CHARACTERIZATION OF NONTUBERCULOUS *MYCOBACTERIUM* SPECIES DETECTED FROM SPUTUM SAMPLES OF SUBJECTS IN NORTHERN VIETNAM

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Abstract. Nontuberculous mycobacteria (NTM) are opportunistic infections that can proliferate in humans, animals and the environment. NTM and *Mycobacterium tuberculosis* (MTB) are both acid-fast bacilli, therefore conventional tests such as Ziehl-Neelsen staining cannot tell them apart. For the treatment of NTM infection, a macrolide-containing regimen is frequently necessary to overcome innate resistance to first-line antituberculosis medications. Knowing the prevalent species of local NTM, particularly from clinical samples, may suggest suitable treatments when necessary. In order to identify NTM, sequencing the *hsp65* gene, a trustworthy and frequently used method, and qPCR for detection of MTB, was carried out in 1,003 sputum samples from suspected tuberculosis patients in northern Vietnam. Among these, 86 (8.5%) were labeled NTM, while 118 (11.7%) MTB were found. *M. avium* (n = 32, 37%), *M. abscessus* (n = 18, 21%), *M. simiae* (n = 13, 15%), and *M. fortuitum* (n = 12, 14%) were the four largest complex groups among the 86 NTM. In this study, the distribution of common NTM was comparable to other reports from TB-burden countries including China, India and other South East Asian nations. This study provided information on the prevalent NTM species in northern Vietnam, which may aid scientists and physicians in understanding the variety of local mycobacteria.

Keywords: *Mycobacterium abscessus*, *Mycobacterium avium*, *hsp65*, MTB, nontuberculous mycobacteria, NTM

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INTRODUCTION

Nontuberculous mycobacteria (NTM) often inhabit the environment (Falkinham, 2009), and may colonize and/or infect the human body, especially the respiratory tract (Claeys and Robinson, 2018; Yoon *et al*, 2020). Similar to *Mycobacterium tuberculosis* (MTB), NTM can cause diseases in immunodeficiency individuals or chronic conditions such as HIV infection, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (McCarthy *et al*, 2012; Adjemian *et al*, 2018a; Adjemian *et al*, 2018b; Pyarali *et al*, 2018). Also, pulmonary diseases are common features of NTM infections, which is similar to MTB disease (Johnson and Odell, 2014).

Routine testing of sputum with Ziehl-Neelsen staining may detect acid fast bacilli (AFB), but it is unable to distinguish between MTB and NTM as they are similar under microscopic observation (Bayot *et al*, 2023). Although, standard first-line therapy for MTB containing ethambutol, isoniazid, pyrazinamide, and rifampin can treat NTM infection, but this therapy is not optimum for NTM, and there are other recommended drugs such as macrolides and amikacin for treating certain mycobacteria species (Haworth *et al*, 2017; Pathak *et al*, 2022).

In clinical practice, recognizing common NTM in a local setting before initiating therapy is very useful for an empiric treatment. Recently, reports from developed countries such as the United States, and tuberculosis-burden areas (China, India and Tanzania) have shown a variety of NTM in both humans and animals (Katale *et al*, 2014; Adjemian *et al*, 2018a; Adjemian *et al*, 2018b; Sharma and Upadhyay, 2021; Sun *et al*, 2022). According to the abovementioned reports, the dominant common NTM species are *M. abscessus* complex, *M. avium* complex (MAC) and *M. fortuitum*. These data are important for local guidelines of empiric treatment when needed.

In Vietnam, there are limited data on NTM variability from environmental and/or clinical samples. Previously, NTM from Vietnam are included in several multi-site research studies from South East Asia, where HIV-infected adults and children are enrolled for the detection of NTM and MTB (McCarthy *et al*, 2012; Borand *et al*, 2019). In these studies, NTM are detected in 16.7-21.0% of the patients, and 12.0-17.7% with MTB. The common NTM species are *M. abscessus, M. fortuitum, M. kansasii*, and MAC (McCarthy *et al*, 2012; Borand *et al*, 2019).

However, these studies did not provide specific results from Vietnam, and it is important to note that NTM infection cases may extend beyond HIV-infected patients. Therefore, further investigations are required to determine the prevalent NTM species in Vietnam to provide data on their biological properties and medical implications. Quantitative (q)PCR has been reported as a feasible technique to detect of MTB in developing countries such as Colombia and Pakistan (Ikram *et al*, 2015; Casallas-Rivera *et al*, 2017) and sequencing of a mycobacterial *hsp65* amplicon for identification of NTM (Ringuet *et al*, 1999; Chimara *et al*, 2008; Senna *et al*, 2008; Kumar *et al*, 2014). Here, we conducted a study to characterize the NTM species present in sputum samples collected from individuals in northern Vietnam.

MATERIALS AND METHODS

Sample collection

Sputum samples were collected from 1,003 patients with suggestive symptoms of tuberculosis including a persistent cough, exhaustion, chest pain, weight loss, and evening rise fever, attending National Hospital 74, Ha Giang Lung Hospital, Nghe An Friendship General Hospital, Quang Ninh General Hospital, and Thanh Thuy Medical Center, from 2020 to 2021. For the purpose of a cross sectional analysis, a single sample was retrieved from each patient, with written informed consent.

The study was approved by the Ethics Committee, Joint Vietnam-Russia Tropical Science and Technology Research Center (Approval no. 2199/CN-HDDD).

Molecular assay

All samples were tested by quantitative (q)PCR (MTB Real-TM kit; Sacace Biotechnologies, Como, Italy) to identify *M. tuberculosis.* We chose *hsp65* gene as the target of DNA amplification and sequencing using the reference primer pair hsp-65 F 5′ ACCAACGATGGT-GTGTCCAT 3′ and hsp-65 R 5′ CTTGTCGAACCGCATACCCT 3′ (Telenti *et al*, 1993; Kumar *et al*, 2014). The reaction was conducted using 2X PCR Master Mix Solution (i-Taq™, LiliF Diagnostic, iNtRON Biotechnology, Gyeonggi-do, Korea). The thermocycling program of DNA amplification was as follows: 95°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds. PCR amplicon (~441 bp) was separated by 1.5% agarose gel-electrophoresis, observed using Redsafe dye and directly sequenced (First BASE DNA Sequencing Services, Singapore).

DNA sequence was analyzed by both NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and *hsp65* blast tool (http://hsp65blast.phsa.ca/blast/blast.html) to identify Mycobacterium spp. Sequences were submitted to GenBank (accession nos. OQ658413 - OQ658498).

RESULTS

Of the 1,003 sputum samples, 118 (11.7%) were MTB positive based on the MTB qPCR assay (Sacace Biotechnologies, Como, Italy) and excluded, while the remaining samples were subjected to PCR amplification and sequencing of the *Mycobacteria hsp65* amplicons (~441 bp). Eighty-nine (10.0%) samples were successfully amplified and sequenced, of which 86 (8.5% of total samples) were identified as NTM and the remaining three as *Breviabacterium casei, Rothia mucilaginosa* and *Streptomyces coelicolor*. Among the NTM-positive 86 subjects, 58 and 28 were males and females respectively, with a mean age of 58 years old and occupations comprising of farmers (*n* = 39), industrial workers (*n* = 14), official staff (*n* = 1), retirees (*n* = 26), students (*n* = 2), traders (*n* = 3), and unemployed (*n* = 1). The most prevalent detected NTM specimen was *M. intracellulare* (*n* =25, 29.0%), *M. abscessus* (*n* = 10, 11.6%), *M. fortuitum* (*n* = 8, 9.3%), *M. massiliense* (*n* = 8, 9.3%), and *M. simiae* (*n* = 7, 8.1%) (Table 1). In terms of complex groups, the predominant was MAC, followed by *M. abscessus* complex, then *M. simiae* complex, *M. fortuitum* complex, and *M. gordonae*. Six samples did not belong to any of these groups.

DISCUSSION

In this study, for the first time, a description of NTM species from the sputum of MTB-suspected patients in Vietnam was reported. Although the number of samples was limited and restricted to subjects in the northern Vietnam, the results might reflex NTM prevalence in the respiratory tracts of people in the whole country. MAC and *M. abscessus* complexes were most prevalent. It is well-documented that *M. avium* induces a high risk of lung disease among people with chronic conditions (Wang *et al*, 2020) and *M. abscessus* is strongly associated with nosocomial infections (Ghosh *et al*, 2017). Although *M. simiae* and *M. fortuitum* complexes were present at low prevalence and are believed to be less pathogenic than the two aforementioned groups, they can cause disease in individuals with immunosuppression, malignancy or pulmonary fibrosis (Park *et al*, 2008; Jabbour *et al*, 2020). These

Table 1

Nontuberculous mycobacteria from sputum of individuals with suggestive symptoms of tuberculosis in northern Vietnam (2020 to 2021) determined from sequences of a fragment of *Mycobacterium hsp65*

Sample ID	*Mycobacterium* sp (total number)	*Mycobacterium* complex (total number)
N126	*M. avium* (1)	*M. avium* complex (32)
N45, N46, N55, N59, N100	*M. colombiense* (5)	
N3, N5, N8, N14, N15, N20, N28, N38, N39, N43, N47, N49, N52, N57, N60, N88, N89, N90, N99, N106, N107, N111, N113, N125	*M. intracellulare* (25)	
N92	*M. timonense* (1)	
N1, N4, N19, N22, N32, N65, N68, N91, N96, N108	*M. abscessus* (10)	*M. abscessus* complex (18)
N17, N27, N33, N37, N62, N95, N112, N118	*M. massiliense* (8)	
N29	*M. paraense* (1)	*M. simiae* complex (13)
N64, N94, N120	*M. parascrofulaceum* (3)	
N12, N51	*M. parmense* (2)	
N30, N35, N36, N109, N119, N122, N124	*M. simiae* (7)	
N44, N93	*M. conceptionense* (2)	*M. fortuitum* complex (12)
N7, N31, N48, N80, N84, N86, N103, N114	*M. fortuitum* (8)	
N67, N117	*M. senegalense* (2)	
N71	*M. gastri* (1)	*M. gordonae* complex (5)
N87, N212	*M. gordonae* (2)	
N82, N83	*M. paragordonae* (2)	

Table 1 (cont)

Sample ID	*Mycobacterium* sp (total number)	*Mycobacterium* complex (total number)
N11	*M. kansasii* (1)	NA
N53	*M. lentiflavum* (1)	NA
N24, N98	*M. scrofulaceum* (2)	NA
N13, N34	*M. seoulense* (2)	NA

ID: identification; NA: not applicable

results are similar to those reported from China and India (Sharma and Upadhyay, 2021; Sun *et al*, 2022). Acquired immunodeficiency subjects in the same regions show a higher prevalence of *M. kansaii* (McCarthy *et al*, 2012), while our study detected only one sample with *M. kansaii* but the immune status of this subject was not available. Our MTB and NTM detection rates were slightly different from a similar study from Indonesia (Mertaniasih *et al*, 2017), with lower frequency of MTB (11.7% *vs* 18.8%) and higher NTM (8.5% *vs* 5.7%). These differences can be attributed to differences in geographic and social factors, and to the local situation of tuberculosis infection.

Detections of mycobacteria by qPCR and *hsp65* PCR are described as reliable tools (Varma-Basil *et al*, 2013; Ikram *et al*, 2015; Varadaiah *et al*, 2016; Casallas-Rivera *et al*, 2017), bacterial culture is still considered the gold standard for mycobacteria identification (Boldi *et al*, 2023). Unfortunately, our laboratory currently lacks the required biosafety facilities for validating the PCR-based assays. In the future, we will need to acquire facilities to perform mycobacterial cultures if we are to carry out longitudinal monitoring of patients to confirm the presence of NTM-pulmonary disease, as recommended by the American Thoracic Society and Infectious Disease Society of America (Haworth *et al*, 2017; Daley *et al*, 2020).

Identification of the causative pathogen allows appropriate antibiotic treatment rather than a presumptive regimen based on clinical signs and symptoms. For example, antibiotics for the treatment of MAC infections should contain a macrolide, ethambutol and rifamycin; for the treatment of *M. kansasii,* they should consist of azithromycin, ethambutol and rifampicin; and for the treatment of *M. abscessus*, they should be a combination one or two of the following members of the group comprising amikacin, imipenem and tigecycline together with two of the members of the group containing azithromycin, clofazimine and linezolid (Haworth *et al*, 2017; Daley *et al*, 2020; Pathak *et al*, 2022). For the common NTM group detected in this study, a macrolide-containing regimen is recommended to avoid intrinsic resistance to first-line anti-TB drugs (Park *et al*, 2008; Jabbour *et al*, 2020; Pathak *et al*, 2022). In resource-limited settings, inability to detect NTM infections could lead to interpretation of treatment failure with first-line anti-TB drugs as drug-resistance MTB (Tortoli *et al*, 2010). Thus, our findings should alert physicians to prescribing macrolide-containing regimens to treat suspected NTM diseases while lacking specific identification.

The study contains several limitations. Firstly, single samples collected without following up may not identify true NTM infections. In future investigation, a cohort study should be established, in which clinical features of NTM-infected individuals should be described in more detail. Secondly, as only sputum samples were used, the results would not indicate extra-pulmonary NTM infections. Future studies should include lymphoid aspirates and postoperative and joint or soft-tissue infections. Thirdly, assay of NTM based solely on a short sequence of mycobacterial *hsp65* might not be adequate to classify all *Mycobacterium* spp (Kim *et al*, 2019). Most recently, the application of a next generation sequencing (NGS) technique was reported to be as sensitive and specific as Mycobacteria Growth Indicator Tube (MGIT) culture method (Wei *et al*, 2023). In addition, whole genome analysis of NTM can predict drug resistance characteristics, which can inform physicians to the appropriate antibiotic regimens (Solanki *et al*, 2022). With the approach of metagenomic NGS, infections of multiple NTM species are able to be detected simultaneously, which is not easy to resolve using traditional bacterial culture or conventional PCR (Liu *et al*, 2022).

In conclusion, this study confirms, in lieu of mycobacterial cultures, the utility of PCR-based methods in providing insights into the variability of NTM and in identifying the common NTM species present in sputum samples collected in northern Vietnam.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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