Infection and Drug Resistance

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Identification of Significant Pathogenic Nontuberculous Mycobacteria Species from Presumptive TB Patients Using Partial *hsp65* Gene Sequencing

Dyshelly Nurkartika Pascapurnama[1], Nabilla Ghina Zavitri[1], Raspati Cundarani Koesoemadinata[1], Adi Imam Cahyadi[2], Lidya Chaidir[2,3]

[1]Research Center for Care and Control of Infectious Diseases, Universitas Padjadjaran, Bandung, West Java, Indonesia; [2]Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Sumedang, West Java, Indonesia; [3]Center for Translational Biomarker Research, Universitas Padjadjaran, Bandung, West Java, Indonesia

Correspondence: Lidya Chaidir, Faculty of Medicine, Universitas Padjadjaran, Jln. Ir. Soekarno Km. 21 Jatinangor, Sumedang, 45363, West Java, Indonesia, Tel +62-22-8428812, Email lidya.chaidir@unpad.ac.id

Purpose: To date, the diagnosis of nontuberculous mycobacteria (NTM) disease primarily relies on clinical symptoms and radiological features. Our objective was to apply a sequence-based analysis method by using partial gene sequencing of heat shock protein 65 (*hsp65*) to identify NTM species.

Patients and Methods: A total of 32 stored isolates obtained from individuals suspected of having pulmonary NTM infection were subjected to solid Ogawa culture. Genomic DNA from each sample was extracted and used in a conventional polymerase chain reaction (PCR) targeting a specific region of *hsp65* gene. Identified amplicons from the PCR were then subjected to targeted sequencing. Analysis of the obtained *hsp65* sequence was performed using DNA Baser tool. The consensus sequences obtained were compared to references in the GenBank NCBI database to determine NTM species.

Results: We identified several important NTM species which posses opportunistic characteristics. *M. abscessus* and *M. chelonae* are the most frequent NTM species identified in this study (40.63% and 18.75%, respectively). These two species have the potential to cause significant infections in human, ranging from opportunistic pulmonary infection to localized skin infection. Additionally, pathogenic NTM members of *M. fortuitum group (MFG), M. avium, M. intracellulare, M. kansasii*, and *M. celatum* were also found among all identified species.

Conclusion: Sequence-based analysis is a promising method for identifying species of NTM. The *hsp65* gene has a high discriminatory power to identify opportunistic pathogen NTM species in specimens in Indonesia. Consequently, *hsp65* partial gene sequencing is considerable as an alternative and reliable approach for NTM speciation.

Keywords: nontuberculous mycobacteria, species identification, *hsp65* gene, sequence-based analysis

Introduction

The Mycobacterium genus encompasses various species, including the *Mycobacterium tuberculosis* complex, which is responsible for tuberculosis (TB) infections, *Mycobacterium leprae*, which associated with leprosy, and a group of nontuberculous mycobacteria (NTM) species.[1] The NTM species, also known as Mycobacteria other than tuberculosis (MOTT), can be widely found in the common environment, such as soil and water. To this day, over 200 species of NTM are reported. They can be classified into slow-growing mycobacteria (SGM), which the colony formation requires at least seven days and rapid-growing mycobacteria (RGM), which form colonies in less than seven days.[2] As they are widely present in the environment, humans can be infected with NTM through regular activities.

There has been a significant global increase of opportunistic infection due to NTMs since the 1990s, resulting them emerge as important human pathogens.[1] However, unlike TB, diseases associated with NTM infections are not monitored by public

Received: 4 May 2023
Accepted: 27 September 2023
Published: 30 October 2023

health authorities; therefore, the true incidence of NTM infections may be underestimated, despite a steady rise in their prevalence. NTMs can cause infections in a wide variety of body sites. While approximately 90% of NTM infections frequently causes pulmonary diseases, there are also NTM-related skin diseases, lymphadenitis, and disseminated diseases.[3] NTM pulmonary disease (NTM-PD) shares similar symptoms to TB, often leading to misdiagnosis and treatment failures. Furthermore, the NTM infections place some groups at higher risk, such as patients with chronic pulmonary diseases and immunocompromised conditions (for example, patients with HIV and cystic fibrosis).[4] Pulmonary NTM infections are most commonly caused by *Mycobacterium avium* complex (MAC), *Mycobacterium kansasii, Mycobacterium abscessus* complex (MABC) and *Mycobacterium chelonae*.[5]

Currently, diagnostic modalities for identifying NTM infections are very limited. In Indonesia, the NTM infections are typically diagnosed using chest x-ray, liquid cultures, and differential diagnosis of MTB (eg, MPT64 antigen test).[6] However, these techniques only allow to identifying NTM groups, rather than species specific. Generally, NTMs display initial resistance to the first line of the anti-TB drug. Their treatment requires different approaches from anti-TB drug, despite sharing similar symptoms. Moreover, the progression and disease characteristics are completely different.[7] Furthermore, due to variations in treatment options among NTM species, it is necessary to identify the species and determine an appropriate regimen for the disease.[8] Due to these limitations, studies on developing better diagnostic tools and treatments for NTM-related diseases are necessary.

In this study, an alternative approach for identifying mycobacteria was established through heat shock protein 65 (*hsp65*) partial gene sequencing, and the identification of NTM species can be done by sequence-based analysis. Additionally, our objective was to investigate the prevalence rate of each identified NTM species among the confirmed NTM infection based on the obtained result.

Materials and Methods

Selection of NTM Isolates

The samples used in this research were selected from retrospective samples in the form of stored isolates characterized as NTM suspects. All samples involved in the study categorized as "NTM suspects" were obtained from previous clinical research in which the inclusion is patient-based, and sputum sampling was carried out twice. For this research, samples underwent a thorough data screening process, ensuring the availability of relevant information such as demographic data, confirmation of positive Acid-Fast Bacilli (AFB) result through microscopic examination, negative result of MPT64 antigen test to differentiate between MTBC and NTM, and formation of cord factor in liquid bacterial culture. All stored isolates were sourced from a sample biobank in the Research Center for Care and Control of Infectious Diseases, Faculty of Medicine, Universitas Padjadjaran, Indonesia.

Mycobacterial Culture and DNA Extraction

Retrospective samples were retrieved and grown in Ogawa solid medium. After colonies emerged, total genome extraction was performed using the Cetyltrimethylammonium bromide (CTAB) based method adapted from Somerville et al in 2005.[9] The sample was then put in 200 µL oTris-EDTA buffer and boiled at 85°C to kill the bacteria, followed by incubation in −20°C for 15 minutes and addition on 40 µL lysozyme (20 mg/mL). Following incubation in 37°C for 1 hour, the bacterial membrane and protein was broken down using SDS 1% and Proteinase K (250 µg/mL final concentration) with continuous agitation and incubation in 65°C. After incubation; the pellet and suspension phases were separated using 1% N-acetyl-N, N, N-trimethyl ammonium bromide (CTAB reagent) and NaCl. DNA was then precipitated with ethanol and eluted in 60 µL of nuclease-free water. The extracted DNA was stored at −20°C for other purposes.

PCR *hsp65* Gene and DNA Sequencing

A conventional polymerase chain reaction (PCR) was performed on the DNA of 32 selected isolates. A mix consists of 1x PCR Master Mix Solution (HotStar Taq Master Mix Kit (Qiagen, Hilden, Germany), 0.3 µM of each forward-reverse *hsp65* primers and 1.5 µL DNA template. The *hsp65* primers used for the PCR (Forward: 5'- GGT CAA GGA AGT TGC CAA GA-3' and reverse: 5'- ACC AGC AGG ATG TAG GGA TC-3') were from in silico design. The PCR was performed with the following

conditions: 95°C for 15 minutes, 35 cycles at 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 20 seconds and finalized at 72°C for 10 minutes. The amplicon visualization was performed using electrophoresis with 2% agarose gel. Negative (Nuclease-Free Water) and *M. tuberculosis* H37Rv controls were added in each reaction to detect cross reaction. The PCR product was then sequenced using the Sanger sequencing method with Applied Biosystems BigDye® Terminator Cycle Sequencing Kit v3.1. The sequencing results were then analyzed, and gene profiling was carried out to determine the feasibility of the fragment as a DNA barcode. Sequence data was then imported in .abi and FASTQ files format, then trimming and base editing of the sequences results through alignment and adjustment with sequencing chromatograms using DNA Baser software (www.dnabaser.com). The results of sequence edits are saved in notepad format for later analysis for species identification. Sequence analysis and editing used DNA Baser software, while species identification used NCBI BLAST.

The study was approved by the Ethics Committee, Faculty of Medicine, Universitas Padjadjaran (434/UN6.KEP/EC/2020).

Results

A total of 2476 adult patients with suspected pulmonary TB were subjected to data screening; 1424 (57.5%) had positive culture results. Based on the screening of MPT64 antigen test results and formation of cord factor in the liquid culture, which differentiates between MTBC and NTM, 32 (2.2%) patients were determined as suspected NTM cases during the study period. Table 1 shows the demographic distribution of patients included in this study, with a majority being female (n = 19; 59.4%). The median age was 43.4 years old, with a higher distribution of patients aged 36–45 (28.1%). Some of the samples were collected from patients with previous history and treatment (40.6%). Only a few with HIV or DM (3.1% and 18.8%, respectively).

The *hsp65* gene fragments (446 bp) were successfully amplified for all the isolates. The sequence analysis identified some pathogenic NTM species (93.7%) and non-pathogenic species (6.3%) among 32 NTM isolates. *M. abscessus* (n = 13) and *M. chelonae* (n = 6) were the most prominent species identified in this study. Other pathogenic NTM species can also be found, such as members of the MAC complex (*M. avium* (n = 2), *M. intracellulare* (n = 1)), *M. kansasii* (n = 2), *M. celatum* (n = 2), and member of MFG (*M. fortuitum* (n = 2), *M. arcueilense* (n = 1), and *M. conceptionense* (n = 1)). The result also showed the identification of non-pathogenic NTM, *M. vanbaaleni* (n = 2). The highest prevalence from the sample population was *M. abscessus* (40.63%), followed by *M. chelonae* (18.75%), *M. fortuitum* group (12.51%), and MAC (*M. avium* complex) (9.38%) (Table 2).

NTM speciation data can also be related to the type of colony growth, as well as patient data such as age, history of TB disease and DM status. NTM species were determined based on their presentation of similarity to the *hsp65* reference sequence (Table 3).

Table 1 Participant Sociodemographic Characteristics (N = 32)

Characteristics	N	(%)
Age (Median)	**43.4**	
Sex		
Male	13	38.2
Female	19	61.8
Category		
Patient with TB history	13	38.2
Patient with HIV positive	1	2.9
Patient with DM positive	6	17.6

Table 2 Identified NTM Species Based on *hsp65* Partial Gene Sequencing

Species	No. of Identified Samples	%	Note
Mycobacterium abscessus	13	40.63%	
Mycobacterium chelonae	6	18.75%	
Mycobacterium fortuitum	2	6.25%	*M. fortuitum, M. arcueilense and M. conceptionense* are part of MFG (*M. fortuitum* group)
Mycobacterium arcueilense	1	3.13%	
Mycobacterium conceptionense	1	3.13%	
Mycobacterium avium	2	6.25%	*M. avium* and *M. intracellulare* are grouped as MAC (*M. avium* complex)
Mycobacterium intracellulare	1	3.13%	
Mycobacterium kansasii	2	6.25%	
Mycobacterium celatum	2	6.25%	
Mycobacterium vanbaalenii	2	6.25%	
Total	32	100%	

Table 3 Identified NTM Species Based on *hsp65* Partial Gene Sequencing, Looking into Subject's Demographic and History Data

No.	Isolate No.	Age	Sex	History of Previous TB	DM Status	Colony Growth Type	Species Based on *hsp65* Seq Analysis	% identity with *hsp65* Seq
1	41100518	26,6	Male	No	No	Fast	*M. abscessus*	100%
2	41100246	37,0	Female	Yes	No	Fast	*M. abscessus*	100%
3	41100864	28,4	Male	No	No	Fast	*M. abscessus*	100%
4	41101080	39,7	Female	No	No	Fast	*M. abscessus*	100%
5	41100689	30,0	Female	No	No	Fast	*M. abscessus*	100%
6	41100174	42,5	Female	Yes	No	Fast	*M. abscessus*	100%
7	41100967	42,8	Male	No	No	Fast	*M. abscessus*	100%
8	41101057	53,2	Female	Yes	Yes	Slow	*M. abscessus*	100%
9	41101072	39,0	Male	Yes	NA	Slow	*M. abscessus*	100%
10	41100525	27,2	Male	Yes	No	Slow	*M. abscessus*	99.51%
11	41100709	38,7	Female	Yes	No	Slow	*M. abscessus*	98.44%
12	41100841	47,0	Male	Yes	No	Fast	*M. abscessus*	96%
13	41100459	48,5	Female	No	Yes	Fast	*M. abscessus*	94.65%
14	41100721	62,9	Female	No	No	Fast	*M. chelonae*	100%
15	41100194	48,0	Male	Yes	Yes	Fast	*M. chelonae*	100%
16	41100318	53,0	Female	No	No	Fast	*M. chelonae*	100%

(*Continued*)

Table 3 (Continued).

No.	Isolate No.	Age	Sex	History of Previous TB	DM Status	Colony Growth Type	Species Based on *hsp65* Seq Analysis	% identity with *hsp65* Seq
17	41100560	29,0	Female	No	No	Fast	*M. chelonae*	100%
18	41100213	65,7	Female	No	No	Fast	*M. chelonae*	100%
19	41100593	32,4	Female	No	No	Fast	*M. chelonae*	99.78%
20	41100104	21,2	Female	Yes	No	Fast	*M. fortuitum*	99.33%
21	41100125	27,9	Male	No	No	Fast	*M. fortuitum*	99.33%
22	41100116	49,3	Male	No	No	Fast	*M. arcueilense*	98.89%
23	41100105	47,2	Female	No	Yes	Fast	*M. conceptionense*	99.08%
24	41100434	65,3	Female	Yes	No	Slow	*M. avium*	100%
25	41100530	57,0	Male	No	Yes	Slow	*M. avium*	100%
26	41100842	57,0	Male	Yes	No	Slow	*M. intracellulare*	99.48%
27	41100838	56,4	Female	No	Yes	Slow	*M. kansasii*	99.72%
28	41100441	28,6	Male	No	No	Slow	*M. kansasii*	99.53%
29	41100235	39,9	Male	Yes	No	Slow	*M. celatum*	98.21%
30	41100097	40,5	Female	No	No	Slow	*M. celatum*	98.21%
31	41100166	44,0	Female	Yes	No	Slow	*M. vanbaalenii*	95.98%
32	41100173	64,1	Female	No	No	Slow	*M. vanbaalenii*	95.98%

Discussion

NTM species known as opportunistic pathogen, which can cause human disease in complex clinical context, such as chronic pulmonary diseases. Giving symptoms which are similar to TB, NTM infections might be misdiagnosed as TB, or drug-resistant TB. Various factors, such as age, gender, HIV status, or history of chronic pulmonary diseases are associated with the susceptibility to NTM infection.[10]

As the burden of NTM infection among individual with presumptive TB cases is increasing, it is crucial to develop a reliable diagnostic approach for NTM disease and implement screening of NTM infection for presumptive TB patients with specific risk factors such as age and gender, and prior pulmonary disease.[5] Furthermore, the development of tools to identifying NTM species will enhance our understanding of prevailing species and possible treatment given, as different NTM species require specific drug regimens, which may affect the effectiveness of therapy.

This study specifically focused on identifying NTM species from stored specimens obtained from individuals suspected of having NTM infections using partial *hsp65* gene sequencing and discovering the prevalence of each identified NTM species in adult patients. The *hsp65* gene, also known as the *gro-EL2* gene, codes for a 65 KDa heat shock protein, which is present in all the species of mycobacteria.[11] This protein contains epitopes that are unique as well as common to various species of mycobacteria. While it has hypervariable regions which are very polymorphic, its conserved nature allows differentiation of mycobacteria using primers common to all mycobacteria.

The identification method for Mycobacterium species using the *hsp65* gene was first implemented by Telenti et al.[11] At that time, the gene was used in a PCR pattern restriction enzyme analysis (PRA) method, employing a 441-bp fragment of the 1600-bp complete gene. Forty reference strains of Mycobacterium were used to establish a diagnostic algorithm. Later, the algorithm was applied to 290 clinical samples. Restriction enzyme digestion of those PCR products using *BstEII* and *HaeIII* resulted in the differentiation of Mycobacterium species based on their pattern of molecular size.

This resulted identification of *M. tuberculosis complex, M. gordonae, M. intracellulare, M. malmoense, M. avium, M. kansasii, M. szulgai, M. fortuitum complex, M. chelonae*, and *M. abscessus*. Since then, the *hsp65* gene has been very reliable in various Mycobacterium diagnostic methods.

In this research, a specifically designed primer set targeting the *hsp65* partial gene was utilized, and it underwent the in-silico optimization step. In contrast to the primers which are widely used,[11] where the primer can still detect *M. tuberculosis*, the primer designed for this study excludes the *M. tuberculosis complex*, discriminate RGM and SGM, making it suitable for the specific identification of NTM. Our findings demonstrated that *the hsp65* gene can unambiguously identify RGM species responsible for human infection, such as *M. abscessus, M. chelonae, M. fortuitum*, and *M. peregrinum*. The *hsp65* gene offers an advantage over *16S rRNA* gene sequencing in accurately identifying RGM species, which has been challenging to distinguish.[12]

The *hsp65* gene is also effective in distinguishing different subsets within the SGM groups, such as the *M. avium* complex (MAC). Previous study used sequencing of hypervariable 3' region of *hsp65* to distinguish among subsets of MAC, which has been difficult to identify due to several factors, including the ambiguous phenotypic and genotypic assays as well as limitations in human and avian strains, which appear to be distinct.[13] The obtained sequences indicate that *M. intracellulare* is genetically divergent from *M. avium* organisms, and distinct sequevars were identified for *M. avium* subsets, including *M. avium subsp. avium* (bird type), *M. avium subsp. hominissuis*, and *M. avium subsp. paratuberculosis*.

In this study, the *hsp65* gene was selected as the target gene due to its presence in all Mycobacterium and has a considerable variation in its gene sequence, which has the potential to identify a bacterial species genetically. The *hsp65* protein is also one of the most expressed Mycobacterium antigens, supported by a phylogenetic analysis which demonstrated the *hsp65* gene capability to perform separation with a high level of confidence.[11,13]

This study highlights the effectiveness of partial *hsp65* gene sequencing in identifying various NTM species, which are known as opportunistic pathogens causing human disease. Of the 32 samples used in this study, *M. abscessus* was the most predominant species identified (40.63%). This finding is consistent with other previous studies, where *M. abscessus* was the most common rapidly growing mycobacteria identified from respiratory specimens.[14,15] A study conducted in Singapore found 49.9% of *M. abscessus-chelonae* group identified among 2026 NTM isolates.[15] Similarly, *M. abscessus* was also predominant in a study conducted in Thailand, where 45 of 129 NTM specimen were identified as *M. abscessus*. [16]

M. abscessus is a rapid-growing NTM pathogen that can cause lung disease, especially in susceptible patients with a history of cystic fibrosis, bronchiectasis, and tuberculosis.[17,18] It is also common to find *M. abscessus* in the environment, such as water and soil. At least 50% of cases of *M. abscessus* infection manifest as lung disease and after tuberculosis treatment,[19] followed by *M. chelonae* (18.75%) and species of the MFG group (identified species consist of *M. fortuitum* (6.25%), *M. arcueilense* (3.13%) and *M. conceptionense* (3.13%)) which have also been well documented in the causation of NTM-PD. Moreover, recovered SGM species recovered, such as MAC (*M. avium* (6.25%), *M. intracellulare* (3.13%)) and *M. kansasii* (6.25%), have commonly been associated with pulmonary disease. Our research also discovered *M. celatum* (6.25%) and *M. vanbaalenii* (6.25%) among the NTM isolates. *M. celatum*, even though it is uncommon to cause human infection, can cause localized infections in the lungs and lymph nodes, mainly in immunocompromised hosts. In contrast, *M. vanbaaleni* is not known to be pathogenic.

Considering demographic and clinical history data, 10 out of 13 patients with a history of TB were suspected to be infected with RGM, and eight were identified as *M. abscessus*. This NTM species can cause pulmonary disease, especially in people with underlying structural lung disease and a history of tuberculosis.[17] Although *M. abscessus* is considered as RGM species, this study found three out of 13 isolates identified as *M. abscessus* showed slow growth when were observed in liquid culture observation (Isolate 41101057, 41101072, 41100525, and 41100709). Those results are possibly correlated with each patient's history of TB, as isolates of the *M. abscessus complex*, especially those from patients treated with multiple antimicrobial agents over time, often grow very slowly in liquid culture, even more than 5 days.[20] However in this study, the detail of each patient's medication were not enclosed. Meanwhile, the risk factor for gender cannot be determined in this study because it requires a larger sample size.

In Indonesia, study related to NTM infections, particularly regarding species identification and the prevalence of each species, is limited. Previous research has been conducted in various settings with different research focuses and methods. Studies conducted Surakarta by Saptawati et al analyzed the 5-year laboratory data obtained from a lung hospital (Balai

Besar Kesehatan Paru Masyarakat/BBKPM), Surakarta, Indonesia to calculate the prevalence of NTM and came up with a percentage of 15% among positive *Mycobacterium* culture, and 3.5% among suspected TB patients.[6] Study from Mertaniasih et al, reported 5.78%of NTM infection positivity rate within TB suspect patients in Dr. Soetomo Hospital, Surabaya, Indonesia, which were diagnosed with niacin accumulation test and TB Ag MPT 64 (SD Bioline) test without providing species-specific information of each NTM isolates.[6,21], A study focusing on NTM identification had been done in Jakarta on analyzing 15 samples obtained from pulmonary specimens, skin, and soft tissues and showed the identification of *M. abscessus, M. intracellulare, M. avium*, and *M. cookii* by using 16S rRNA sequence analysis.[22]

Despite being the second study conducted in Indonesia that applies molecular approach to identify NTM species, our research is the first study in Indonesia which demonstrates the use of partial *hsp65* gene sequencing for the identification of NTM species and present the distribution of each species identified and expected to provide broader epidemiological data on NTM in the region, contributing to a more comprehensive understanding of the NTM prevalence among patients with pulmonary infection. To expand our understanding, further studies about species identification using sequence-based analysis are needed, preferably with a larger sample size that covers a wider geographic area of Indonesia. The low proportion of NTM found in our study reflects the study subjects (study population) which were recruited from primary health care facilities with a high likelihood of being TB disease. The findings might be different in the hospitals or lung clinics where patients come with more severe disease and have risk factors for NTM infection. Conducting a prospective cohort study embedded with TB cohort would be ideal to t to maximize the benefits for the patients.

Conclusion

In conclusion, partial *hsp65* gene sequencing has a high discriminatory power to identify NTM species in specimens from Indonesia. This research successfully identified several significant NTM species known to be opportunistic pathogen through sequence-based analysis of the *hsp65* gene. *M. abscessus* became the most prevalent species among the NTM specimens analyzed in this study, *followed by M. chelonae, M. fortuitum group* (MFG), *M. avium complex* (MAC), *M. celatum, M. vanbaalenii*. The identification of NTM species can help clinicians in selecting appropriate treatment strategies, reducing the risk of misdiagnosis with TB. Based on our findings, partial *hsp65* gene sequencing holds a potential to serve as an appropriate first-line identification method for NTM isolated from human samples.

Acknowledgments

We thank Bachti Alisjahbana, MD, PhD, for providing access to the samples used for this study. This study was financially supported by the Health Science and Technology Research Grants by The National Institute of Health Research and Development (BALITBANGKES Ministry of Health of the Republic of Indonesia) (HK.03.01/1/727/2020). The funding sources had no role in the study's design, conduct, analysis or reporting.

Disclosure

The authors report no conflicts of interest in this work.

References

1. To K, Cao R, Yegiazaryan A, Owens J, Venketaraman V. General overview of nontuberculous mycobacteria opportunistic pathogens: *Mycobacterium avium* and *Mycobacterium abscessus*. *J Clin Med*. 2020;9(8):2541. PMID: 32781595; PMCID: PMC7463534. doi:10.3390/jcm9082541
2. Kim CJ, Kim NH, Song KH, et al. Differentiating rapid- and slow-growing mycobacteria by difference in time to growth detection in liquid media. *Diagn Microbiol Infect Dis*. 2013;75(1):73–76. PMID: 23114094. doi:10.1016/j.diagmicrobio.2012.09.019
3. Atkins BL, Gottlieb T. Skin and soft tissue infections caused by nontuberculous mycobacteria. *Curr Opin Infect Dis*. 2014;27(2):137–145. PMID: 24464139. doi:10.1097/QCO.0000000000000041
4. Bar-On O, Mussaffi H, Mei-Zahav M, et al. Increasing nontuberculous mycobacteria infection in cystic fibrosis. *J Cyst Fibros*. 2015;14(1):53–62. PMID: 24917112. doi:10.1016/j.jcf.2014.05.008
5. Johnson MM, Odell JA. Nontuberculous mycobacterial pulmonary infections. *J Thorac Dis*. 2014;6(3):210–220. PMID: 24624285; PMCID: PMC3949190. doi:10.3978/j.issn.2072-1439.2013.12.24
6. Saptawati L. Prevalence of Nontuberculous Mycobacteria (NTM) in Surakarta, Indonesia: higher Than Expected. The 1st International Conference on Health, Technology and Life Sciences. KnE Life Sciences; 2019: 132.
7. Pennington KM, Vu A, Challener D, et al. Approach to the diagnosis and treatment of nontuberculous mycobacterial disease. *J Clin Tuberc Other Mycobact Dis*. 2021;8(24):100244. PMID: 34036184; PMCID: PMC8135042. doi:10.1016/j.jctube.2021.100244

8. Maya TG, Komba EV, Mensah GI, et al. Drug susceptibility profiles and factors associated with nontuberculous mycobacteria species circulating among patients diagnosed with pulmonary tuberculosis in Tanzania. *PLoS One*. 2022;17(3):e0265358. PMID: 35324922; PMCID: PMC8947393. doi:10.1371/journal.pone.0265358
9. Somerville W, Thibert L, Schwartzman K, Behr MA. Extraction *of Mycobacterium tuberculosis* DNA: a question of containment. *J Clin Microbiol*. 2005;43(6):2996–2997. PMID: 15956443; PMCID: PMC1151963. doi:10.1128/JCM.43.6.2996-2997.2005
10. Pavlik I, Ulmann V, Weston RT; Clinical relevance and environmental prevalence of Mycobacterium fortuitum Group Members. Comment on Mugetti et al. Gene sequencing and phylogenetic analysis: powerful tools for an improved diagnosis of fish mycobacteriosis caused by Mycobacterium fortuitum Group Members. Microorganisms 2021, 9, 797. *Microorganisms*. 2021;9(11):2345. PMID: 34835470; PMCID: PMC8622867. doi:10.3390/microorganisms9112345
11. Telenti A, Marchesi F, Balz M, Bally F, Böttger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol*. 1993;31(2):175–178. PMID: 8381805; PMCID: PMC262730. doi:10.1128/jcm.31.2.175-178.1993
12. Ringuet H, Akoua-Koffi C, Honore S, et al. *hsp65* sequencing for identification of rapidly growing mycobacteria. *J Clin Microbiol*. 1999;37(3):852–857. PMID: 9986875; PMCID: PMC84584. doi:10.1128/JCM.37.3.852-857.1999
13. Turenne CY, Semret M, Cousins DV, Collins DM, Behr MA. Sequencing of hsp65 distinguishes among subsets of the Mycobacterium avium complex. *J Clin Microbiol*. 2006;44(2):433–440. PMID: 16455896; PMCID: PMC1392684. doi:10.1128/JCM.44.2.433-440.2006
14. Koh WJ, Kwon OJ, Jeon K, et al. Clinical significance of nontuberculous mycobacteria isolated from respiratory specimens in Korea. *Chest*. 2006;129(2):341–348. doi:10.1378/chest.129.2.341
15. Zhang ZX, Cherng BPZ, Sng LH, Tan YE. Clinical and microbiological characteristics of non-tuberculous mycobacteria diseases in Singapore with a focus on pulmonary disease, 2012–2016. *BMC Infect Dis*. 2019;19(1):436. doi:10.1186/s12879-019-3909-3
16. Chetchotisakd P, Kiertiburanakul S, Mootsikapun P, Assanasen S, Chaiwarith R, Anunnatsiri S. Disseminated nontuberculous mycobacterial infection in patients who are not infected with HIV in Thailand. *Clin Infect Dis*. 2007;45(4):421–427. doi:10.1086/520030
17. Griffith DE, Aksamit T, Brown-Elliott BA, et al; ATS Mycobacterial Diseases Subcommittee; American Thoracic Society; Infectious Disease Society of America. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med*. 2007;175(4):367–416. Erratum in: Am J Respir Crit Care Med. 2007 Apr 1, 175(7):744–745. Dosage error in article text. PMID: 17277290. doi:10.1164/rccm.200604-571ST
18. Lee MR, Yang CY, Chang KP, et al. Factors associated with lung function decline in patients with nontuberculous mycobacterial pulmonary disease. *PLoS One*. 2013;8(3):e58214. PMID: 23483998; PMCID: PMC3590167. doi:10.1371/journal.pone.0058214
19. Redelman-Sidi G, Sepkowitz KA. Rapidly growing mycobacteria infection in patients with cancer. *Clin Infect Dis*. 2010;51(4):422–434. PMID: 20617902. doi:10.1086/655140
20. Brown-Elliott BA, Woods GL, Kraft CS. Antimycobacterial susceptibility testing of nontuberculous mycobacteria. *J Clin Microbiol*. 2019;57(10):e00834–19. PMID: 31315954; PMCID: PMC6760954. doi:10.1128/JCM.00834-19
21. Mertaniasih NM, Kusumaningrum D, Koendhori EB, Kusmiati T, Dewi DN, Dewi DSS. Nontuberculous mycobacterial species and *Mycobacterium tuberculosis* complex coinfection in patients with pulmonary tuberculosis in Dr. Soetomo Hospital, Surabaya, Indonesia. *Int J Mycobacteriol*. 2017;6(1):9–13. doi:10.4103/2212-5531.201894
22. Yasmon A, Agnes A, Febriani R, Kusumaningrum A, Sudarmono P. Identification of Nontuberculous Mycobacteria (NTM) species isolated from the sputum, skin, and soft tissue of patients in Jakarta, Indonesia. *Southeast Asian J Trop Med Public Health*. 2021;52(5):644–650.