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Evaluation of Peptide Nucleic Acid Probe-Based Fluorescence In Situ Hybridization for the Detection of *Mycobacterium tuberculosis* Complex and Nontuberculous Mycobacteria in Clinical Respiratory Specimens

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Background: Tuberculosis is globally the most important cause of death from single pathogen. Rapid and accurate identification of mycobacteria is essential for the control of tuberculosis. We evaluated a fluorescence in situ hybridization (FISH) method using peptide nucleic acid (PNA) probes for the differentiation of *Mycobacterium tuberculosis* complex (MTB) and nontuberculous mycobacteria (NTM) in direct smears of sputum specimens.
Methods: The cross-reactivity of MTB- and NTM-specific PNA probes was examined with reference strains of *M. tuberculosis* ATCC 13950, *Mycobacterium kansasii* ATCC 12479, *Mycobacterium fortuitum* ATCC 6841, several clinical isolates of mycobacteria (*Mycobacterium abscessus, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium gordonae* and *Mycobacterium chelonae*), and 11 frequently isolated respiratory bacterial species other than mycobacteria. A series of 128 sputa (89 MTB culture positive, 29 NTM culture positive, and 10 under treatment culture negative) with grades of trace to 4+ were used to evaluate the performance of the method.
Results: The MTB- and NTM-specific PNA probes showed specific reactions with the reference strains of MTB and *M. kansasii* and clinical isolates of mycobacteria except *M. fortuitum* ATCC 6841, and no cross-reactivity with other tested bacteria. The PNA probe-based FISH assay for detection of MTB had a sensitivity and specificity of 100%, respectively. The sensitivity and specificity of the NTM-specific PNA probe was 100%. The smear grades of the PNA FISH test were same as with those of the fluorescence AFB stain in 2+ or higher grade.
Conclusion: Detection and differentiation based on PNA FISH is sensitive and accurate for detecting mycobacteria and for differentiating MTB from NTM in clinical sputum smears. **(Ann Clin Microbiol 2015;18:37-43)**

Key Words: Fluorescence, In situ hybridization, *Mycobacterium tuberculosis*, Nontuberculous mycobacteria, Peptide nucleic acids

INTRODUCTION

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB) and remains a major global health problem, with an estimated 8.6 million new cases in 2012 and 1.3 million TB deaths [1]. Infections by nontuberculous mycobacteria (NTM) are common opportunistic diseases in immunocompromised patients, especially those with AIDS [2,3]. Anti-TB drugs are often ineffective against NTM infection. Therefore, rapid and accurate identification of mycobacteria in clinical settings is crucial for planning treatment [4]. Detection of mycobacteria usually depends on acid-fast bacilli (AFB) staining, culture, and polymerase chain reaction (PCR). The conventional microscopic examination of an AFB stain is simple and fast, and a positive AFB stain is the first indication of possible TB. However, the AFB stain yields poor positive predictive values for TB in clinical settings in which NTM is frequently isolated, because it does not allow differentiation MTB from NTM [5]. Recently, several investigators have used peptide nucleic acid (PNA) probes for the identification of MTB and NTM in various specimens, in-

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cluding cultures, sputum, and tissue [6-12]. PNA probes are artificially synthesized DNA analogues with an uncharged peptide backbone, which have more favorable hybridization properties and chemical, thermal, and biological stability than previous probes [13,14]. The uncharged nature and the high conformational flexibility of PNA probes allows hybridization to complementary DNA or RNA with high specificity [15]. The hydrophobic structure of PNA probes gives better penetrance through the mycobacterial cell wall, yielding higher sensitivity [8,9].

In the current study, we developed a PNA probe-based fluorescence in situ hybridization (FISH) assay and evaluated its diagnostic applicability for the differentiation between MTB and NTM in sputum specimens.

MATERIALS AND METHODS

1. Synthesis of PNA probes

Fluorescence-labeled PNA probes targeting the MTB and *Mycobacterium avium* 16S rRNA sequences in GenBank and EMBL databases were synthesized by PANAGENE (Daejeon, Korea) with searching for sequence similarity by BLAST. Probes were purified by reverse-phase high-performance liquid chromatography at 50°C and characterized using an AXIMA-CFR MALDI-TOF MS instrument (Shimadzu Biotech, Columbia, MD, USA). The probes specific for MTB and NTM are as follows: MTB, NH_2-CTTAGGAATTTTCGG-Lys-Flu-H; NTM, NH_2-CGGTCGCCCATTACG-Ala-Ala-Flu-H [9].

2. Strain preparation for checking cross reaction

To rule out cross reactions, we used three standard reference strains, *M. tuberculosis* ATCC 13950, *Mycobacterium kansasii* ATCC 12479 and *Mycobacterium fortuitum* ATCC 6841 and several clinical isolates of mycobacteria (*Mycobacterium abscessus, M. avium, Mycobacterium intracellulare, Mycobacterium gordonae,* and *Mycobacterium chelonae*). Also, we used 11 frequently isolated respiratory bacterial species (*Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Streptococcus pneumoniae, Nocardia asteroides, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Proteus mirabilis, Legionella pneumophila* and *Pseudomonas aeruginosa*). The sources and numbers of strains of each species are described in detail in Table 1.

A bacterial suspension was prepared as follows. The mycobac-

Table 1. Results of the PNA probe-based FISH assay for mycobacteria and other ordinary bacteria

Type and clinical strains (no. of strains)*	Results obtained with the following probes	
	M. tuberculosis-specific	NTM-specific
Mycobacteria		
M. tuberculosis ATCC 13950	P	N
M. kansasii ATCC 12479	N	P
M. fortuitum ATCC 6841	N	N
M. intracellulare (11)	N	P
M. abscessus (6)	N	P
M. avium (5)	N	P
M. gordonae (1)	N	P
M. chelonae (1)	N	P
Gram-positive bacteria		
Enterococcus faecalis ATCC 29212	N	N
Staphylococcus aureus ATCC 29213	N	N
Streptococcus pneumoniae ATCC 49619	N	N
Enterococcus faecium (1)	N	N
Nocardia asteroides (1)	N	N
Gram-negative bacteria		
Escherichia coli ATCC 25922	N	N
Haemophilus influenzae ATCC 9007	N	N
Klebsiella pneumoniae ATCC 13883	N	N
Pseudomonas aeruginosa ATCC 27853	N	N
Proteus mirabilis (1)	N	N
Legionella pneumophila (1)	N	N

*All clinical strains were isolated in Pusan National University Yangsan Hospital. Numbers in parentheses indicate number of clinical isolates. Abbreviations: PNA, peptide nucleic acid; FISH, fluorescence in situ hybridization; NTM, nontuberculous mycobacteria; P, positive; N, negative.

teria were grown on 3% Ogawa medium (Asan Pharmaceutical, Seoul, Korea) and in BacT/ALERT MP liquid medium (BioMérieux, Durham, NC, USA) at 37°C until visible colonies or positive growth signals were observed. After culture on solid medium, a loop of colonies was suspended in 500 μL of phosphate-buffered saline (PBS). After liquid medium culture, the bottles were vortexed for 10 to 15 s, and 1 mL of the cultures was centrifuged for 2 min at 12,000 × *g*. The supernatant liquid was removed, and the sediment was resuspended in 500 μL of PBS. For ordinary bacteria, all strains were grown on blood agar plates at 37°C, 5% CO_2 for 24 hr. A loop of colonies was suspended in 500 μL of PBS. Twenty microliters of all suspensions were added to microscope slides. The smear was air dried and heated at 80°C for 2 hr.

3. Specimen preparation for evaluating the PNA probe-based FISH assay

Sputum specimens with a grade of trace to 4+ on the Centers for Disease Control and Prevention (CDC)-recommended scale were collected at Pusan National University Yangsan Hospital from June 2012 to March 2013 [16]. Finally, 128 samples were collected after checking of the result of culture except for contamination. Routine laboratory practices were applied for smear microscopy and culture. Briefly, all sputum specimens were liquefied, decontaminated with N-acetyl-L-cysteine-2% NaOH, and concentrated by centrifugation at 3,000 × *g* for 15 min. The sediment was resuspended in 1.5 mL of PBS, and part of the sediment from each specimen was used for fluorescence AFB stain and culture in BacT/ALERT MP (BioMérieux) and 3% Ogawa medium. (Asan Pharmaceutical) A positive culture isolate was confirmed by AFB staining and identified by an immunochromatographic assay kit (BIOLINE SD *M. tuberculosis* Ag MPT64 Rapid; Standard Diagnostics, Yongin, Korea) and a PCR-restriction fragment-length polymorphism (RFLP) method. After routine processing of the specimens for microscopy and culture, samples of resuspended specimens were stored at −70°C for later use. Subsequently, culture in BacT/ALERT MP liquid medium (BioMérieux) yielded 89 MTB culture-positive specimens from 48 patients, 29 NTM culture-positive specimens from 20 patients, and 10 under treatment culture-negative specimens from 4 patients, which were selected for PNA FISH analysis. The respective specimens in −70°C deep freezer were retrieved and used for preparation for the assay. Twenty microliters of all suspensions were added to microscope slides. The smears were air dried and heated at 80°C for 2 hr.

4. In situ hybridization

Smears were immersed in 80% ethanol for 30 min and subsequently air dried and covered with approximately 20 μL of hybridization solution containing 20 mM Na_2HPO_4 pH 7.4, 20 mM Tris HCl pH 7.4, 60% formamide, 2× SSC, salmon sperm DNA 0.1 μg/mL (all reagents from Biosesang, Seongnam, Korea) and 100 nM fluorescein-labeled PNA probe. The slides were covered with coverslips and placed in a heated moisture chamber in the dark at 55°C for 1.5 hr. Handling of the smears and solutions was carried out using gloved hands to avoid ribonuclease contamination. Following hybridization, the coverslips were removed, and the slides were submerged in warm 0.1% Triton X-100 pH 10, 15 mM NaCl pH 10, and 5 mM Tris HCl (all from Biosesang) in a moist chamber at 55°C and washed for 30 min. The slides were then briefly immersed in distilled water. The sputum smears were finally mounted with one drop of DAPI IV mounting solution (Vysis, Abbott Park, IL, USA) and covered with coverslips. Microscopic examinations were undertaken using a fluorescence microscope (Zeiss, Obercochen, Germany) equipped with a 100×/1.30 oil objective, an HBO 100W mercury lamp, and an FITC/Texas Red dual band filter set (Chroma Technology Corp., Brattleboro, VT, USA).

5. Interpretation of results

By using two PNA probe hybridization reactions in parallel, the analyzed samples were categorized as MTB or NTM according to a positive reaction with one probe and a negative reaction with the other. The PNA FISH and routine AFB stain results were graded from negative to 4+ using a CDC-recommended scale and compared.

6. Ethics statement

This research was approved by a full committee of the Institutional Review Board at Pusan National University Yangsan Hospital (no. 04-2012-025).

RESULTS

1. Probe specificity

The MTB-specific PNA probe hybridized with DNA of the *M. tuberculosis* type strain and not with DNA of the tested NTM species. The NTM-specific PNA probe hybridized with DNA of the tested NTM species except *M. fortuitum* and not with DNA of the MTB species. *M. fortuitum* were double neg-

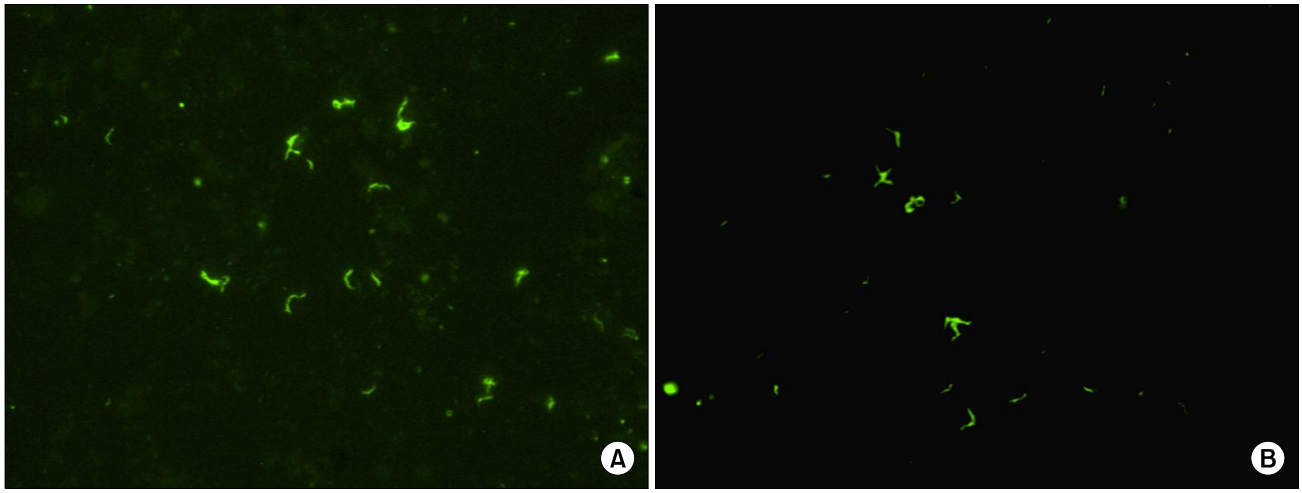

Fig. 1. Positive peptide nucleic acid probe-based fluorescence in situ hybridization for *Mycobacterium tuberculosis* (A) and *M. kansasii* (B) (×100).

Table 2. PNA FISH results of sputum smears compared with culture results

PNA FISH results	Culture results			Total
	Negative	MTB	NTM*	
Negative	10	0	0	10
MTB+/NTM-	0	89	0	89
MTB-/NTM+	0	0	29	29
Total	10	89	29	128

*Eleven *M. intracellulare,* seven *M. kansasii,* six *M. abscessus* and five *M. avium* isolates were included.
Abbreviations: PNA, peptide nucleic acid; FISH, fluorescence in situ hybridization; MTB, *Mycobacterium tuberculosis*; NTM, nontuberculous mycobacteria.

Table 3. Smear grade of routine fluorescent AFB strain and PNA FISH test

Grade of PNA FISH test	Grade of routine fluorescent AFB stain						Total
	Negative	Trace	1+	2+	3+	4+	
Negative	0	10*	0	0	0	0	10
Trace	0	6	4†	0	0	0	10
1+	0	0	15	0	0	0	15
2+	0	0	2‡	34	0	0	36
3+	0	0	0	0	33	0	33
4+	0	0	0	0	0	26	26
Total	0	16	21	34	33	26	128

*Under-treatment culture-negative specimens. †Nontuberculous mycobacteria. ‡*Mycobacterium tuberculosis*.
Abbreviations: PNA, peptide nucleic acid; FISH, fluorescence in situ hybridization; AFB, acid-fast bacillus.

ative with both probes. When testing with the 11 common pathogens, the two probes did not bind any bacteria (Table 1). In all cases, true-positive staining was morphologically distinguishable from the background of the PNA FISH reaction. Morphologic evaluation of PNA FISH was simple in cases with a high bacterial load, even at low magnification, whereas a prolonged high magnification scan was required in cases with a paucity of organisms. Both MTB and NTM were observed on the basis of bright fluorescence and morphology of rod-shaped bacilli (Fig. 1).

2. PNA-FISH reactions in clinical specimens

Eighty-nine MTB culture-positive sputum specimens showed positive results with the MTB-specific PNA probe. Twenty-nine NTM culture-positive sputum specimens were positive with the NTM-specific PNA probe. Ten under-treatment culture-negative sputa showed a positive reaction with a grade of trace. With the culture as the gold standard, MTB- and NTM-specific PNA FISH probes resulted in sensitivity of 100% (89/89; 29/29) and specificity of 100% (39/39; 89/89), respectively (Table 2). The smear grades of the PNA FISH test were the same as those of the routine fluorescent AFB stain in smear grades of 2+ or higher. In trace to 1+ specimens, the smear grade of the PNA FISH test were concordant with those of routine fluorescent AFB stains in 21 specimens. Ten under-treatment culture-negative showed negative reactions in the PNA FISH test. Four NTM specimens were grade 1+ in the routine fluorescent AFB stain, but only a trace was found in the PNA FISH test. Two MTB specimens were grade 1+ in the routine AFB stain, but 2+ was found in the PNA FISH test (Table 3).

DISCUSSION

For the rapid and effective treatment of TB, identification and differentiation of MTB and NTM is critical. In order to reduce the time needed for identification of MTB, stain-based methods that differentiate MTB and NTM in sputum smears are beneficial. Conventional methods for microscopic examination of an AFB stain are simple and fast but are unable to differentiate MTB from NTM. The PNA probe-based FISH assay is rapid and simple for the detection of mycobacteria by smear microscopy. The use of PNA FISH probes that target rRNA makes direct detection of single cells possible without amplification procedures, and no additional enzymatic permeabilization step was required for efficient in situ hybridization [8,17]. In our study, the major advantage of PNA FISH was that it allowed morphologic evaluation of the positive signals, which contributes significantly to avoidance of false-positive results and is as simple as microscopy with less-advanced laboratory facilities, given that a fluorescence microscope and an incubator are already available. The interpretation of the result was possible with few or no problems in most cases, and background artifacts were rarely seen and could easily be distinguished from true-positive signals on the basis of difference in structure.

In previous studies, PNA probe-based FISH assays were able to differentiate between MTB and NTM in culture [6-8,17]. In one study, results obtained with 30 smears of cultured isolates were compared with AccuProbe mycobacterium identification kits (Gen-Probe, San Diego, CA, USA), which revealed 84% diagnostic sensitivities for the MTB-specific PNA probe and 91% for the NTM-specific PNA probe. The specificity of both probes was 100% [8]. Another study using 53 smears of LJ solid cultures and 77 smears of Mycobacteria Growth Indicator Tube (MGIT) liquid cultures (BBL Microbiology Systems, Cockeysville, MD, USA) showed 98-99% diagnostic sensitivities, whereas the diagnostic sensitivities of the NTM probe were 57-100%. The specificity of both probes in both studies was 100% [17].

We report an MTB- and NTM-specific probe-based PNA FISH assay applied to smears of 128 clinical sputum specimens with a grade of trace or higher. By our methods, the MTB- and NTM-specific PNA FISH probes resulted in sensitivity of 100% (89/89; 29/29) and specificity of 100% (39/39; 89/89), respectively, taking culture results as the gold standard. The smear grades of the PNA FISH test were as same as those of routine fluorescent AFB stains in 2+ or higher specimens. Ten under-treatment culture-negative specimens were negative in the PNA FISH test. The result was probably caused by the destruction of rRNA by treatment of TB. Smear grades of trace to 1+ specimens showed lower grades in the PNA FISH test than in a routine fluorescent AFB stain, probably because of the damage to rRNA during specimen processing.

The limitation of our study is that we applied MTB- and NTM-specific PNA probes on two smear slides for each sputum specimen because we designed MTB- and NTM-specific probes labeled with same color of fluorescein. A study has been published in which multicolor multiplex PNA FISH has been used for identification of mixed bacteria [18]. In that study, a mixture of four organisms was simultaneously hybridized with four PNA probes labeled with different fluoresceins. Further work using different fluorescein-labeled PNA probes for simultaneous identification of MTB and NTM on a single smear slide is necessary for rapid and effective identification and differentiation of MTB and NTM. Another problem is that in the current study, NTM probe cannot cover all NTM species such as *M. fortuitum, M. flavescens, M. marinum, M. peregrinum, M. vaccae,* and *M. xenopi* [7,8]. That's why *M. fortuitum* were double negative with both probes.

In mixed mycobacterial sputum specimens with MTB and NTM, culture results may be incorrectly determined to NTM because rapid growing mycobacteria would grow early. Performing a molecular biological technique like a PNA FISH test can solve these problems. Unfortunately however, among the 128 samples in the current study, mixed mycobacterial sputum specimens with MTB and NTM were not included. So, we could not demonstrate the possibility to detect MTB and NTM simultaneously in those specimens.

In addition, we intentionally included the smear positive specimens in the current study because we aimed to compare the smear results between the conventional and PNA FISH methods. Therefore, the calculated sensitivity and specificity of the PNA FISH method are relative performance in smear positive specimens, compared with the conventional AFB staining method. Also, the current study did not cover other species of *M. tuberculosis* complex such as *M. bovis* or *Mycobacterium bovis* BCG strain, and other bacterial and viral species and acid-fast bacteria that may cause respiratory infections not infrequently, especially atypical pneumonia pathogens. Therefore, it was not clear whether the response of the probes react correctly to the untested pathogens.

In conclusion, PNA FISH assay is a useful method for rapid and accurate identification of mycobacteria in 2+ or higher

grade of AFB stain as a new stain-based method that can differentiate MTB and NTM with direct smears of sputum samples.

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=국문초록=

임상 객담검체에서 Peptide Nucleic Acid Probe를 이용한 결핵과 비결핵 항산균의 구분

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배경: 결핵은 전세계적으로 단일 원인균으로는 가장 높은 치사율을 가진 질병이다. 결핵의 치료에 있어서 마이코박테리아를 빠르고 정확하게 확인하는 것이 필수적이다. 본 연구에서는 임상 객담 검체의 도말 슬라이드에서 결핵균과 비결핵 항산균의 감별을 위해 Peptide Nucleic Acid (PNA) Probe를 이용한 FISH assay를 평가하고자 한다.

방법: 결핵균과 비결핵 항산균의 16s rRNA를 타겟으로 한 PNA probe를 합성하였다. 각 probe의 특이도는 표준 균주인 *Mycobacterium tuberculosis* ATCC 13950, *M. kansasii* ATCC 12479, 임상 검체에서 분리된 마이코박테리아 3종(*M. abscessus, M. avium,* and *M. intracellulare*) 그리고 호흡기계에서 흔히 분리되는 10종류의 박테리아를 이용하였다. Centers for Disease Control and Prevention (CDC)의 Acid fast bacili (AFB) 염색 기준상 trace 이상인 128개의 임상 객담 검체를 이용하여 Probe의 성능을 평가하였다. PNA FISH와 항산균 염색결과는 CDC 기준에 따라 단계를 분류하고 서로 비교하였다.

결과: 결핵균과 비결핵 항산균 특이 PNA probe는 결핵균과 *M. kansasii* 표준 균주 그리고 임상검체에서 분리된 3종의 마이코박테리아에 대해 특이적인 반응을 보였고 다른 박테리아와 교차반응을 보이지 않았다. 또한 89개의 결핵균 배양 양성 객담 검체와 29개의 비결핵 항산균 배양 양성 객담 검체에 각각 특이적인 반응을 보였고 CDC 기준에 따라 분류한 PNA FISH와 AFB염색 결과는 2+ 이상의 검체에서 서로 잘 일치하였다.

결론: PNA FISH 방법은 임상 객담 검체에서 결핵을 진단하고, 항산균과 비결핵 항산균을 구분하는데 있어서 민감하고 정확한 결과를 보였다. [Ann Clin Microbiol 2015;18:37-43]

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