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Evaluation of EZplex MTBC/NTM Real-Time PCR kit: diagnostic accuracy and efficacy in vaccination

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Purpose: Tuberculosis (TB) is mainly caused by *Mycobacterium tuberculosis,* which is a pathogenic mycobacterial species grouped under *Mycobacterium tuberculosis* complex (MTBC) with four other pathogenic mycobacterial species. The mycobacteria not included in MTBC are known as nontuberculous mycobacteria (NTM), and cause several pulmonary diseases including pneumonia. Currently, NTM occurrences in TB-suspected respiratory specimens have increased, due to which, precise detection of MTBC and NTM is considered critical for the diagnosis and vaccination of TB. Among the various methods available, real-time PCR is frequently adopted for MTBC/NTM detection due to its rapidness, accuracy, and ease of handling. In this study, we evaluated a new real-time PCR kit for analytical and clinical performance on sputum, bronchial washing, and culture specimens.

Materials and Methods: For assessing its analytical performance, limit of detection (LOD), reactivity, and repeatability test were performed using DNA samples. To evaluate clinical performance, 612 samples were collected and clinically tested at a tertiary hospital.

Results: LOD was confirmed as 0.584 copies/μL for MTBC and 47.836 copies/μL for NTM by probit analysis (95% positive). For the reactivity test, all intended strains were detected and, in the repeatability test, stable and steady results were confirmed with coefficient of variation ranging from 0.36 to 1.59. For the clinical test, sensitivity and specificity were 98.6%-100% and 98.8%-100% for MTBC and NTM, respectively.

Conclusion: The results proved the usefulness of the kit in TB diagnosis. Furthermore, it could be adopted for the assessment of vaccine efficacy.

Keywords: Tuberculosis, *Mycobacterium tuberculosis* complex, Nontuberculous mycobacteria, Real-time polymerase chain reaction, BCG vaccine

Introduction

Tuberculosis (TB) is a bacterial infection caused by *Mycobacterium tuberculosis* complex (MTBC). Despite its existence over a couple of millennia in human history, it remains an unresolved global health issue. With approximately 10 million people affected worldwide, TB is ranked higher than human immunodeficiency virus/acquired immune deficiency syndrome with respect to single infection death [1]. It is a serious health threat in Korea as well, with the incidence and associated death rate being the highest among the Organization for Economic Cooperation and Development (OECD) nations [2].

TB is mainly caused by *Mycobacterium tuberculosis* (MTB) which is a very small, aerobic, non-motile bacillus [3]. It is grouped with *M. bovis, M. afrcanum, M. canetti*, and *M. microti* in the MTBC [4]. Incidence of tuberculosis caused by other MTBC species (except MTB) is not much common, although observed in some African regions [5-8]. The mycobacteria, not included in MTBC, are known as nontuberculous mycobacteria (NTM), and approximately 140 species including *M. leprae, M. avium*, and *M. kansassi* have been reported [9,10]. Although NTM is not related to TB, it is a causative agent of several NTM infections such as disseminated disease, lymphnoditis, and other lung diseases [10].

Several studies have indicated an increase in the frequency of NTM isolation from respiratory specimens in worldwide [9,11-13]. Since prescribed drugs are different for MTBC-mediated and NTM-induced diseases [9], there is an increased necessity of accurately distinguishing an infection caused by MTBC from that caused by NTM [9,10].

Diagnosis of MTBC infection has been implemented by multiple methods including chest X-ray, sputum culture, acid-fast bacilli test, and interferon gamma release assay (IGRA) [14,15]. Chest X-ray has limitations in detecting MTBC activity and needs to compare with previous X-ray result [2]. Sputum culture is a more confirmatory test of TB, but takes more than six weeks to complete [16]. Acid-fast bacilli method cannot distinguish between MTBC and NTM [2]. IGRA also lacks accuracy in MTBC diagnosis [17].

Currently, nucleic acid amplification test (NAAT)-based assays are being actively conducted for the detection of MTBC and NTM, due to its advantage of rapid and precise target detection [18-20]. Several national authorities recommend their use for TB diagnosis, along with other conventional methods [2,21]. Of the several NAATs, however, conventional polymerase chain reaction (PCR) and hybridization assay need an additional confirmatory step, such as electrophoresis, for the amplified DNA product. Sometimes it may pose a time-consuming step to the user and lead to contamination in the test, thereby generating inappropriate result [22,23].

Real-time PCR assay has minimized these inconveniences; it does not require additional confirmatory steps and can report more accurate result in relatively shorter time than other conventional NAATs. More recently, TaqMan probe method (Applied Biosystems, Foster City, CA, USA) has been developed that enables multiple target detections retaining the accuracy and rapid performance of real time PCR assay [24,25]. Therefore, it has been frequently adopted for TB diagnosis along with many other related assays commercially available [26].

The current study aimed to evaluate the commercial EZplex MTBC/NTM Real-Time PCR kit (Genetree Research Inc., Seoul, Korea) for MTBC and nontuberculous mycobacteria, which was approved by the Ministry of Food and Drug Safety, Korea (MFDS).

Materials and Methods

Clinical sensitivity and specificity

Six hundred and twelve samples of sputum, bronchial washing fluid, and sputum culture were collected from Samkwang Medical Laboratories (Seoul, Korea) and sent to Samsung Medical Center (Seoul, Korea) for clinical test. Out of those, 216 samples were positive for MTBC, 139 were positive for NTM, and the remaining 257 samples were negative for both MTBC and NTM. All specimens were confirmed as positive or negative by acid-smear, culture, or PCR test; samples were considered positive if more than one result from those three assays were positive. This study was conducted with approval from the Institutional Review Board of the Samsung Medical Center.

DNA extraction

DNA was extracted from the specimens using Chelex-100 resin (Bio-Rad, Hercules, CA, USA) as per manufacturer's instruction. In case of sputum and bronchial washing specimens, all samples were pre-treated with the same volume of 1 N NaOH as the sample itself.

Real-time PCR

All tests were conducted as per manufacturer's instruction. PCR master mix for a single sample was prepared with 12.5 μL of 2× reaction mixture, 9 μL of probe primer, 0.1 μL of internal control DNA, 0.9 μL of distilled water and 2.5 μL of template DNA. PCR was conducted with the following program: 50°C for 2 minutes and 95°C for 10 minutes in the first cycle, followed by 95°C for 20 seconds and 66°C for 1 minute repeated over 45 cycles. By adding an additional internal control DNA into every sample, the real-time PCR status, during or after the test, could be monitored. The EZplex assay is designed to detect MTBC on HEX (4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein), NTM on FAM (6-carboxyfluorescein), and internal control on Cy5 Channel.

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Sequencing

In case of discrepancy between the evaluation kit and reference assays, additional confirmatory test (Sanger sequencing) was performed in the same institute as the clinical test, and considered as reference result at the end of the test, only if those matched >90%. The test was in accordance with the institution's approved protocol and results were analyzed using National Center for Biotechnology Information (NCBI)-BLAST.

Limit of detection

MTBC positive control and NTM positive control from Vircell (Granada, Spain) were used for assessing the limit of detection (LOD) of MTBC and NTM respectively. Initial concentration of both DNA controls was set to 1×10^4 copies/μL from original controls. MTBC control was serially diluted into 100, 50, 10, 5, 1, 0.5, 0.1, and 0.05 copies/μL and NTM control was serially diluted into 500, 100, 75, 50, 25, 10, 5, and 1 copies/μL. In all those concentrations, repetitive tests were performed 40 times and each LOD was calculated by the probit analysis for 95% positive result.

Analytical reactivity

Five strains of MTBC and 20 strains of NTM were selected for reactivity test, whose details are shown in Table 1. Every strain was prepared with DNA material approximately 10 times higher than LOD concentration.

Repeatability

For positive controls, 100 copies/μL controls were set as mid-concentration and 10 copies/μL controls were set as low-concentration control in repeatability test. The test was repeated 20 times, performed twice a day, and every single test was repeated twice for 5 days. For assessing repeatability, the mean, standard deviation, and coefficient of variation (CV) of Ct value were calculated from the test results.

Statistical analysis

For the LOD result, 95% positive probits were calculated by PASW Statistics for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA). Repeatability tests were analyzed by Microsoft Excel 2013 (Microsoft, Redmond, WA, USA). Clinical tests were analyzed by MedCalc (bvba, Ostend, Belgium).

Ethics statement

The study protocol for clinical sensitivity and specificity was approved by the institutional review board of Samsung Medical Center (IRB No. SMC 2016-11-004). Informed consent was waived by the IRB.

Table 1. DNA samples for reactivity test

Group	Strain	Reference
MTBC	*Mycobacterium tuberculosis*	Vircell (MBC034)
	Mycobacterium bovis	Plasmid DNA
	Mycobacterium bovis BCG	Plasmid DNA
	Mycobacterium africanum	Plasmid DNA
	Mycobacterium microti	Plasmid DNA
NTM	*Mycobacterium abscessus*	Plasmid DNA
	Mycobacterium fortuitum	KCTC 9510
	Mycobacterium scrofulaceum	Plasmid DNA
	Mycobacterium intracellulare	KCTC 9514
	Mycobacterium marinum	Plasmid DNA
	Mycobacterium chimera	Plasmid DNA
	Mycobacterium smegmatis	KCTC 9108
	Mycobacterium massiliense	Plasmid DNA
	Mycobacterium mucogeicum	Plasmid DNA
	Mycobacterium triviale	Plasmid DNA
	Mycobacterium malmoense	Plasmid DNA
	Mycobacterium gordonae	Plasmid DNA
	Mycobacterium ulcerans	Vircell (MBC094)
	Mycobacterium chelonae	Plasmid DNA
	Mycobacterium avium	Vircell (MBC086)
	Mycobacterium kansasii	Vircell (MBC095)
	Mycobacterium szulgai	Plasmid DNA
	Mycobacterium terrae	Plasmid DNA
	Mycobacterium celatum	KCTC (19714)
	Mycobacterium interjectum	KCTC (19649)

Twenty-five strains were selected for the test (5 strains of MTBC and 20 strains of NTM).
MTBC, *Mycobacterium tuberculosis* complex; NTM, nontuberculous mycobacteria.

Results

Limit of detection

The results of LOD for MTBC and NTM are shown in Table 2. Probit analysis of 95% positivity in 40 replicates with 6 concentrations for MTBC and NTM revealed MTBC to have LOD of 0.584 copies/μL and NTM to have 47.836 copies/μL. In MTBC, 40 repetitive tests from 100 copies/μL to 1 copy/μL showed 100% positive rate; however, the positive rate reduced to 87.5% at 0.5 copies/μL, and nothing was detectable at 0.05 copies/μL. In NTM, all repeated tests were positive till 50 copies/μL, positive rates gradually decreased to 25 copies/μL, and nothing was detectable at 1 copy/μL.

Table 2. Limit of detection

Pathogen	DNA concentrations (copy/μL)	Reactions	Positive	Positive rate (%)	95% Probit result (copy/μL)
MTBC (*Mycobacterium tuberculosis*)	100	40	40	100	0.584
	50	40	40	100	
	10	40	40	100	
	5	40	40	100	
	1	40	40	100	
	0.5	40	35	87.5	
	0.1	40	16	40	
	0.05	40	0	0	
NTM (*M. intracellulare*)	500	40	40	100	47.836
	100	40	40	100	
	75	40	40	100	
	50	40	40	100	
	25	40	26	65	
	10	40	19	47.5	
	5	40	7	17.5	
	1	40	0	0	

MTBC, *Mycobacterium tuberculosis* complex; NTM, nontuberculous mycobacteria.

Table 3. Result of reactivity test

Group	Organism	HEX	FAM
MTBC	*Mycobacterium tuberculosis*	+	-
MTBC	*Mycobacterium bovis*	+	-
MTBC	*Mycobacterium bovis* BCG	+	-
MTBC	*Mycobacterium africanum*	+	-
MTBC	*Mycobacterium microti*	+	-
NTM	*Mycobacterium abscessus*	-	+
NTM	*Mycobacterium fortuitum*	-	+
NTM	*Mycobacterium scrofulaceum*	-	+
NTM	*Mycobacterium intracellulare*	-	+
NTM	*Mycobacterium marinum*	-	+
NTM	*Mycobacterium chimera*	-	+
NTM	*Mycobacterium smegmatis*	-	+
NTM	*Mycobacterium massiliense*	-	+
NTM	*Mycobacterium mucogeicum*	-	+
NTM	*Mycobacterium triviale*	-	+
NTM	*Mycobacterium malmoense*	-	+
NTM	*Mycobacterium gordonae*	-	+
NTM	*Mycobacterium ulcerans*	-	+
NTM	*Mycobacterium chelonae*	-	+
NTM	*Mycobacterium avium*	-	+
NTM	*Mycobacterium kansasii*	-	+
NTM	*Mycobacterium szulgai*	-	+
NTM	*Mycobacterium terrae*	-	+
NTM	*Mycobacterium celatum*	-	+
NTM	*Mycobacterium interjectum*	-	+

All purposed 25 strains were detected in each group's fluorescent channel.
HEX (4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein) for *Mycobacterium tuberculosis* complex (MTBC); FAM (6-carboxyfluorescein) for nontuberculous mycobacteria (NTM).

Table 4. Result of repeatability test

Pathogen	Concentration	Reaction	Positive	Mean±SD	CV (%)
MTBC (*M. tuberculosis*)	Medium	20	20	32.43±0.35	1.06
	Low	20	20	37.23±0.59	1.59
	Negative	20	0	Neg	Neg
NTM (*M. intracellulare*)	Medium	20	20	33.90±0.12	0.36
	Low	20	20	37.93±0.55	1.45
	Negative	20	0	Neg	Neg

All positives were detected in medium and low concentration of positive control. All negatives were confirmed by negative controls. In positive tests, repeatability was confirmed by CV, ranging from 0.36 to 1.59 (%).
CV, coefficient of variation; MTBC, *Mycobacterium tuberculosis* complex; Neg, negative; NTM, nontuberculous mycobacteria.

Analytical reactivity

All intended strains of MTBC and NTM were detected in each fluorescent channel using commercial panel, synthesized DNA, and DNA prepared from KCTC strains (Table 3).

Repeatability

As a result of 20 repetitive tests with medium and low concentrations of both MTBC and NTM, 100% positive rate was confirmed in each repeat and the CV of Ct value ranged between 0.36 and 1.59 (%). In the negative tests, all results were confirmed as negative (Table 4).

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Table 5. MTBC sensitivity and specificity compared with the reference results of culture, PCR, and sequencing (number of MTBC samples=473)

EZplex MTBC/NTM Real-time PCR	Reference results (culture, PCR, sequencing)			Sensitivity (95% CI, %)	Specificity (95% CI, %)	PPV[a] (95% CI, %)	NPV[a] (95% CI, %)
	Positive	Negative	Total				
Positive	213	3	216	98.6 (95.6-99.6)	98.8 (96.3-99.7)	98.6 (96.0-99.7)	98.8 (96.6-99.8)
Negative	3	254	257				
Total	216	257	473				

MTBC, *Mycobacterium tuberculosis* complex; PCR, polymerase chain reaction; NTM, nontuberculous mycobacteria; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.
[a]The PPV and NPV are the proportion of positive and negative results in diagnostic tests, considered as true positive and true negative results.

Table 6. NTM sensitivity and specificity compared with the reference results of culture, PCR, and sequencing (number of MTBC samples=396)

EZplex MTBC/NTM Real-time PCR	Reference results (culture, PCR, sequencing)			Sensitivity (95% CI, %)	Specificity (95% CI, %)	PPV[a] (95% CI)	NPV[a] (95% CI)
	Positive	Negative	Total				
Positive	139	0	139	100 (96.6-100)	100 (98.2-100)	100 (97.4-100)	100 (98.6-100)
Negative	0	257	257				
Total	139	257	473				

NTM, nontuberculous mycobacteria; PCR, polymerase chain reaction; MTBC, *Mycobacterium tuberculosis* complex; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.
[a]The PPV and NPV are the proportion of positive and negative results in diagnostic tests, considered as the true positive and true negative results.

Clinical sensitivity and specificity

In the clinical test of 612 specimens, sensitivity and specificity for MTBC were confirmed as 98.6% (95% confidence interval [CI], 95.6 to 99.6) and 98.8% (95% CI, 96.3 to 99.7), respectively. For NTM, both sensitivity and specificity were confirmed to be 100% (sensitivity: 95% CI, 96.6 to 100; specificity: 95% CI, 98.2 to 100). All NTM results were perfectly matched with reference, but those of MTBC had six discrepancies (Tables 5, 6). The positive predictive value (PPV) for MTBC was 98.8% (95% CI, 96.3 to 99.7) and negative predictive value (NPV) was 98.8% (95% CI, 96.6 to 99.8) (Table 5), whereas for NTM, both PPV (95% CI, 97.4 to 100) and NPV were 100% (95% CI, 98.6 to 100) (Table 6).

Discussion

Since frequencies of NTM isolation, from patients suspected with TB, have increased [11-13], precise detection of MTBC and NTM is important for TB diagnosis. The need for accurate diagnosis is yet more important since the anti-drug between MTB and NTM is different [9]. Furthermore, therapeutic effects cannot be expected when inappropriate drugs are used based on incorrect diagnosis.

To evaluate whether the EZplex assay is appropriate for the accurate diagnosis of TB, analytical test was performed with clinical samples. In the LOD test for evaluating analytical sensitivity, MTBC corresponded to 0.584 copies/μL and NTM to 47.836 copies/μL. Comparing the results from different commercial assay kits using 10 copies/μL of MTB and 100 copies/μL of NTM from another study [27], the kit tested in the present study is more sensitive for detecting MTBC and NTM. In the reactivity test, it was able to detect all the 25 species. However, further validation using actual strain isolated from some tested species may be required, since it was tested on synthesized DNA. Repeatability was evaluated from the CV of the repeated Ct values. Twenty repetitive tests showed its steady and stable repeatability with 0.36%-1.45% CV.

Clinical performance ranged from 98.6% to 100% in MTBC and NTM. Of the 473 specimens in the MTBC test, there were six discrepancies and sequencing had confirmed them as three false positives and three false negatives, using the evaluation kit (Table 5). However, in NTM, all results were fully matched with the references (Table 6). According to previous studies on other assays, the MTBC sensitivity/specificity and NTM sensitivity/specificity were 71.4%-97.2%/95.8%-100% [19,28-31] and 33.3%-76.5%/89.6%-98.4% [20,32,33], respectively. Previous studies had shown that NTM performances were relatively lower than that of MTBC. Compared to other assays for NTM performance, the EZplex assay shows greater potential; however, to assess a more precise comparison, ad-

ditional test might be required with several assays performed on same specimens.

In the 21st century, medicines are not only focused onto disease treatment, but also for disease prevention [34], as a result of which, the importance of diagnosis has increased further. Moreover, precise diagnosis may help to develop potent targets for vaccines, which has contributed significantly to the prevention of TB, such as in bacillus Calmette-Guerin (BCG) vaccine [35].

Although BCG vaccines are widely used for newborn infants, a recent study has shown that the effectiveness of BCG vaccine is almost halved in an adult, hence suggesting the need for re-vaccination or development of new vaccines [36,37]. For the latter, accurate diagnosis may be able to provide new pathways toward vaccine evaluation and offer new candidate vaccines [38].

Moreover, NAAT has shown accurate detection of BCG substrains and ability to suggest the degree of immunity in prior-vaccinated individuals [39,40]. With such potential, the NAAT-based diagnostic technique may be adopted for assessing vaccine efficacy.

Through the high diagnostic performance of the EZplex, we confirmed its possibility of use in vaccine studies, but it was not found its usefulness in actual vaccine studies. Further research will be needed to confirm that the EZplex has usefulness in the search for new biomarkers and vaccine candidates.

In conclusion, the current study confirmed that the EZplex assay has high sensitivity and specificity in distinctly detecting MTBC and NTM. Therefore, it may be useful for TB diagnosis and contribute to vaccination.

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