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Performance Evaluation of *In Vitro* Diagnostic Reagents for *Mycobacterium tuberculosis* and Non-tuberculous Mycobacteria by FDA Approval

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미국 FDA 허가사례를 통해 본 결핵균 및 비결핵 항산균 체외진단용 시약의 성능평가

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Tuberculosis (TB) is a bacterial infection disease caused by members of the species *Mycobacterium tuberculosis* (MTB) complex. Approximately one third of the world's population is infected with TB. In Korea, approximately 40,000 new patients are identified each year. Moreover, infections from non-tuberculous mycobacteria (NTM) have also increased. In the diagnosis of TB and NTM, traditional bacterial cultures are required for 3 to 4 weeks. Therefore, rapid and accurate diagnostic tests for TB and NTM are needed. To distinguish between TB and NTM, a range of diagnostic methods have been developed worldwide. In vitro diagnostic assays are constantly being developed to meet the increasing need for the rapid and accurate identification for TB and NTM. On the other hand, the performance evaluations of in vitro diagnostic reagents for TB and NTM are lacking. Recently, the Korea Food and Drug Administration (KFDA) issued a guideline for in vitro diagnostic reagents for MTB and NTM. Here, this study analyzed the performance of currently developed in vitro diagnostic reagents for TB and NTM in the US FDA. This analysis of US FDA approved molecular assays could serve as a useful reference for an evaluation of the reagent performance of TB and NTM.

Key words: Tuberculosis, Sensitivity, Specificity, Diagnostic test approval, Food and drug administration

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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 l 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

approved were collected and analyzed.

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. kansasii*, *M. gordonae*, *Corynebacterium* spp., *Gordona sputi* and *Rhodococcus bronchialis* ($>10^5$ ~ 10^8/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 *rpo*B 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[a]	Real-time PCR	*rpo*B	93.8% (439/468)[d], 94.7% (18/19)[e]	98.7% (620/628)[d], 99.0% (404/408)[e]
	Amplified *Mycobacterium tuberculosis* Direct Test	P940034	Class II[b]	Transcription mediated amplification (TMA) and Hybridization protection assay (HPA)	rRNA	93.2% (109/114)	98.8% (414/419)
	Amplicor *Mycobacterium tuberculosis* test	P940040	Class II[c]	PCR, Hybridization	16S rRNA	95% (134/141)	100% (48/48)
	SNAP *M. tuberculosis* complex	K900292	Class I	NAAT, DNA probe	NR	NR	NR
	BDProbetec ET *Mycobacterium tuberculosis* complex culture identification kit	K000884	Class I	NAAT, DNA probe	16S-23S rRNA ITS	99.6% (226/227)	95.6% (473/495)
	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 otitidiscaviarum*. 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/Productsand Medical Procedures/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 lifescience 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]
		Kampen SC et al.	2015	5,611 sp, utum specimens	93.1	96.4	[13]
		Geleta DA et al.	2015	227 sputum specimens	65.5	96.3	[14]
		Detjen AK et al.	2015	4,768 respiratory specimens	62.0	98.0	[15]
		Antonenka U et al.	2013	121 respiratory specimens	74.6	96.2	[16]
	Amplified *Mycobacterium tuberculosis* Direct test	Chen X et al.	2012	178 sputum specimens	95.2	97.9	[17]
		Papaventsis D et al.	2012	152 clinical specimens	100.0	85.0	[18]
		Guerra RL et al.	2007	1,151 respiratory specimens	91.7	98.7	[19]
		David WD et al.	2003	499 respiratory specimens	99.6	99.7	[20]
	Cobas® Amplicore MTB test	Fegou E et al.	2005	Sputum (684) BAL (1473) SAB (625) TA (296) Pleural (189) Gastric (23) fluids(124)	77.5[a], 45.6[b]	88.1[a], 98.0[b]	[21]
		Mitarai S et al.	2001	Sputum (1088)	61.8	97.4	[22]
		Choi WS et al.	2006	807 respiratory specimens	93.3%[c], 100.0%[d], and 50.0%[e]	83.3%[c], 89.0%[d], and 95.7%[e]	[23]
FDA not-approved	Cobas® Taqman® MTB test	Cho WH et al.	2015	9,728 respiratory specimens 2,401 non-respiratory specimens	67.2	98.4	[24]
		Huh HJ et al.	2015	629 respiratory specimens	78.8	99.5	[25]
		Lee M et al.	2013	586 respiratory specimens	82.7	96.5	[26]
		Moon JW et al.	2005	111 pleural effusion specimens	17.5	98.1	[27]
		Lim TK et al.	2003	168 respiratory specimens	88.0	97.0	[28]

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. Abbreivation; 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

		Author	Year	Sample	Sample size	Specificity	Reference
FDA approved	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%[a], 94.1%[b]	[30]
FDA not-approved	GenoType *Mycobacterium* CM/AS	Lebrun L et al.	1992	Culture	134	95.2%	[31]
		Makinen J et al.	2006	Culture	219	94.4~100%	[32]
		Richter E et al.	2006	Culture	148	92.6%[c], 89.9%[d]	[33]
		Lee AS et al.	2009	Culture (solid)	131	90.8%	[34]
		Singh AK et al.	2013	Culture	219	98.33%	[35]
	INNO-LiPA *Mycobacterium* V2	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.

요 약

결핵(TB)은 *Mycobacterium tuberculosis* (MTB) 복합체의 구성원에 의한 세균 감염 질병이다. 결핵은 전 세계 인구의 1/3이 감염된 것으로 알려져 있으며, 한국에서는 매년 약 4만 명의 새로운 결핵환자가 발생한다. 또한, 비결핵 항상균 감염이 증가하고 있는 추세이다. 전통적인 결핵 및 비결핵 항상균 진단방법은 세균 배양으로 3~4주 이상이 소요된다. 따라서, 신속하고 정확한 결핵균(TB) 및 비결핵 항상균(NTM) 진단법의 필요성이 요구되고 있다. 결핵균 및 비결핵 항상균을 구분하기 위하여, 전 세계적으로 다양한 진단 방법이 개발되고 있다. 특히, 결핵균과 비결핵 항상균을 신속하고 정확한 동정의 요구가 증가함에 따라, 정확하고 신속하게 진단하기 위한 체외 진단 방법이 개발 되고 있다. 그러나 현재 결핵과 비결핵 항상균에 대한 체외 진단 시약의 성능 평가는 부족한 실정이다. 최근 식약청은 결핵균 및 비결핵 항상균 체외 진단 시약에 대한 가이드 라인을 발표했다. 본 연구에서는, 미국 FDA에 승인을 받은 결핵균 및 비결핵 항산균에 대한 체외 진단 시약의 성능을 검토하였다. 이 검토는 결핵균 및 비결핵 항상균 체외 진단 시약 평가에 유용한 참고 자료가 될 것으로 사료된다.

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