ORIGINAL ARTICLE

Identification of Nontuberculous Mycobacteria in Patients with Multidrug-resistant Tuberculosis in Quezon City, Philippines, Using Multiplex PCR

Michelle M. Cabanatan, MSPH,[1] Alice Alma C. Bungay, DVM, MVS,[1]
Sharon Yvette Angelina M. Villanueva, MMSc, PhD, RMicro,[1] Marohren C. Tobias-Altura, MSc,[1]
Dario D. Defensor, MD[2] and Maria Margarita M. Lota, MD, MHPEd[1]

[1]*Department of Medical Microbiology, College of Public Health, University of the Philippines Manila, Manila, Philippines*
[2]*Department of Pathology and Laboratory Medicine, Lung Center of the Philippines, Quezon City, Philippines*

ABSTRACT

Background and Objective. Nontuberculous mycobacteria (NTM) lung disease appears like tuberculosis infection but is resistant to primary anti-tuberculosis drugs. Hence, patients whose sputum sample tests positive for acid-fast bacilli (AFB) and bacterial culture for several times should be assessed for colonization or infection with NTM in a damaged lung secondary to TB. In such cases, though drug-resistant TB may be adequately treated, treatment may need to be directed towards the NTM as well. In NTM therapy, the duration and choice of treatment agent is based upon the specific organism and disease extent. This study used one-step multiplex PCR (mPCR) assay for rapid differentiation of solid cultures in Ogawa medium as *Mycobacterium tuberculosis* (MTB) and/or NTM.

Methods. A total of 80 stocked isolates obtained from the Lung Center of the Philippines from January to December 2018 were screened for NTM in terms of growth in Ogawa medium, acid fastness, and MPT64 TB antigen test result. These were from sputum specimens of multidrug-resistant tuberculosis (MDR-TB) patients. DNA was extracted from cultures (n=55) grown in Ogawa medium and one-step mPCR was performed to identify NTM to the species level.

Results. Out of 80 samples screened, a total of 55 isolates were identified as NTM. One-step mPCR identified 12.73% (7/55) as *M. abscessus*, 34.55% (19/55) as *M. massiliense*, 1.82% (1/55) as *M. kansasii*, and 50.91% (28/55) were identified only up to genus *Mycobacteria* spp. Neither *M. avium* complex nor *M. intracellulare* was identified among the samples tested.

Conclusion. One-step mPCR was able to identify isolates as MTB or NTM coinciding with the initial screening using MPT64 TB antigen test. Multiplex PCR has given a more specific identification to the species level. The use of mPCR in identifying MTB and clinically significant NTM's is suitable for the adequate treatment of mycobacterial infection.

Keywords: multiplex PCR, mycobacteria, multidrug-resistant tuberculosis

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Corresponding author: Michelle M. Cabanatan, MSPH
Department of Medical Microbiology
College of Public Health
University of the Philippines Manila
625 Pedro Gil St., Ermita, Manila 1000, Philippines
Email: mmcabanatan@up.edu.ph
ORCiD: https://orcid.org/0000-0001-6301-1514

INTRODUCTION

Nontuberculous mycobacteria (NTM) have about 150 known species and are considered as potentially pathogenic mycobacteria known to cause both pulmonary[1] and extra-pulmonary infection.[2] They are neither considered as members of *Mycobacterium tuberculosis* complex (MTBc)[3,4] nor *M. leprae*[4] and are found ubiquitous in the environment particularly in soil and water[5]. Hence, infection to humans can be due to environmental exposures with no human-to-human transmission documented as of the present. Moreover, since it does not cause communicable infection, there is limited data regarding the prevalence of NTM diseases. The most common pathogenic species reported were the following:

M. avium complex (MAC), *M. fortuitum*, *M. kansasii*,[5] *M. abscessus,* and *M. chelonae*.[6]

The presence of NTM in respiratory samples does not automatically mean that a person is suffering from NTM lung disease. Sometimes it can be due to environmental contaminants or transient infection.[7,8] Patients are currently considered as having pulmonary NTM disease if the clinical, radiological, and microbiological characteristics, as defined by the 2007 American Thoracic Society and Infectious Disease Society of America (ATS/ IDSA) statements, are met.[5] Patients whose sputum sample tests positive for acid-fast bacilli and bacterial culture for several times should be assessed for colonization or infection with nontuberculous mycobacteria in a damaged lung secondary to TB.[9] NTM lung disease appears like tuberculosis infection but is resistant to primary anti-tuberculosis drugs.[10] In NTM therapy, the duration and choice of treatment agent is based upon the specific organism and disease extent,[1] hence, it is important to identify NTM by molecular characterization for diagnosis and treatment[11].

Sputum smear microscopy is a simple, rapid, and inexpensive method primarily used in the diagnosis of pulmonary tuberculosis[12] wherein Ziehl-Neelsen method (ZN) is the most used staining method[13]. Conventional method of identification such as biochemical testing has low sensitivity, labor intensive, and time-consuming.[14] Rapid identification, on the other hand, can be done using antigen detection tests through the detection of MTB complex specific protein such as MPT64 and MPB64.[15] MPT64 Ag detection test is a rapid immunochromatographic assay used to differentiate MTB complex from nontuberculous mycobacteria. It detects a specific protein known as MPT64 that is known to be secreted only by strains of MTB complex.[16] Due to the improvement in culture and molecular techniques, more than 150 species of Mycobacteria have already been discovered. The detection of the differences within 16S rRNA gene allowed species differentiation of Mycobacteria.[1,17] Molecular techniques such as conventional, real-time, and multiplex PCR, hybridization, as well as oligonucleotide array allow rapid and accurate detection and identification of MTB and NTM that are already available. Additionally, PCR restriction fragment length polymorphism analysis (PRA) of the hsp6, groES and rpoB genes is useful in the identification of NTM to the species level. However, this technique does not have the ability to differentiate patterns of mycobacterial species in samples with mixed infection.[18]

Data are limited regarding the distribution of NTM in Asia. Mycobacterium avium complex (MAC) is common in countries such as Japan, South Korea, North America, and parts of Europe. The second most frequently isolated species in pulmonary samples from Taiwan, China, and Singapore are the Rapidly Growing Mycobacteria (RGM) (16%).[19] In the Philippines, Rivera AB et al. conducted a nationwide prevalence study of nontuberculous mycobacteria in 2001. The prevalence of NTM is 31.9/1000 population.[20] Species identification, however, was not carried out. This study, therefore, utilized multiplex PCR to detect and identify NTM to the species level; however, records of patients to show clinical significance of the isolates were not reviewed. Further species-specific identification was carried out in representative samples using standard sequencing of amplified PCR products.

MATERIALS AND METHODS

Organisms and Media

A total of 80 stock isolates that were collected from January to December 2018 were obtained from the Department of Pathology and Laboratory Medicine, Lung Center of the Philippines. These were isolated from multidrug-resistant tuberculosis (MDR-TB) patients enrolled under the Programmatic Management of Drug-Resistant Tuberculosis (PMDT). Patients' identity and records were kept confidential and accessible only to the researchers. Clinical isolates from sputum samples were included in the study. Cultures were considered as NTM based on colony morphology in Ogawa medium within eight weeks of incubation, tested positive in AFB smear, and negative for MPT64 Antigen Detection Test. Clinical isolates from urine, fine needle aspirates, gastric aspirates, pus, cerebrospinal fluid, peritoneal fluid, synovial fluid, and pericardial fluid were not included in the study.

Stock isolates were maintained in Middlebrook 7H9 with glycerol at -70°C. Reference strains such as *M. tuberculosis* H37Rv, *P. aeruginosa* ATCC 27853, *M. kansasii* ATCC 12478, and *M. avium* subsp. *avium* ATCC 15769 were included in the study and served as control organisms. These strains were used because they are well-known standards in microbiology, representing key species important for both tuberculosis and nontuberculous mycobacterial infections. NTM stock isolates were subcultured at 100 µl per Middlebrook 7H9 at 37°C for 2 to 4 weeks until the liquid medium went turbid. The suspension was mixed thoroughly using a vortex mixer and 200 µl was inoculated into each of two of Ogawa slants. Slants were incubated in a slanted rack at 35–37 °C with caps loosened for seven days to ensure complete absorption of the inoculum. Caps were tightened after seven days and continued incubation for eight weeks. Slants were examined for growth weekly for a span of eight weeks. Colonies with characteristic morphology of TB were confirmed by preparing Ziehl-Neelsen (ZN) smears to confirm the presence of AFB.

Ziehl-Neelsen Method

The smears were stained by Ziehl-Neelsen staining (TB-color; Merck, Darmstadt, Germany). Slide smears were placed on a staining rack with finger-width apart. Slides were covered completely with carbolfuchsin and heated from below until steam has risen. Slides were left for 10 min after heating. Each slide was gently rinsed with water and tilted to drain off excess water. Acid alcohol was added to decolorize the slide

for 3 min. Again, each slide was gently rinsed with water and tilted to drain off excess water. Lastly, each slide was covered with methylene blue for 60 seconds and gently rinsed with water. Each slide was tilted to drain off excess water. Back of slides were cleaned with moist paper and were examined after they have dried.

MPT64 TB Antigen Detection Test

Bacterial colonies that tested positive in AFB smear were examined for the presence of MPT64 antigen. About 200 µl of extraction buffer (SD Bioline Kit, Standard Diagnostics, Inc., Korea) was dispensed in a sterile 2 mL microtube with glass beads (Scientific industries, Bohemia, N.Y., USA; Glasstechnique Mfg, Wertheim, Germany). From the Ogawa medium, 1 µl of bacteria (equivalent to 1 mm diameter of inoculating loop) was inoculated into the extraction buffer. Microtube was closed and mixed thoroughly using a vortex mixer. About 200 µl of the extraction buffer suspension was placed on the specimen well of the test cartridge (SD Bioline Kit, Standard Diagnostics, Inc., Korea). The result was interpreted after 15 minutes.

Multiplex Polymerase Chain Reaction Assay

Procedure for the multiplex PCR Assay was adopted from the method developed by Chae H, et al. with slight modification.[21] The eight-primer sets used in this study are shown in Table 1.

DNA Extraction

A loop of bacterial culture was inoculated in a 200 µl DNA extraction solution (Chelex 100 Resin, Bio-Rad) and was vortexed for 1 minute. It was then placed in a VS-251D1 block heater (Vision Scientific, Korea) with a temperature of 86°C for 20 minutes. A mini-beadbeater-16 was used to disrupt microbial cells through agitation for 20 seconds. After which, samples were boiled for 20 minutes using a water bath and was centrifuged at 13,000 rpm for 3 min at RT. Supernatant liquid was transferred in a 2 mL microtube. The quality and quantity of DNA were analyzed by a spectrophotometer (Thermo Scientific NanoDrop 2000).

PCR Amplification

Polymerase Chain Reaction was performed using the following PCR mixture: 5 µl of 2X Prime Taq Premix (GeNet Bio, Daejeon, Korea), 1.92 µl of primer mix containing 0.06 µl of *DT1* and *mtbk_20680*, 0.8 µl of *Rv0577*, and 0.2 µl of *16S rRNA*, *IS1311, mass_3210, RD9* and *mkan_rs12360* primer sets (Integrated DNA Technologies, Coralville, IA, USA), 1 µl of DNA template, and 2.08 µl of RNASE free water (*QIAGEN*). PCR tubes were placed in a preheated thermal cycler (Bio-Rad T100 thermal cycler) set at 95°C and proceeded to PCR reaction. The PCR cycles (20 cycles) comprised the following: initial denaturation at 95°C for 10 min, followed by denaturation at 96°C for 45 sec, annealing at 54°C and 66°C for 45 sec, extension at 72°C for 40 sec and final extension steps at 72°C for 5 min.

Agarose Gel Electrophoresis

About 3 µl PCR product, 1 µl 100 bp PCR size marker (VC 100bp DNA ladder, Vivantis) on the first lane, and 1 µl 1kb PCR size marker (BenchTop 1kb DNA ladder, Promega) on the last lane were placed on a 1.5% agarose gel (Invitrogen). The assay products were electrophoresed for 40 min at 75 V in 0.5X TAE buffer (Invitrogen, Thermo ref. 15558-042). The gel was post-stained with GelRed (Biotium 41003, Fremont, California, USA). Result of the gel electrophoresis was read with the use of a UV transilluminator (Bio-Rad Laboratories Inc. USA). For validation, representative sample of amplified products were submitted for sequencing. Around 10 µl of PCR products as well as 20 µl of primers were prepared and

Table 1. Specific Primers Used in the Multiplex PCR Assay *(Chae, H. et al., 2017)*

	Target Gene	Target Organism(s)	Primer Sequence (Forward and Reverse)	Amplicon Length	Reference
1	*16S* rRNA gene	All mycobacterial species	5` GAGATACTCGAGTGGCGAAC 3` 5` CAACGCGACAAACCACCTAC 3`	506 bp	[21]
2	*rv0577*	All *M. tuberculosis* complex	5` ATGCCCAAGAGAAGCGAATACA 3` 5` AATGTCAGCCGGTTCCGCA 3`	705 bp	[21]
3	*RD9*	*M. tuberculosis*	5` GTGTAGGTCAGCCCCATCC 3` 5` GTAAGCGCGTGGTGTGGA 3`	369 bp	[21]
4	*mtbk_20680*	*M. tuberculosis* Beijing family	5` TTATGCCAGAAATACACCCGCG 3` 5` AATCGCGGGCTTGTGGCTAC 3`	231 bp	[21]
5	*IS1311*	*M. avium complex*	5` TCGATCAGTGCTTGTTCGCG 3` 5` CGATGGTGTCGAGTTGCTCT 3`	600 bp	[21]
6	*DT1*	*M. intracellulare*	5` AAGGTGAGCCCAGCTTTGAACTCCA 3` 5` GCGCTTCATTCGCGATCATCAGGTG 3`	106 bp	[21]
7	*mass_3210*	*M. abscessus* *M. massiliense*	5` GCTTGTTCCCGGTGCCACAC 3` 5` GGAGCGCGATGCGTCAGGAC 3`	310 bp 1145 bp	[21]
8	*mkan_rs12360*	*M. kansasii*	5` ACAAACGGTGTGTCGCAATGTGCCA 3` 5` TGTCGAGCAGACGTTCCAGGACGGT 3`	199 bp	[21]

submitted to Macrogen, Inc. (South Korea). Samples and primers were placed in individual 0.5 ml microtubes, packed in a sealed 50 ml container, and placed in a Ziploc.

Sequence Data Analysis

The resulting DNA sequence was processed using BioEdit software, a biological sequence alignment editor, and analyzed using Basic Local Alignment Search Tool (BLAST) gene sequence analysis.

Ethics Statement

This research study was approved by the Lung Center of the Philippines Institutional and Ethics Review Board (LCPIERB) and University of the Philippines Manila Research Ethics Board (UPMREB).

RESULTS

The assay employed an eight-target multiplex PCR, and the presence of specific amplicons was visualized on 1.5% agarose gel. The sizes of the resulting PCR products and the groups that they identify are as follows: a 506-bp amplicon specific to the 16S rRNA gene for all mycobacteria; a 705-bp amplicon specific to *rv0577* for MTBC species; a 369-bp amplicon specific to RD9 for *M. tuberculosis*; a 231-bp amplicon specific to *mtbk_20680* for the *M. tuberculosis* Beijing family; a 600-bp amplicon specific to IS*1311* for MAC; a 106-bp amplicon specific to DT1 for *M. intracellulare*; a 310-bp amplicon and a 1,145-bp amplicon specific to *mass_3210* for *M. abscessus* and *M. massiliense*, respectively; and a 199-bp amplicon specific to *mkan_rs12360* for *M. kansasii* (Figures 1A to 1G).

From the 80 isolates subcultured in Ogawa medium, 92.5% (74/80) had growth, 3.75% (3/80) had no growth, and 3.75% (3/80) were contaminated. Of the 74 isolates with growth in Ogawa medium and tested for AFB, about 75.68% (56/74) were smear positive. The absence or faintness of bands in some lanes can be attributed to several factors such as sample degradation, suboptimal loading of DNA, or technical variations during the gel electrophoresis process.[22] To differentiate *Mycobacterium tuberculosis* complex from nontuberculous mycobacteria, smear positive organisms were tested for MPT64 TB antigen. Of which, 98.21% (55/56) tested negative for MPT64 antigen.

A total of 55 clinical isolates were identified to the species level using one-step mPCR assay. These isolates have growth in Ogawa medium, tested positive in AFB smear, and tested negative in MPT64 antigen test. From the 55 isolates tested, majority of the clinically significant NTM identified belongs to *Mycobacterium abscessus* complex (i.e., *M. abscessus* subsp. *abscessus* referred herewith as *M. abscessus*), *M. abscessus* subsp. *massiliense* (referred herewith as *M. massiliense*). About 12.73% (7/55) were identified as *M. abscessus*, 34.55% (19/55) were *M. massiliense*, 1.82% (1/55) were *M. kansasii*, and 50.91% (28/55) were identified as other mycobacteria. There were no species of *M. avium* complex and *M. intracellulare* identified among the samples tested (Figure 2). Reference strains such as *M. tuberculosis* H37Rv, *M. kansasii* ATCC 12478, *M. avium* subsp. *avium* ATCC 15769, and *P. aeruginosa* ATCC 27853 were also identified.

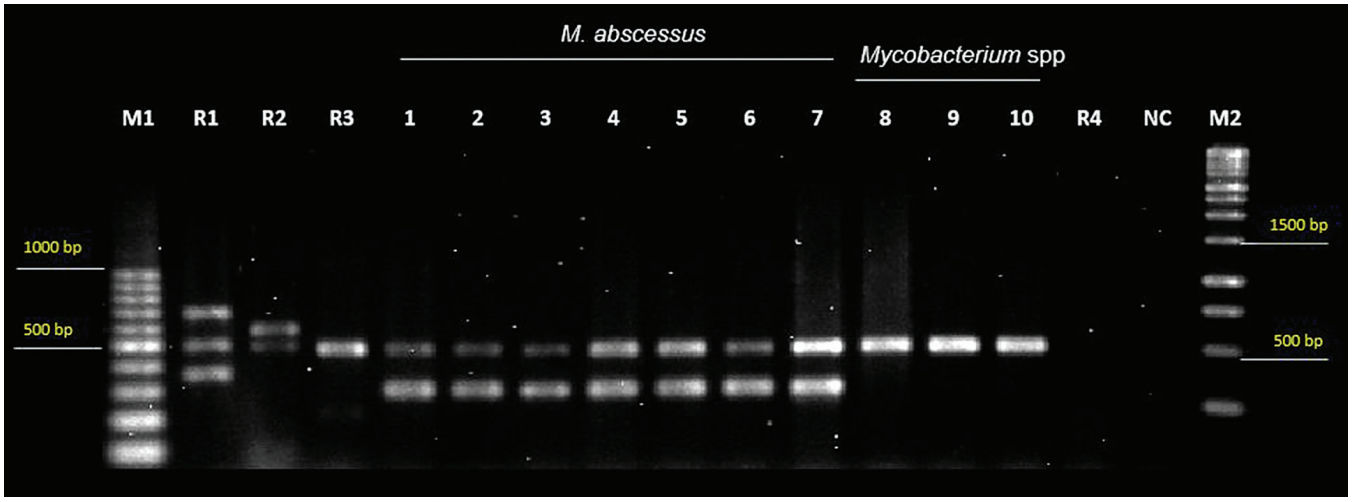

Figure 1A. ***Representative result of mPCR using reference strains and clinical isolates.*** The amplified products were of the following sizes: **Lane M1** (100 bp) and **M2** (1 kb), DNA ladder; **Lane R1**, 506 bp (16S), 705 bp (*rv0577*), and 369 bp (RD9) for *M. tuberculosis* H37Rv; **R2**, 506 bp (16S) and 600 bp (IS*1311*) for *M. avium* ATCC 15769; **R3**, 506 bp (16S) and 199 bp (*mkan_rs12360*) for *M. kansasii* ATCC 12478; **R4**, no band for *P. aeruginosa* ATCC 27853; **Lane 1 to 7**, 506 bp (16S) and 310 bp (*mass_3210*) for *M. abscessus*; **Lane 8 to 10**, 506 bp (16S) for all mycobacterial species; and **Lane NC**, for negative control.

M – marker, R – reference strain, and NC – negative control

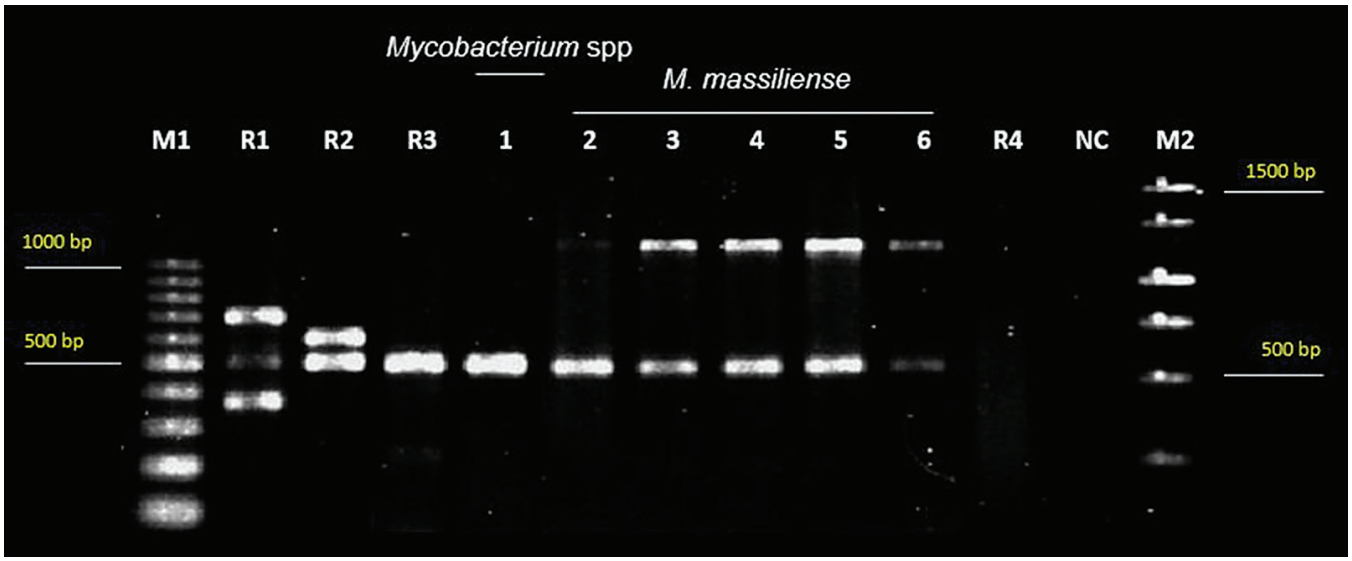

Figure 1B. ***Representative result of mPCR using reference strains and clinical isolates.*** The amplified products were of the following sizes: **Lane M1** (100 bp) and **M2** (1 kb), DNA ladder; **Lane R1**, 506 bp (16S), 705 bp (*rv0577*), and 369 bp (RD9) for *M. tuberculosis* H37Rv; **R2**, 506 bp (16S) and 600 bp (IS*1311*) for *M. avium* ATCC 15769; **R3**, 506 bp (16S) and 199 bp (*mkan_rs12360*) for *M. kansasii* ATCC 12478; **R4**, no band for *P. aeruginosa* ATCC 27853; **Lane 1**, 506 bp (16S) for all mycobacterial species; **Lane 2 to 6**, 506 bp (16S) and 1145 bp (*mass_3210*) for *M. massiliense*; and **Lane NC**, for negative control.

M – marker, R – reference strain, and NC – negative control

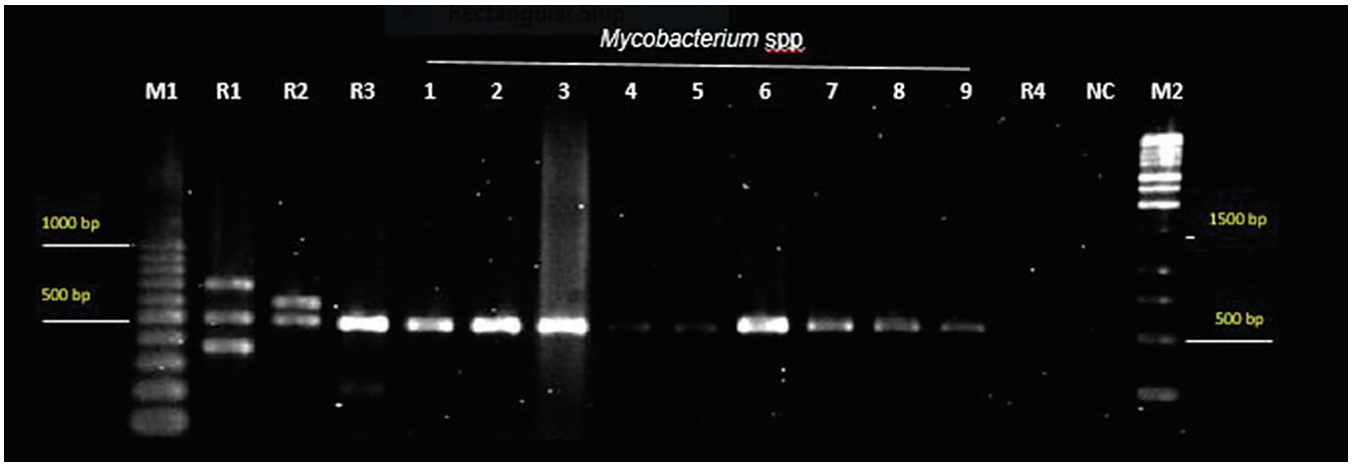

Figure 1C. ***Representative result of mPCR using reference strains and clinical isolates.*** The amplified products were of the following sizes: **Lane M1** (100 bp) and **M2** (1 kb), DNA ladder; **Lane R1**, 506 bp (16S), 705 bp (*rv0577*), and 369 bp (RD9) for *M. tuberculosis* H37Rv; **R2**, 506 bp (16S) and 600 bp (IS*1311*) for *M. avium* ATCC 15769; **R3**, 506 bp (16S) and 199 bp (*mkan_rs12360*) for *M. kansasii* ATCC 12478; **R4**, no band for *P. aeruginosa* ATCC 27853; **Lane 1 to 9**, 506 bp (16S) for all mycobacterial species; and **Lane NC**, for negative control.

M – marker, R – reference strain, and NC – negative control

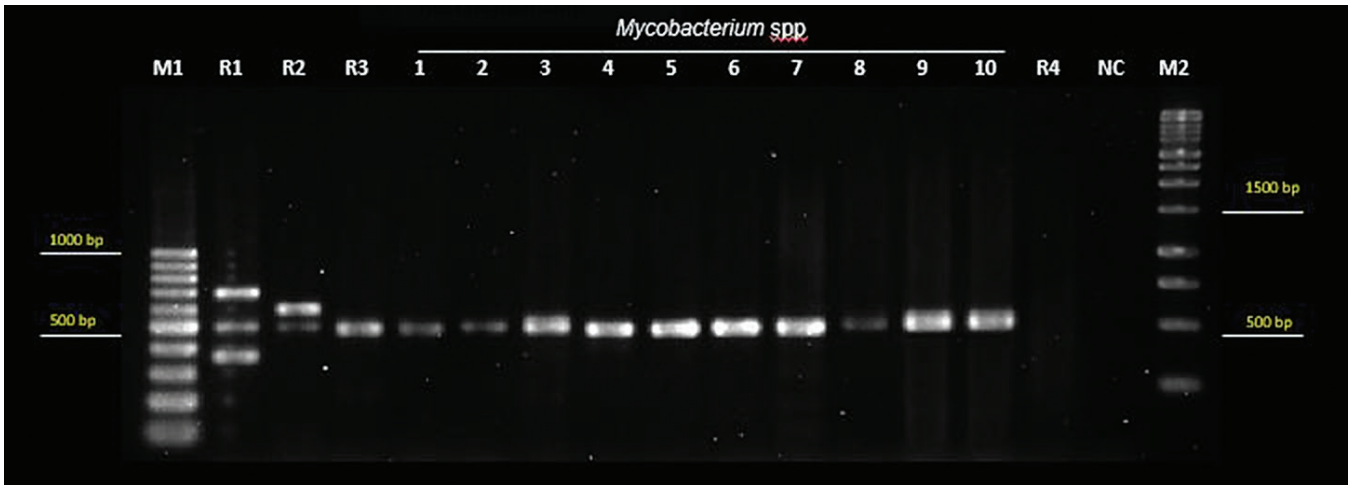

Figure 1D. ***Representative result of mPCR using reference strains and clinical isolates.*** The amplified products were of the following sizes: **Lane M1** (100 bp) and **M2** (1 kb), DNA ladder; **Lane R1**, 506 bp (16S), 705 bp (*rv0577*), and 369 bp (RD9) for *M. tuberculosis* H37Rv; **R2**, 506 bp (16S) and 600 bp (IS*1311*) for *M. avium* ATCC 15769; **R3**, 506 bp (16S) and 199 bp (*mkan_rs12360*) for *M. kansasii* ATCC 12478; **R4**, no band for *P. aeruginosa* ATCC 27853; **Lane 1 to 10**, 506 bp (16S) for all mycobacterial species; and **Lane NC**, for negative control.

M – marker, R – reference strain, and NC – negative control

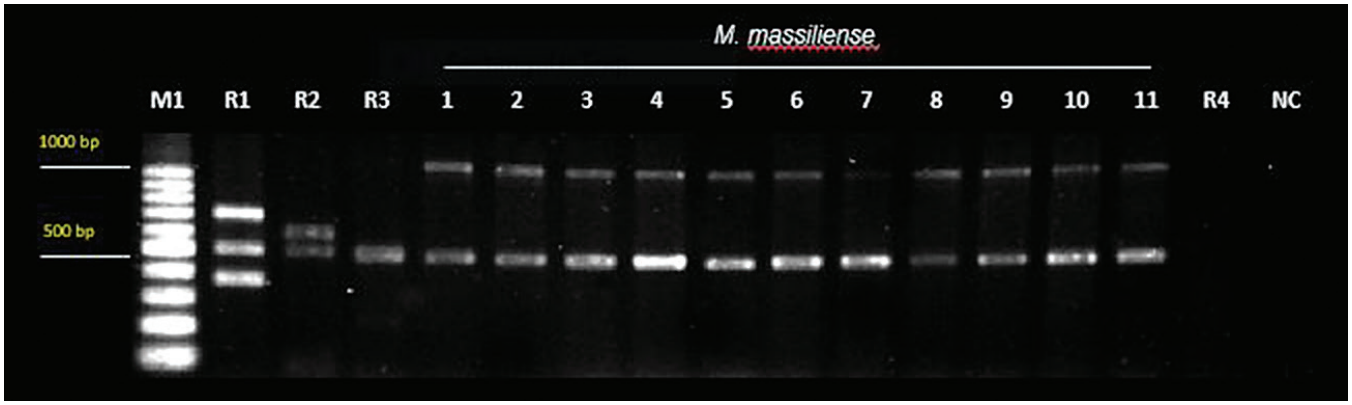

Figure 1E. ***Representative result of mPCR using reference strains and clinical isolates.*** The amplified products were of the following sizes: **Lane M1** (100 bp), DNA ladder; **Lane R1**, 506 bp (16S), 705 bp (rv0577), and 369 bp (RD9) for *M. tuberculosis* H37Rv; **R2**, 506 bp (16S) and 600 bp (IS*1311*) for *M. avium* ATCC 15769; **R3**, 506 bp (16S) and 199 bp (*mkan_rs12360*) for *M. kansasii* ATCC 12478; **R4**, no band for *P. aeruginosa* ATCC 27853; **Lane 1 to 11**, 506 bp (16S) and 1145 bp (*mass_3210*) for *M. massiliense*; and **Lane NC**, for negative control.

M – marker, R – reference strain, and NC – negative control

The identities of the amplified products were confirmed by Macrogen (Seoul, South Korea) through DNA standard sequencing. BLAST gene sequence analysis of representative samples revealed that the analyzed products have high similarity (95-100%) with *M. abscessus* complex species and Mycobacterium spp. (Table 2).

DISCUSSION

From the 55 clinical isolates tested, seven isolates were identified as *M abscessus*, 19 isolates as *M. massiliense*, one isolate as *M. kansasii*, and 28 isolates were only identified up to the genus level, *M. tuberculosis* spp. Band at 506-bp which is specific to the 16S rRNA gene for all mycobacteria was present in all 55 clinical isolates as well as in the 3 reference strains of *M. tuberculosis* H37Rv, *M. avium* complex ATCC 15769, and *M. kansasii* ATCC 12478. This coincided with the initial screening done for NTM which include having

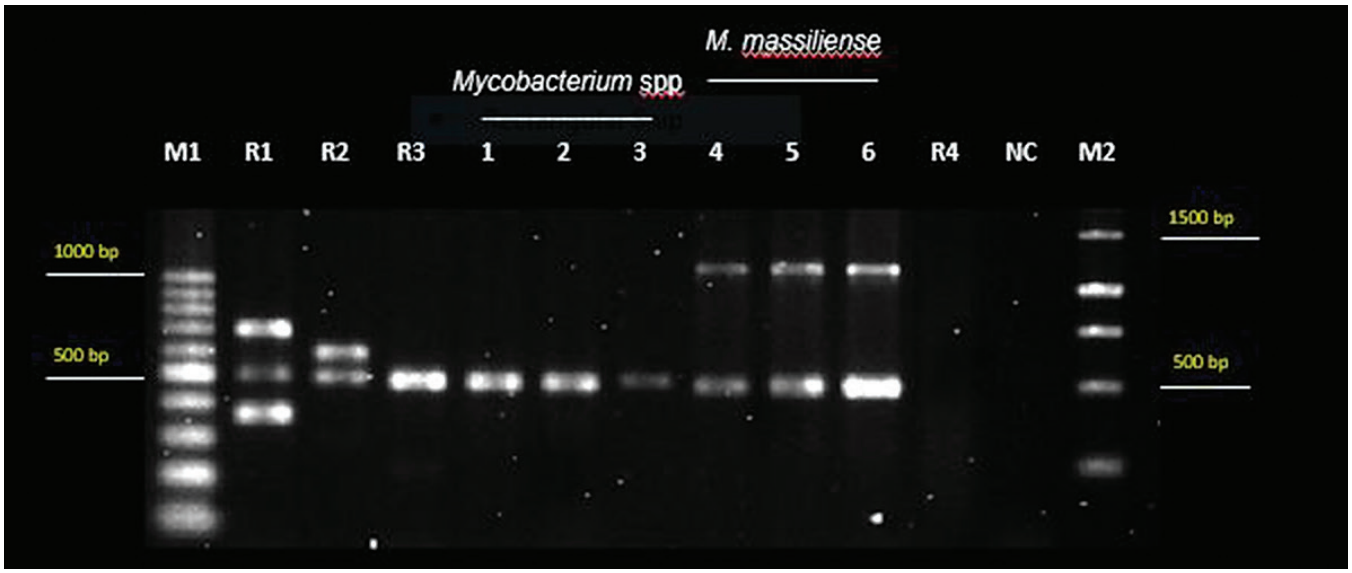

Figure 1F. ***Representative result of mPCR using reference strains and clinical isolates.*** The amplified products were of the following sizes: **Lane M1** (100 bp) and **M2** (1 kb), DNA ladder; **Lane R1**, 506 bp (16S), 705 bp (*rv0577*), and 369 bp (RD9) for *M. tuberculosis* H37Rv; **R2**, 506 bp (16S) and 600 bp (IS*1311*) for *M. avium* ATCC 15769; **R3**, 506 bp (16S) and 199 bp (*mkan_rs12360*) for *M. kansasii* ATCC 12478; **R4**, no band for *P. aeruginosa* ATCC 27853; **Lane 1 to 3**, 506 bp (16S) for all mycobacterial species; **Lane 4 to 6**, 506 bp (16S) and 1145 bp (*mass_3210*) for *M. massiliense*; and **Lane NC**, for negative control.

M – marker, R – reference strain, and NC – negative control

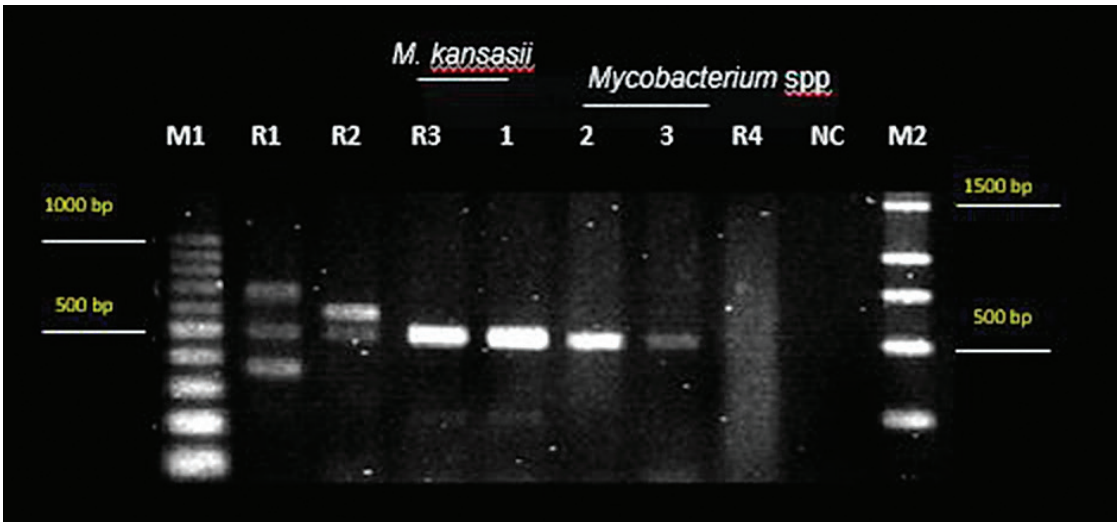

Figure 1G. ***Representative result of mPCR using reference strains and clinical isolates.*** The amplified products were of the following sizes: **Lane M1** (100 bp) and **M2** (1 kb), DNA ladder; **Lane R1**, 506 bp (16S), 705 bp (*rv0577*), and 369 bp (RD9) for *M. tuberculosis* H37Rv; **R2**, 506 bp (16S) and 600 bp (IS*1311*) for *M. avium* ATCC 15769; **R3**, 506 bp (16S) and 199 bp (*mkan_rs12360*) for *M. kansasii* ATCC 12478; **R4**, no band for *P. aeruginosa* ATCC 27853; **Lane 1**, 506 bp (16S) and 199 bp (*mkan_rs12360*) for *M. kansasii*; **Lane 2 to 3**, 506 bp (16S) for all mycobacterial species; and **Lane NC**, for negative control.

M – marker, R – reference strain, and NC – negative control

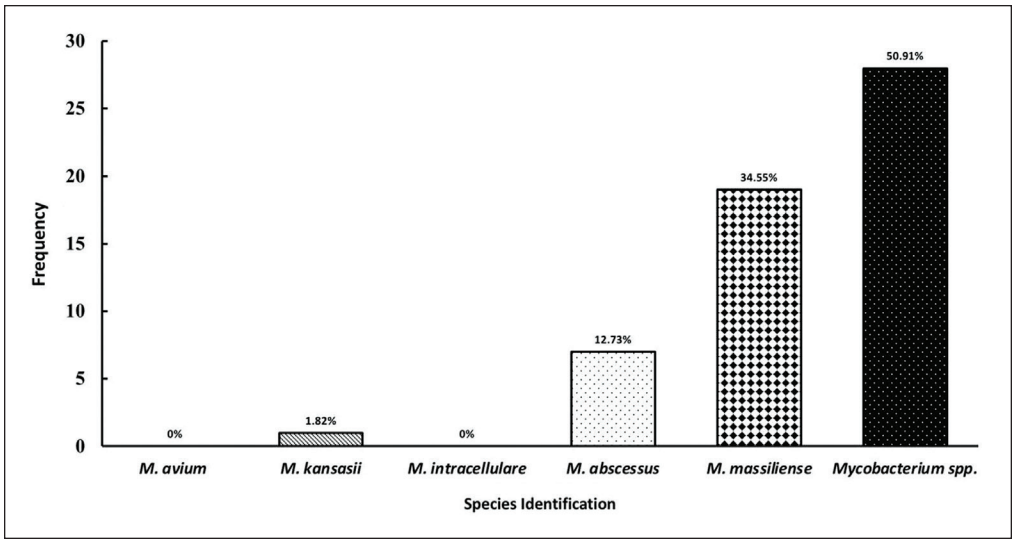

Figure 2. Frequency of NTM species recovered from stock isolates (n=55).

Table 2. Sequence Analysis of Representative Samples Using BLAST Sequence Alignment

Query		Subject			Score		Identities Pct. (%)
Name	Length	Gene	Accession	Length	Max	EValue	
176774_mass_3210	284	*Mycobacterium abscessus* subsp. *abscessus* strain GD69A chromosome, complete genome	CP065269.1	5166737	481	2.E-113	99.25
566_mass_3210	1100	*Mycobacterium abscessus* subsp. *massiliense* strain 381 chromosome, complete genome	CP022230.1	4681624	1338	0	95.41
756_mass_3210	280	*Mycobacterium abscessus* subsp. *abscessus* strain GD69A chromosome, complete genome	CP065269.1	5166737	422	1.E-113	97.22
321_mass_3210	240	*Mycobacterium abscessus* subsp. *massiliense* strain 381 chromosome, complete genome	CP022230.1	4681624	342	7.E-90	97.04
814_mass_3210	60	*Mycobacterium abscessus* strain FDAARGOS_678 chromosome	CP050978.1	5094912	111	3.E-21	100
9358_16S	95	Mycobacterium sp. BA-241 gene for ribosomal RNA, partial sequence	LC546451.1	807	176	2.E-40	100

growth in Ogawa medium, testing positive in AFB smear, and testing negative in MPT64 TB antigen. The result of the DNA standard sequencing through BLAST sequence alignment also revealed high similarity (95-100%) with *M. abscessus* complex and *Mycobacterium* spp. tested.

In this study, the two most identified clinically significant NTM species in the clinical isolates tested were *M. abscessus* and *M. massiliense*. These two organisms belong to *Mycobacterium abscessus* complex (MABSC) and are classified as rapidly growing mycobacteria. Additionally, neither *M. avium* complex nor *M. intracellulare* were identified among the isolates tested. In countries like Japan, South Korea, North America, and parts of Europe, the most isolated mycobacteria belong to *Mycobacterium avium* complex (13%–81%). Also, this organism is the most common cause of NTM lung disease. However, in some parts of Asia like Taiwan, Singapore, and China, the second most frequently isolated species in pulmonary samples are the rapidly growing mycobacteria (16%).[19] And among all the RGM isolates, 31% (582/1,744) were considered clinically relevant and consistent with American Thoracic Society (ATS) diagnostic criteria. Patients are considered as having pulmonary NTM disease if the clinical, radiological, and microbiological characteristics, as defined by the 2007 American Thoracic Society and Infectious Disease Society of America (ATS/IDSA) statements, are met.[19] On this conducted study, however, records of patients to show clinical significance of the isolates were not reviewed.

The group of *M. abscessus* complex composed of *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* are known to cause not only pulmonary infections but also skin and soft tissue infections (SSTIs).[23] Globally, *M. abscessus* has been regarded as the most common RGM which causes chronic inflammatory lung disease among individuals with cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease (COPD). Patients appear to have nodular as well as cavitary granuloma and manifests persistent lung infection. Unlike

other NTM infections, there is no reliable antibiotic regimen for *M. abscessus* infection.[24] Drugs such as clarithromycin and azithromycin are considered as key components of treatment.[25,26] It was also found that the three subspecies of *M. abscessus* exhibit a difference in clarithromycin susceptibility. Although *M. abscessus* subsp. *massiliense* does not exhibit inducible clarithromycin resistance, both *M. abscessus* subsp. *abscessus* and M. *abscessus* subsp. *bolletii* appear to resist clarithromycin through the *erm (41)* gene mechanism.[27] Resistance to macrolides by *M. abscessus* spp. can either be acquired or inducible. Acquired resistance does not entail horizontal transmission of genetic elements such as plasmids. Inducible resistance, on the other hand, has functional *erm (41)* gene but with the presence of thymine in position 28 (T28) and methylation of adenine in 23S rRNA at position 2058. Isolates with inducible resistance requires up to 14 days of incubation for detection, hence, can be mistaken as susceptible in a 3-day susceptibility test.[28]

Lastly, only one isolate of *M. kansasii* was identified and was classified under slow growing mycobacteria. The abovementioned organism as well as *M. gordonae,* and *M. malmoense* are not common in Asia but are commonly isolated in Europe. In a study done by Singh K. et al. in India, 50 isolates were tested using one-step multiplex PCR assay.[2] About 26 were identified as *M. kansasii,* 20 as *M. abscessus*, and four as MAC. According to Guan Q, et al.,[29] NTM disease in South America, South Africa, and Europe is caused by *M. kansasii*. The said organism is known to cause lymph node, skin, and systemic infection. Cases of co-infection with HIV has also been reported. Similarities of *M. kansasii* with *M. tuberculosis* infection has been observed in terms of symptoms and radiographic pattern.[29,30] In several genetic studies, five subspecies have been discovered with type I as the most pathogenic and the other four considered as environmental isolates.[29]

CONCLUSION

One-step multiplex PCR Assay was able to identify reference strains and test isolates as NTM and this coincided with the initial screening done for NTM using MPT64 TB antigen test. Moreover, mPCR has given a more specific identification of the samples to the species and/ or subspecies level. This has been further confirmed by the result of the DNA standard sequencing of the representative sample of amplified products. As clinically significant NTM's causing pulmonary disease such as *M. abscessus*, *M. massiliense*, and *M. kansasii* are being isolated from TB patients, this calls for a need to check possible co-infection and have data regarding the true burden of NTM disease in the country. In this study wherein NTM isolates were tested, records of patients were not reviewed. In future studies, review of records of patients must be done to show clinical relevance of NTM isolated in patients with pulmonary TB. Moreover, replicates of PCR are recommended to ensure consistency and accuracy of results. The use of mPCR in identifying MTB as well as clinically significant NTM's is suitable for the adequate treatment of mycobacterial infection in the Philippines.

Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

All authors declared no conflicts of interest.

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