity, the FDA Guideline suggests that *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, and *M. caprae* corresponding to the MTB complex are all detected. However, recent studies on the genome differences among MTB complexes have been conducted [39], and methods for differentiating *M. tuberculosis* and *M. bovis* from the MTB complex have been developed [40].

The currently developed in vitro diagnostic reagents for TB and NTM in US FDA was actively perform to end of TB worldwide. This analysis of US FDA approved molecular assays could serve as a useful reference for evaluation of reagent performance of TB and NTM.

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