The Lung Microbiota in Korean Patients with Non-tuberculous Mycobacterial Pulmonary Disease

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Research

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Abstract

Background: The role of the lung microbiota in non-tuberculous mycobacterial pulmonary disease (NTM-PD) remains poorly known. We obtained protected specimen brushing (PSB) and bronchial washing specimens to investigate the microbiota of patients with NTM-PD via a bronchoscopic approach.

Results: Eleven subjects with indications for NTM-PD on chest computed tomography (CT) were enrolled in the NTM-PD group, and 10 subjects with endobronchial lesions on chest CT that were not typical of NTM-PD, tuberculosis, malignancy, or any known lower respiratory tract disease or condition were enrolled in the control group. Samples from NTM-PD patients had lower microbial richness/diversity compared with the control group. Principal component analysis showed that the PSBs and bronchial washings exhibited similar patterns within each group but differed between the two groups. In the NTM-PD group, PSBs exhibited lower alpha and beta diversities than those of bronchial washings.

Conclusion: NTM-PD patients exhibited a unique microbiotic distribution that was low in richness/diversity. PSB samples can serve as surrogates of lower airway samples when evaluating the airway microbiota of NTM-PD patients, as they accurately reflect lower airway status.

Background

Nontuberculous mycobacterial (NTM) pulmonary disease (NTM-PD) is caused by infection by a range of pathogenic species of NTM.[1–4] NTM-PD can affect subjects of any age but is more common in those at least 50 years. [4–7] The clinical features are generally nonspecific, consisting of a cough, increased mucous production, weight loss, low-grade fever, hemoptysis, and dyspnea, and may be indistinguishable from symptoms of tuberculosis and other chronic lung diseases.[8–10] When NTM-PD is suspected, chest computed tomography (CT) should be performed; the fibrocavitary and nodular bronchiectatic forms of NTM-PD are the two most common radiological findings.[8, 11, 12] When chest CT indicates NTM-PD, a diagnosis generally requires microbiological confirmation. However, this may be difficult because NTM occur naturally in the environment and are associated with a very high risk of sputum or bronchoscopic sample contamination.[4, 8] Moreover, a positive finding may reflect endobronchial colonization only and not the inflammation characteristic of NTM-PD.[4, 8] To overcome this microbiological issue, NTM growth from two or more sputum specimens collected at different times is now required before NTM-PD can be diagnosed.[4, 5, 8] Alternatively, growth from only one specimen extracted via bronchoalveolar lavage (BAL) is acceptable, as is a culture-positive lung biopsy.[4, 5, 8]

The pathogenesis of NTM-PD remains unclear. The outcome of NTM-PD is likely to reflect a complex interplay among the extent of exposure, organism involved, and host; few data are available.[4, 13–17] The microorganisms (bacteria, viruses, and fungi) residing in the human body are collectively called the “microbiota”.[18–20] These participate in diverse cellular processes including metabolism and affect the development of various diseases. Recent studies have found that various populations of microbiota living in diverse cellular compartments modulate immune system function, the aberrant metabolism that

triggers chronic inflammation, and cellular transformation.[19–25] However, any role of the microbiota in respiratory disease remains poorly known. Only a few reports have evaluated the role of the microbiota in NTM-PD.[20, 26] Here, we used a bronchoscopic approach to explore the role played by the microbiota in NTM-PD by analyzing protected specimen brushing (PSB) and bronchial washing samples.

Results

Clinical characteristics

A total of 14 and 10 subjects were enrolled in the NTM-PD and control groups, respectively. However, three NTM-PD patients were excluded because NTM were not identified in acid-fast bacillary culture of bronchial washings. Respiratory samples from 21 subjects (11 in the NTM-PD and 10 in the control groups) were finally analyzed. The demographic and clinical characteristics are shown in Table 1. The median age [range] was 57 [23–83] years, and 7 subjects (33.3%) were female and 14 (66.7%) male. Age, sex, smoking status, medical history, and comorbid status did not differ between the groups. BMI was significantly lower in the NTM-PD than the control group (20.4 [18.1–22.0] vs. 25.7 [17.6–31.3 kg/m²], $p = 0.012$). *Mycobacterium avium*, *M. intracellulare*, and *M. kansasii* were cultured from six (54.4%), three (27.3%), and one (9.1%) NTM-PD patients, respectively (Supplementary Table 1). NTM could not be cultured in one patient (9.1%).

Table 1
Demographic and clinical characteristics of the study subjects.

Variable	All subjects (n = 21)	NTM-PD group (n = 11)	Control group (n = 10)	p-value*
Age, years	57 [23–83]	57 [23–74]	57.5 [33–83]	0.863
Female (%)	7 (33.3)	8 (72.7)	6 (60.0)	0.537
BMI (kg/m ²)	20.5 [17.6–31.3]	20.4 [18.1–22.0]	25.6 [17.6–31.3]	0.012
Height (cm)	162.0 [140.0–183.0]	159.8 [152.5–183.0]	162.4 [140.0–172.0]	1.000
Weight (kg)	55.0 [45.0–85.1]	52.0 [45.0–63.0]	60.6 [46.4–85.1]	0.051
Smoking status (%)				0.709
Never-smoker	15 (71.4)	8 (72.7)	7 (70.0)	
Ex-smoker	3 (14.3)	1 (9.1)	2 (20.0)	
Current smoker	3 (14.3)	2 (18.2)	1 (10.0)	
Smoking level (pack-years)	11.4 [10.0–60.0]	35.0 [10.0–60.0]	11.4 [10.0–40.0]	0.800
Comorbidity				
Hypertension	2 (9.6)	1 (9.1)	1 (10.0)	1.000
Hyperlipidemia	1 (4.8)	1 (9.1)		1.000
Parkinson's disease	1 (4.8)		1 (10.0)	0.476
Past medical history				
Pneumonia	1 (4.8)	1 (9.1)		1.000
Tuberculosis	3 (14.4)	1 (9.1)	2 (20.0)	1.000
Pulmonary embolism	1 (4.8)		1 (10.0)	0.476
Canine space abscess	1 (4.8)	1 (9.1)		1.000
Colon polyp	1 (4.8)	1 (9.1)		1.000
Data are shown as medians (with ranges) or frequencies (with % values). *p-values < 0.05 are shown in bold for comparisons between the NTM-PD and control groups. NTM-PD, nontuberculous mycobacterial pulmonary disease; BMI, body mass index.				

RNA concentrations of the sequencing libraries in the various samples

Figure 1 presents the RNA concentrations of the sequencing libraries in the various samples. We excluded the possibility of environmental contamination introduced by the bronchoscopic channel by constructing 16S sequencing libraries from the negative control samples. However, as shown in Fig. 1, most of the negative control samples contained very little RNA, preventing sequencing. We thus assumed that environmental contamination was minimal during sample acquisition. Bronchial washing samples yielded libraries with higher RNA concentration compared with PSB samples.

The richness/diversity of NTM-PD microbiota

Figure 2 shows the microbial richness and alpha diversity of each sample based on the OTU number and Chao1 richness index. NTM-PD samples had fewer OTUs (Fig. 2A) and lower Chao1 richness values (Fig. 2B) compared with the control samples, although the difference was not statistically significant. Figure 3 shows the weighted UniFrac distances between all sample pairs within each sample group, reflecting the beta diversity. In both bronchial washing and PSB samples, significantly lower beta diversity was observed in the NTM-PD group ($p = 2.25E-6$) than in the control group ($p = 4.13E-4$). In the control group, the microbiotic composition had lower beta diversity in PSBs than bronchial washings ($p = 0.0024$). In the NTM-PD group, beta diversity was lower in the PSBs than bronchial washings, but the differences were not statistically significant.

Differences in the microbiota between the NTM-PD and control groups

The PCoA plots for bronchial washings (Fig. 4A) and PSBs (Fig. 4B) were similar within each group. In the NTM-PD group, the bronchial washing and PSB samples were clustered closely, and most could be distinguished from those of the control group, the patterns of which were disperse. The proportions of individual phyla in bronchial washings varied (Fig. 5A and B). Compared with the control group, the proportions of Ignavibacteriae, Deinococcus–Thermus, Actinobacteria, and Gemmatimonadetes were increased (2.67 vs. 0.48%, 3.16 vs. 0.36%, 5.61 vs. 2.18%, and 0.19 vs. 0.03%, respectively), and those of Firmicutes and the unassigned fractions were decreased (5.84 vs. 15.27% and 7.16 vs. 27.06%, respectively) in NTM-PD bronchial washings. The PSBs yielded similar results (Fig. 5C and D), with minor changes in a few phyla (Firmicutes, Actinobacteria, and Gemmatimonadetes).

The microbiotic fractions at the genus level (or higher for OTUs lacking genus-level taxonomic assignments) in bronchial washings and PSBs were compared between the NTM-PD and control groups (Fig. 6). The proportions of various genera differed between the groups and also between bronchial washings and PSBs. In bronchial washings, the proportions of Ignavibacterium, Pseudomonas, Meiothermus, Rhodococcus, Dechloromonas, Cytophagaceae family, Alcaligenaceae family, Phos-Hee51 family, Blastocatellaceae family Hyphomicrobium, Candidatus and Nomurabacteria classes, Comamonadaceae family, and Nitrospira and JG30-KF-CM45 orders were increased, whereas those of Propionibacterium, Burkholderia–Paraburkholderia, Dermacoccaceae, Enhydrobacter, and the unassigned fractions were decreased in the NTM-PD compared with control groups. In PSBs, the proportions of

Ignavibacterium, Pseudomonas, Meiothermus, Rhodococcus, Dechloromonas, Cytophagaceae family, Alcaligenaceae family, Blastocatellaceae family, Comamonadaceae family, and Pirellula were increased, whereas those of Burkholderia–Paraburkholderia, Sphingomonas, Staphylococcus, and the unassigned fractions were decreased in the NTM-PD compared with control groups. The proportions of Pseudomonas, Rhodococcus, Cytophagaceae, and Alcaligenaceae were consistently higher in bronchial washings and PSBs from the NTM-PD group compared with the control group (Fig. 7).

Discussion

We found that NTM-PD exhibited a predominance in middle-aged females. The BMI was significantly lower in the NTM-PD than control groups. Thinner and older females appear to be more susceptible to NTM-PD.[27] One recent study investigated the microbiomes of the upper and lower respiratory tracts of patients with suspected NTM-PD. The BMI was lower in those whose sputa were positive, compared with negative, for NTM cultures.[28] Most NTM that we detected belonged to the *Mycobacterium avium* complex (MAC), including *M. avium* and *M. intracellulare*. *M. kansasii* was cultured from one subject only, and NTM from another patient were not identified. As most NTM infections involve the MAC, previous studies evaluating the NTM-PD microbiome focused on the MAC.[20, 28]

We found that 16S rRNA sequencing identified mycobacteria in only 54.5% of bronchial washings and 27.3% of PSBs from NTM-PD patients (Supplementary Table 2). Previous studies reported that 16S rRNA sequencing identified NTM in only 47% of BAL samples from subjects whose sputum was positive for NTM cultures despite use of a nested mycobacterial microbiome approach. Thus, 16S rRNA gene sequencing is not adequately sensitive, and technical improvements are needed.[28, 29]

Respiratory samples (especially PSBs) from NTM-PD patients tended to exhibit lower alpha diversities compared with those from control subjects. In a previous study, the alpha diversities of sputum samples from females with NTM-PD and those with both NTM-PD and breast cancer were significantly lower than those of samples from healthy females, consistent with our results.[20] However, in another study, the alpha diversities in oral washes, sputum, and BAL samples did not differ in NTM-positive versus -negative sputum cultures.[28] Further studies are needed to explore whether microbial diversity is decreased in NTM-PD patients. In a previous study, oral washings exhibited higher alpha diversities than those of sputum, which in turn exhibited a bacterial load of approximately 100-fold greater than that of BAL samples.[28] Thus, previous work and our current data indicate that the bacterial richness/load are higher in the upper airway than lower airway.

We found that both bronchial washings and PSBs exhibited significantly lower beta diversities in the NTM-PD than control groups. In the control group, PSBs exhibited significantly lower beta diversity than that of bronchial washings. However, the PCoA plots of bronchial washings and PSBs were similar within each group. A previous study explored beta diversity using both unweighted and weighted UniFrac PCoA methods. Significant differences in taxonomic distributions were apparent in females with both NTM-PD and breast cancer compared with control females; the distribution of females with NTM-PD only

compared with control females also varied somewhat, but the difference was not statistically significant.[20] In another study, oral washings exhibited significant beta diversity differences between subjects with NTM-positive versus -negative cultures; neither sputum nor BAL samples differed significantly.[28] Interestingly, sputum was more similar to oral washings than to BAL in both the UniFrac distance and PCoA analyses, although all three samples exhibited significantly different beta diversities, indicating that sputum cannot serve as a surrogate of lower airway samples when evaluating the airway microbiota of NTM-PD.[28] However, both bronchial washings and PSBs do serve as adequate surrogates, exhibiting similar PCoA patterns within the NTM-PD and control groups of our present work.

When generic distributions were compared among groups, *Veilonella* was significantly less common in females with NTM-PD compared with the control group, and *Streptococcus* and *Leptotrichia* were less common in females with NTM-PD and in those with breast cancer, but the phylum distributions did not differ significantly.[20] In another study, BAL from patients with NTM-positive cultures tended to contain oral commensals including *Veilonella*, *Leptotrichia*, and *Prevotella*; the presence of these microbiota was correlated significantly with neutrophil numbers and the levels of several cytokines including IL-6, IL-17, IL-23, and Fractalkine, suggesting that oral commensals can migrate to the lower airway to induce inflammation in the lungs of patients with NTM-PD.[28]

However, those two cited works focused on major microbiota (minimum average abundance 1%); minor microbiota including NTM per se may have been overlooked.[20, 28] Standard 16S rRNA gene sequencing identified *Mycobacterium* in only 27% of samples from patients who were positive for NTM cultures and thus was not adequately sensitive. The findings were not correlated with the level of any inflammatory biomarker.[28] We found that, at the genus level, the proportions of various microbiota including *Ignavibacterium*, *Pseudomonas*, *Meiothermus*, *Rhodococcus*, *Dechloromonas*, and *Cytophagaceae* were decreased in the NTM-PD compared with control groups. At the family level (and also at the class level for *Nomurabacteria*), the proportions of *Alcaligenaceae*, *Phos-Hee51*, *Blastocatellaceae*, *Hyphomicrobium*, *Candidatus*, *Nomurabacteria*, *Comamonadaceae*, and *Nitrospira* were decreased. At the order level, the proportions of *JG30-KF-CM45* and *Pirellula* were increased, whereas those of *Propionibacterium*, *Burkholderia*–*Paraburkholderia*, *Dermacoccaceae*, *Enhydrobacter*, *Sphingomonas*, and *Staphylococcus* were decreased. At the phylum level, the proportions of *Ignavibacteriia*, *Deinococcus*–*Thermus*, *Actinobacteria*, and *Gemmatimonadetes* were increased, whereas that of *Firmicutes* was decreased, in the NTM-PD compared with control groups. Of these taxa, *Rhodococcus*, *Comamonadaceae*, *Nitrospiraceae*, *Actinobacteria*, *Firmicutes*, and *Burkholderia* are abundant in groundwater, treated water, and biofilms and may act as opportunistic pathogens, similar to NTM.[30]

Our work had certain limitations. First, the BMI of the subjects differed between the NTM-PD and control groups, and we cannot exclude the possibility that this difference affected the lower respiratory tract microbiome irrespective of the NTM-PD status. Microbiome studies in patients with different BMIs have focused largely on the gut microbiome, however BMI-associated changes in the gut microbiome remain poorly known, although gut bacteria associated with both low and high BMIs have been reported.[31, 32] Further work on any possible link between BMI differences and the lung microbiome of NTM-PD patients,

particularly whether a low BMI is a risk factor for NTM-PD development, is warranted. Second, as we did not evaluate upper airway samples (oral washes and sputum), we do not know whether the upper airway microbiome differed between the groups. Finally, we did not measure the levels of inflammatory biomarkers (such as cytokines) or determine neutrophil or lymphocyte counts. Thus, we cannot conclude that the microbiotic components that are increased in NTM-PD patients in fact cause lung inflammation.

Despite these limitations, our study had certain merits. We compared bronchial washings and PSBs within subjects. PCoA revealed that the microbiome distributions were similar, suggesting that both bronchial washings and PSBs reflect the lower airway status of NTM-PD patients. The PSB samples exhibited the lowest alpha and beta diversities and lacked oral commensals such as Firmicutes, thus optimally reflecting lower airway status. Also, several microbes differed between the NTM-PD and control groups; some may promote or inhibit NTM-PD development.

Conclusion

NTM-PD patients exhibited a unique microbiome that was less rich/diverse compared with that of the control subjects. Further studies are required to verify these findings and to determine if the microbes interact (positively or negatively) with NTM to provoke inflammation and/or antibiotic resistance independent of the NTM-PD status. PSBs can serve as surrogates of lower airway samples when evaluating the airway microbiota of NTM-PD patients. The microbial pattern thereof is similar to that of bronchial washings, albeit of lower richness/diversity, thus specifically reflecting a lower airway status.

Materials And Methods

Study subjects and sample collection

Patients who underwent bronchoscopy due to a suspicion of NTM-PD on chest CT in a 1,600-bed tertiary university medical center in Incheon, Republic of Korea between August 2017 and August 2018 were prospectively enrolled in the NTM-PD group after providing written informed consent. Patients who underwent bronchoscopy to examine suspicious endobronchial lesions that were not typical of NTM-PD, tuberculosis, malignancy, or any known disease or condition of the lower respiratory tract were enrolled in the control group. The exclusion criteria were malignancy at any site; an infection or serious disease of the neural, cardiovascular, renal, hepatobiliary, gastrointestinal, hematological, or respiratory system; use of any antibiotic within the month prior; perceived vulnerability; and refusal to participate. All patients underwent bronchoscopy, and we collected PSB and bronchial washing samples. Prior to bronchoscopy, all subjects underwent topical anesthesia (lidocaine delivered via a nebulizer) and sedation with midazolam and fentanyl. The bronchoscopic channels were washed with 5 mL sterile 0.9% (w/v) saline (negative control samples). In the NTM-PD group, respiratory specimens were collected from lesional bronchi using a protected brush; each brush was chopped into small pieces (using a sterile wire cutter) suspended in 5 mL sterile 0.9% (w/v) saline and then vortexed. Lesional bronchi were also washed with 5 mL sterile saline. In the control group, PSB and bronchial washing samples were collected from random

bronchi. We recorded demographic and clinical data including age, sex, height, weight, body mass index (BMI), smoking status, smoking level, comorbidities, and past medical history.

DNA extraction

DNA was extracted from respiratory specimens on the day of bronchoscopy using the PowerSoil DNA Isolation Kit from Mo Bio Laboratories Inc. (Carlsbad, CA, USA) according to the manufacturer's instructions (manual ver. 07272016).

PCR and sequencing

The V3–4 regions of bacterial 16S rRNA genes were amplified by PCR. PCR was conducted in a 25- μ L total volume containing 2.5 μ L DNA extract, 12.5 μ L KAPA HiFi Hotstart readyMix (Kapa Biosystems, Boston, MA, USA), 5.0 μ L 1 M forward primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'), and 5.0 μ L 1 M reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). PCR featured initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. Template size distributions were explored using the Agilent Technologies 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) fitted with a DNA 1000 chip. The library was sequenced from both ends using the Illumina MiSeq sequencer based at Macrogen (Seoul, Republic of Korea). FASTQ files were generated from the base-calls using the Illumina software package bcl2fastq.

Sequence analysis and microbial diversity

The analytical tools of QIIME ver. 1.9.1 were used to process sequence data and perform taxonomic analysis [33]. Paired reads were merged using FLASH ver. 1.2.11[34] (minimum overlap 10 bp; maximum overlap 100 bp; maximum allowed mismatch:overlap length ratio 0.25). Chimera detection and operational taxonomic unit (OTU) clustering were performed using the cd-hit-dup and cd-hit-otu programs of CD-HIT ver. 4.5.4[35]. Sequences were aligned using the parallel_align_seqs_pynast.py script and taxonomic assignment conducted using the assign_taxonomy.py script and UCLUST[36] method. SILVA ver. 128[37] served as the reference database for sequence alignment and taxonomic assignment. The Chao1 richness index was evaluated using QIIME, which yields both the alpha and beta diversities (the latter calculated using the Weighted UniFrac distances). We employed QIIME to compare dissimilarity among the samples. UniFrac is a phylogenetic distance metric used to compare phylogenetic distances among different samples.[20] We employed principal coordinate analysis (PCoA) to plot these phylogenetic metrics.[20]

Statistical analysis

Continuous variables were compared using the t-test, and frequencies were compared using Fisher's exact test. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using Matlab ver. R2019a (MathWorks, Natick, MA, USA).

Abbreviations

BMI

body mass index

CT

computed tomography

NTM

nontuberculous mycobacterium

NTM-PD

nontuberculous mycobacterial pulmonary disease

OTU

operational taxonomic unit

PCoA

principal coordinate analysis

PSB

protected specimen brushing

Declarations

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Authors' contributions

The project was conceived by SM Lee. SM Lee and S-Y Kang collected respiratory samples from the study subjects and wrote the manuscript. S Jung performed sample processing, bioinformatics analyses, and writing of the manuscript. H Kim assisted with 16S rRNA gene profiling, the sample organization, data management, sample processing, and analyses. The authors read and approved the final manuscript.

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Availability of data and material

We will provide data and material to investigators if they contact us by email (sangminlee77@naver.com)

Ethics approval and consent to participate

The study protocol was reviewed and approved by the institutional review board of our institution (IRB approval number: GAIRB2017-065). The study was registered at clinicaltrials.gov (no. NCT04079400). Informed consents were obtained for all participants

Consent for Publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Haworth CS, et al. British Thoracic Society Guideline for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). *BMJ Open Respir Res.* 2017;4(1):e000242.
2. Diel R, et al. Burden of non-tuberculous mycobacterial pulmonary disease in Germany. *Eur Respir J.* 2017;49(4).
3. Krieger D, et al. Is delamanid a potential agent in the treatment of diseases caused by *Mycobacterium avium-intracellulare*? *Eur Respir J.* 2016;48(6):1803-1804.
4. Chalmers J, et al. Non-tuberculous mycobacterial pulmonary infections. *Pulmonol.* 2018;24(2):120-31.
5. Griffith DE, et al. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med.* 2007;175(4):367-416.
6. Hwang JA, et al. Natural history of *Mycobacterium avium* complex lung disease in untreated patients with stable course. *Eur Respir J.* 2017;49(3).
7. Koh WJ, et al. Outcomes of *Mycobacterium avium* complex lung disease based on clinical phenotype. *Eur Respir J.* 2017;50(3).
8. Tveiten H, Brantsaeter AB, and Mengshoel AT. Non-tuberculous mycobacterial pulmonary infections. *Tidsskr Nor Laegeforen.* 2018;138(19).

9. Koh WJ, et al. Pulmonary TB and NTM lung disease: comparison of characteristics in patients with AFB smear-positive sputum. *Int J Tuberc Lung Dis.* 2006;10(9):1001-7.
10. Kim YK, et al. Comparable characteristics of tuberculous and non-tuberculous mycobacterial cavitary lung diseases. *Int J Tuberc Lung Dis.* 2014;18(6):725-9.
11. Jeong YJ, et al. Nontuberculous mycobacterial pulmonary infection in immunocompetent patients: comparison of thin-section CT and histopathologic findings. *Radiology.* 2004;231(3):880-6.
12. Chung MJ, et al. Thin-section CT findings of nontuberculous mycobacterial pulmonary diseases: comparison between *Mycobacterium avium-intracellulare* complex and *Mycobacterium abscessus* infection. *J Korean Med Sci.* 2005;20(5):777-83.
13. Yoo JW, et al. Mycobacterial diseases developed during anti-tumour necrosis factor-alpha therapy. *Eur Respir J.* 2014;44(5):1289-95.
14. Fletcher LA, et al. Survival of *Mycobacterium abscessus* isolated from people with cystic fibrosis in artificially generated aerosols. *Eur Respir J.* 2016;48(6):1789-1791.
15. Griffith DE and Aksamit TR. Understanding nontuberculous mycobacterial lung disease: it's been a long time coming. *F1000Res.* 2016;5:2797.
16. Kwon YS and Koh WJ. Diagnosis and Treatment of Nontuberculous Mycobacterial Lung Disease. *J Korean Med Sci.* 2016;31(5):649-59.
17. Stout JE, Koh WJ, and Yew WW. Update on pulmonary disease due to non-tuberculous mycobacteria. *Int J Infect Dis.* 2016;45:123-34.
18. Agusti A, et al. Treatable traits: toward precision medicine of chronic airway diseases. *Eur Respir J.* 2016;47(2):410-9.
19. Chan AA, et al. Characterization of the microbiome of nipple aspirate fluid of breast cancer survivors. *Sci Rep.* 2016;6:28061.
20. Philley JV, et al. Microbiome Diversity in Sputum of Nontuberculous Mycobacteria Infected Women with a History of Breast Cancer. *Cell Physiol Biochem.* 2019;52(2):263-279.
21. Hosgood HD, 3rd, et al. The potential role of lung microbiota in lung cancer attributed to household coal burning exposures. *Environ Mol Mutagen.* 2014;55(8):643-51.
22. Goedert JJ, et al. Investigation of the association between the fecal microbiota and breast cancer in postmenopausal women: a population-based case-control pilot study. *J Natl Cancer Inst.* 2015;107(8).
23. Coburn B, et al. Lung microbiota across age and disease stage in cystic fibrosis. *Sci Rep.* 2015;5:10241.
24. Hieken TJ, et al. The Microbiome of Aseptically Collected Human Breast Tissue in Benign and Malignant Disease. *Sci Rep.* 2016;6:30751.
25. Wu J, et al. Sputum microbiota associated with new, recurrent and treatment failure tuberculosis. *PLoS One.* 2013;8(12):e83445.

26. Sulaiman I, et al. The mycobacteriome: A nested approach to identify non-tuberculous mycobacterium. *American Journal of Respiratory and Critical Care Medicine* 2018;197.
27. Chan ED and Iseman MD. Slender, older women appear to be more susceptible to nontuberculous mycobacterial lung disease. *Gend Med*. 2010;7(1):5-18.
28. Sulaiman I, et al. Evaluation of the airway microbiome in nontuberculous mycobacteria disease. *Eur Respir J*. 2018;52(4).
29. Macovei L, et al. The hidden 'mycobacteriome' of the human healthy oral cavity and upper respiratory tract. *J Oral Microbiol*. 2015;7:26094.
30. Kaestli M, et al. Opportunistic pathogens and large microbial diversity detected in source-to-distribution drinking water of three remote communities in Northern Australia. *PLoS Negl Trop Dis*. 2019;13(9):e0007672.
31. Davis SC, et al. Gut microbiome diversity influenced more by the Westernized dietary regime than the body mass index as assessed using effect size statistic. *Microbiologyopen*. 2017;6(4).
32. Stanislawski MA, et al. Gut microbiota phenotypes of obesity. *NPJ Biofilms Microbiomes*. 2019;5:18.
33. Caporaso JG, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 2010;7(5):335-6.
34. Magoc T and Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*. 2011;27(21):2957-63.
35. Fu L, et al. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics*. 2012;28(23):3150-2.
36. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 2010;26(19):2460-1.
37. Glockner FO, et al. 25 years of serving the community with ribosomal RNA gene reference databases and tools. *J Biotechnol*. 2017;261:169-176.

Figures

Figure 1

RNA concentrations in the sequencing libraries from the different samples. The concentrations are grouped by the sample acquisition protocol regardless of the group. CH, bronchoscopic channel washings (negative controls); PSB, protected specimen brushing; WA, bronchial washing.

Figure 2

Microbial richness in bronchial washing and PSB samples from the control and NTM-PD groups. Three samples (one bronchial washing and one PSB from the control group and one bronchial washing from the NTM-PD group) with over 900 OTUs and a Chao1 richness index >1,000 were omitted as outliers. (A) The numbers of OTUs (a measure of microbial richness) in each group. (B) The Chao1 richness index (a measure of alpha diversity) in each group. OTU, operational taxonomic unit; CR, control; WA, bronchial washing; PSB, protected specimen brushing; NTM-PD, non-tuberculous mycobacterial pulmonary disease.

Figure 3

The beta diversities of each group. Each dot represents a weighted UniFrac distance between the microbial compositions of two samples in the same group. CR, control; WA, bronchial washing; PSB, protected specimen brushing; NTM-PD, non-tuberculous mycobacterial pulmonary disease.

Figure 4

PCoA plots for the bronchial washing and PSB samples from the NTM-PD and control groups, based on weighted UniFrac distances. (A) Bronchial washing samples. (B) PSB samples. NTM-PD, non-tuberculous mycobacterial pulmonary disease; PSB, protected specimen brushing.

Figure 5

The phylum fractions in bronchial washing (A and B) and PSB (C and D) samples from the NTM-PD and control groups. The volcano plots show the relative fold changes and whether they were significant. The p-values of all phylum fractions in the NTM-PD compared with the control group are shown (A, C). NTM-PD, non-tuberculous mycobacterial pulmonary disease; PSB, protected specimen brushing.

Figure 6

Comparisons of genus (or higher-level) OTU classifications in the absence of genus-level taxonomic data in bronchial washing and PSB samples from the NTM-PD and control groups. (A) Bronchial washing samples. (B) PSB samples. The volcano plots show the relative fold changes and whether they were significant. The p-values for each genus (or higher-level OTU in the absence of genus-level taxonomic data) present in the NTM-PD group compared with the control group are shown. NTM-PD, non-tuberculous mycobacterial pulmonary disease; PSB, protected specimen brushing.

Figure 7

The genera and families exhibiting significant increases in bronchial washing and PSB samples from the NTM-PD compared with control groups. (A) *Pseudomonas*, (B) *Rhodococcus*, (C) *Cytophagaceae*, and (D) *Alcaligenaceae*. NTM-PD, non-tuberculous mycobacterial pulmonary disease; PSB, protected specimen brushing.

Supplementary Files

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- [SupplementaryTable12tomicrobiome.docx](#)