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Original Article

Detecting circulating microbial cell-free DNA by next-generation sequencing in patients with *Mycobacterium avium* complex-lung disease: A pilot study

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Abstract

Objectives: Determining a diagnosis for non-*Tuberculous mycobacterium* (NTM)-lung disease (LD) remains difficult. The value of circulating cell-free DNA (cfDNA) secreted from microbes has been established in the detection of pathogens in septic patients. However, it is unknown whether NTM-derived cfDNA is detectable in plasma from patients with NTM-LD and whether this is associated with the disease status of NTM-LD, especially in patients with *Mycobacterium avium* complex (MAC)-LD. **Materials and Methods:** In this pilot study, from 2018 to 2019, we enrolled adult patients with MAC-LD at Taipei Veterans General Hospital in Taiwan for the detection of circulating cfDNA. We performed cfDNA extraction from plasma, next-generation sequencing (NGS) for nonhuman cfDNA, and sequence matching to a microbial database and then assessed the association between pathogen cfDNA and MAC-LD. **Results:** Two (40%) plasma samples from MAC-LD patients had detectable MAC-specific cfDNA, namely one instance of DNA polymerase III alpha subunit and one instance of ATP-binding cassette transporters permease. The plasma samples from the three other MAC-LD cases and the one tuberculosis control were negative for either NTM-derived cfDNA or tuberculosis-related cfDNA. In addition to MAC-specific cfDNA, *Ralstonia solanacearum*, *Staphylococcus aureus*, and *Pasteurella multocida* were the most observed bacteria in our patients. The two patients with MAC-cfDNA positivity yielded higher radiographic scores ($P = 0.076$) and presented a higher number of nonhuman reads than those without MAC-cfDNA positivity ($P = 0.083$). **Conclusion:** Using NGS method, we demonstrated MAC-cfDNA was detectable in patients with MAC-LD. Further large-scale research is warranted to assess the clinical value of detecting MAC-specific cfDNA in MAC-LD patients.

Keywords: *Circulating cell-free DNA, Microbial circulating cell-free DNA, Mycobacterium avium complex-lung disease, Next-generation sequencing*

Introduction

The global prevalence of non-*Tuberculous mycobacterium* (NTM)-lung disease (LD) has been increasing in the past three decades [1,2]. Among different NTM species, *Mycobacterium avium* complex (MAC) is one of the most common pathogens that cause LD [3]. MAC is an environmental microorganism that is found in soil and water sources [4]. Preexisting structural LDs and immunocompromised conditions are known risk factors for developing MAC-LD [5]. Currently, the diagnosis criteria for NTM-LD include compatible clinical presentations, radiographical findings, and microbiological evidence [6,7]. However, it remains difficult to diagnose NTM-LD in patients whose sputum culture is positive for NTM because NTM pulmonary colonization does not occur among patients exhibiting these risk factors [8]. Since the criteria of radiographic findings and clinical presentations are relatively nonspecific [6], exploring serologic biomarkers that could assist in timely diagnoses of NTM-LD is warranted, especially for patients with MAC infection who are at risk of disease progression [9].

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Circulating cell-free DNA (cfDNA) is fragmented genes in blood that is around 150 base pairs in length and is proposed to come from dead cells in human [10]. Although most circulating DNA fragments are derived from host genes, there is evidence bacterial genes can be present in the blood of healthy people [11]. Regarding short gene fragments, it has recently been reported cfDNA from inhabited microbes can also be detected in human plasma [12]. Interestingly, the level of bacteria-derived cfDNA is higher in patients with cardiovascular disease than in healthy persons, meaning microbial cfDNA is impacted even in noninfectious settings [13]. Importantly, recent studies have demonstrated specific cfDNA targets can serve as a tool for the diagnosis of certain infectious diseases, such as tuberculosis (TB) [14,15]. Despite this, the value of detecting pathogen-derived cfDNA in patients NTM-LD has rarely been studied or reported.

Next-generation sequencing (NGS) is a technology that could rapidly sequence the whole genome. Grumaz *et al*. have found that analysis of the cfDNA by NGS could identify the infectious microorganism within 30 h [16]. Notably, the results of NGS analysis are highly correlated with the results of blood cultures of the pathogens. Using NGS to analyze cfDNA in plasma, Hong *et al.* demonstrated fungal cfDNA is detectable in patients experiencing invasive fungal infection [17]. In this study, using NGS, we conducted a pilot research to detect circulating pathogen-derived cfDNA in patients with MAC-LD. We aimed to find MAC-specific cfDNA in plasma and evaluate its association with the disease status of MAC-LD.

Materials and methods

Study design and follow-up

This is a prospective cohort study that was conducted from August 2018 to April 2019 at Taipei Veterans General Hospital, a tertiary referral medical center in northern Taiwan. We enrolled adult patients aged ≥20 years with MAC-LD who met the diagnostic criteria [18]. We selected patients with MAC-LD if the patient met these criteria: (1) chest high-resolution computed tomography scan showing compatible findings for NTM-LD, (2) sputum culture positive for MAC without mixed infection with other NTM species, and (3) exclusion of other disorders, such as TB. For a control case, we included a patient with TB whose sputum culture was positive for *Mycobacterium tuberculosis*.

We recorded general data, including age, sex, smoking, radiographic score [19], results of sputum acid-fast stain, and underlying diseases. We observed all patients for at least 1 year to discern whether there was the progression of MAC-LD, determined by either new radiographic changes during follow-up before treatment or clinical decline requiring antibiotic therapy [9]. All participants provided written informed consent (Institutional Review Boards of Taipei Veterans General Hospital, No. 2017-12-001C).

Blood sampling and next-generation sequencing

After obtaining informed consent from each patient, peripheral blood samples were collected. We used K2-EDTA vacutainer tubes (BD) to collect blood (8–10 mL) for plasma preparation. The blood was centrifuged for 10 min at 1500 rpm (430 g) to obtain the plasma samples within 2 h. QIAamp Blood DNA Mini Kit (Qiagen) was used to extract cfDNA from 400 μL of each plasma sample. The eluted samples were stored in microcentrifuge tubes at −80°C [15]. cfDNA was used for NGS library construction by performing end-repairing, a-overhang adding, and adaptor ligation [20]. This library preparation was performed using QIAseq Ultralow Input Library Kit (Qiagen), and the library was then pooled equally for sequencing (Illumina MiSeq sequencer, 2 × 150 bp with more than 5M reads). Sequences were trimmed based on quality values (Q30), and the trimmed reads were used in the ensuing analysis. In accordance with the protocol established in prior studies, the relative cfDNA level of each microbial was reported in MCRPM (Microbial cfDNA Reads Per Million quality PE reads) [21].

Taxonomic classification analysis

Kraken 2 is the newest version of the taxonomic classification system. By using exact k-mer matches, accuracy is higher and classification speeds increase [22]. This classifier matches each k-mer within a query sequence to the lowest common ancestor of all genomes containing the given k-mer. The k-mer assignments inform the classification algorithm. We updated the Kraken database with human reference genome (version GRCh38), 18,832 bacterial genomes, 9332 viral reference genomes, 360 archaea genomes, and 58 fungi genomes. Furthermore, the NCBI BLAST program was installed locally for checking the reads, which were unmapped in the human reference genome. For all BLAST analyses, each read with 100% identity was summarized as described in a previous study [21].

Mycobacterium avium complex subspecies identification

To correlate the results of plasma NGS and subspecies of MAC isolates in patients, we extracted the bacterial DNA from MAC isolates in sputum cultures and performed a polymerase chain reaction (PCR) to amplify the target DNAs, namely the RNA polymerase subunit beta gene and the 16S-23S ribosomal DNA internal transcribed spacer region [23-25]. We used the Basic Local Alignment Search Tool and the National Centre for Biotechnology Information database to determine the sequence of MAC subspecies, including *M. avium*, *Mycobacterium intracellulare* or *Mycobacterium chimaera*, and others [9,25].

Statistics

All data are presented as either a number, percentage, or mean ± standard deviation based on what was deemed to be appropriate. Categorical variables were analyzed using Fisher's exact tests. Two-sided *t*-tests or Mann–Whitney *U*-tests were conducted for continuous variables when comparing two groups. The chemotherapy response rate was compared between the two groups. $P < 0.05$ were statistically significant. All statistical analyses were performed using SPSS software (version 19.0; SPSS Inc., Chicago, IL, USA).

Results

Patients and next-generation sequencing mapping summary

We enrolled five patients with MAC-LD into an NGS survey targeting plasma cfDNA of nonhuman pathogens and included one pulmonary tuberculosis (PTB) case as a reference

control [Table 1]. The mean age of patients with MAC-LD was 75.8 ± 12 years. One patient with MAC-LD was male, as was the one PTB control case. Three of five patients with MAC-LD were sputum smear positive for Acid-fast bacilli. Regarding radiographic findings, one patient with MAC-LD and the PTB patient had cavitary lung lesions. Each patient with MAC-LD had a severe and progressive disease requiring antibiotic treatment.

According to the results of cfDNA sequencing and mapping (MAC-1, MAC-2, MAC-3, MAC-4, MAC-5, and TB-1), the count of reads ranged from nearly one million to 35 million [Table 2]. Specifically, the human classified reads, Homo sapiens, accounted for 99.66%–99.97% among the patients, whereas the nonhuman classified reads accounted for 0.01%–0.02%, and the nonhuman unclassified reads accounted for 0.02%–0.32%. Of note, the nonhuman classified reads (identified microbial cfDNA) were higher than 1000 in MAC-2 and MAC-3 but were lower in MAC-1, MAC-4, MAC-5, and TB-1.

Nonhuman reads and *Mycobacterium avium* complex-specific cell-free DNA

The nonhuman reads were summed and normalized to reflect the relative levels of cfDNA fragments in the plasma [Table 3]. To minimize the influence of nonspecific species, we excluded bacterial species whose MCRMP <0.1 [21]. The most common bacteria among MAC-LD patients and PTB patients were *Ralstonia solanacearum*, *Staphylococcus aureus*, and *Pasteurella multocida*. The pie charts of two patients with MAC-LD (MAC-2 and MAC-3) are illustrated in Figure 1. The number of bacterial species determined by NGS for plasma cfDNA was higher in patients with MAC-LD than in patient with PTB.

Of note, the cfDNA derived from *Mycobacterium spp*. were revealed in patients with MAC-LD (MAC-2, MAC-3), however, the MCRMP was relatively low. The two detectable MAC-specific cfDNAs in the two MAC-LD patients were DNA polymerase III alpha subunit and ATP-binding cassette transporters permease. The sizes of the two cfDNA were 151 bp and 152 bp, respectively [Supplement File]. The plasma from the three other MAC-LD and the one PTB control were negative for any NTM-derived cfDNA or MTB-derived cfDNA. Thus, 40% (2/5) of the MAC-LD patients tested positive for MAC-cfDNA. The subspecies identification of MAC in both patients (MAC-2, MAC-3) was *M. chimaera*, which harbors the sequences of DNA polymerase III alpha subunit and ATP-binding cassette transporters permease.

Features of patients with detectable *Mycobacterium avium* complex-cell-free DNA

As listed in Table 4, patients with or without detectable MAC-cfDNA were similar in age, sex, BMI, underlying diseases, and cavity lesions. However, patients with detectable MAC-cfDNA were more likely to have a smear-positive sputum and a higher radiographic score, although there was no statistical significance due to the limited number of cases (P = 0.400 and P = 0.076). Interestingly, the two patients with MAC-cfDNA positivity presented a higher number of nonhuman reads than did patients without cfDNA positivity (P = 0.083).

Table 1: General data

	MAC-LD (n=5), n (%)	PTB (n=1), n (%)
Age (year)	75.8±12	77
Sex (%)	20 male	0 male
BMI (kg/m^2)	19.20±2.27	24.59
Underlying disease		
DM	40	0
Malignancy	0	0
CKD	0	0
Bronchiectasis	3 (60)	0
Interstitial lung disease	1 (20)	0
Sputum smear positivity	3 (60)	0
Sputum smear grade	1.2±1.3	0
Cavitary lung lesion	20	100

BMI: Body mass index, DM: Diabetes mellitus, CKD: Chronic kidney disease, MAC-LD: *Mycobacterium avium* complex-lung disease, PTB: Pulmonary tuberculosis

Table 2: Mapping summary

Species	Count of reads	Percentage
MAC-1		
Homo sapiens	10,335,278	99.97
Nonhuman classified	590	0.01
Nonhuman unclassified	2194	0.02
MAC-2		
Homo sapiens	18,303,338	99.94
Nonhuman classified	1320	0.008
Nonhuman unclassified	8798	0.05
MAC-3		
Homo sapiens	35,056,279	99.84
Nonhuman classified	4268	0.02
Nonhuman unclassified	50,250	0.14
MAC-4		
Homo sapiens	12,274,815	99.95
Nonhuman classified	700	<0.01
Nonhuman unclassified	5846	0.05
MAC-5		
Homo sapiens	1,483,017	99.66
Nonhuman classified	266	0.02
Nonhuman unclassified	4741	0.32
PTB-1		
Homo sapiens	5,024,817	99.88
Nonhuman classified	287	0.01
Nonhuman unclassified	5577	0.11

PTB: Pulmonary tuberculosis, *M. avium: Mycobacterium avium*, MAC: *M. avium* complex

Discussion

This is the first study to assess the detectability of pathogen-derived cfDNA in patients with MAC-LD. This pilot study reports 40% of patients with MAC-LD had MAC-specific cfDNA as detected by NGS tests. Specifically, the two cfDNA with MAC-compatible sequences were DNA polymerase III alpha subunit and ATP-binding cassette transporters permease.

Using microbial cfDNA as a tool to detect pathogens that cause infectious diseases is a novel approach that is increasing in popularity. A few studies have demonstrated

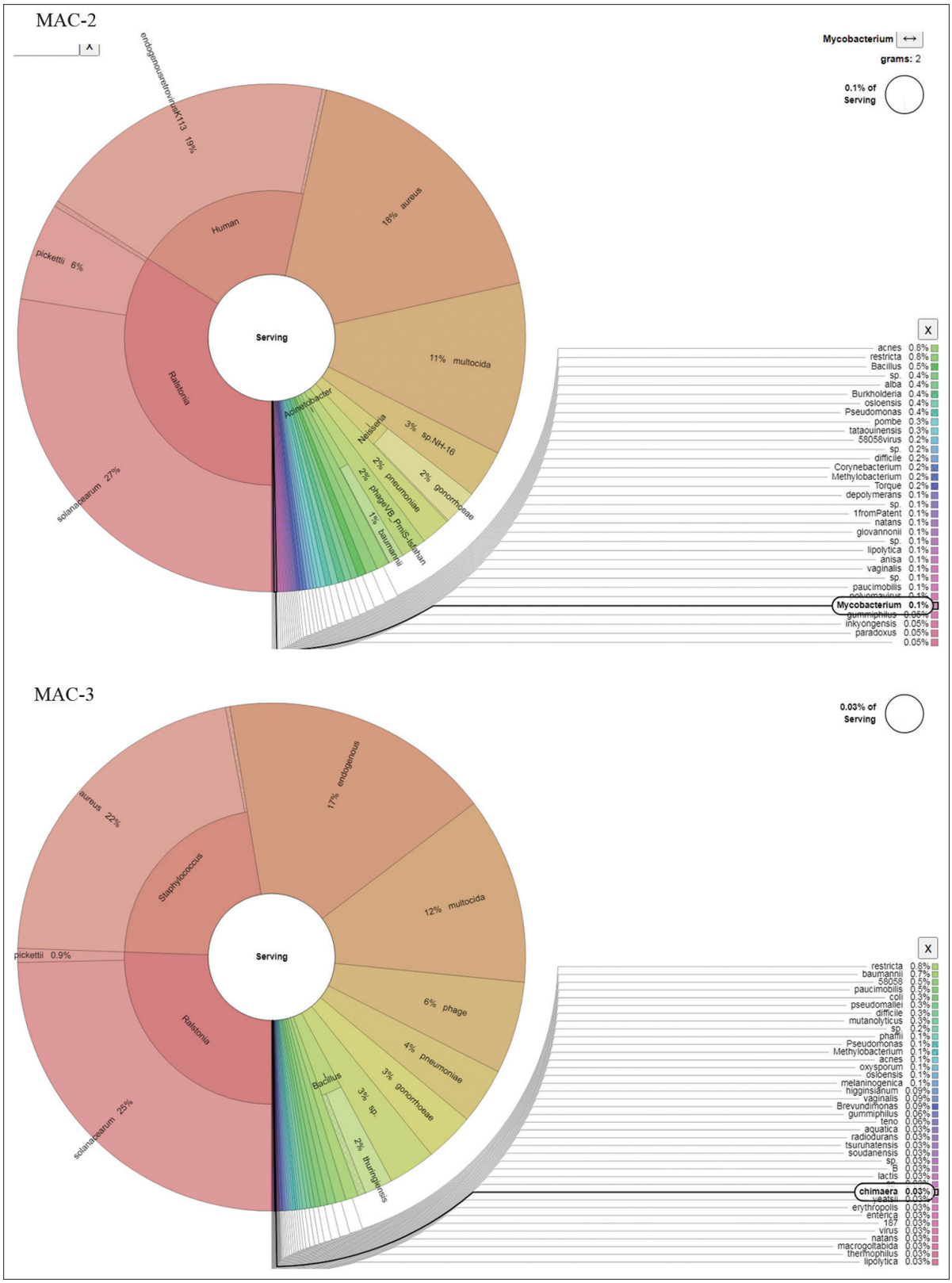

Figure 1: Pie chart of bacterium among MAC-LD (MAC-2 and MAC-3). MAC-LD: *Mycobacterium avium* complex-lung disease

the effectiveness of this approach for detecting mycobacterial infections using PCR-based methods to detect *M. tuberculosis* (MTB)-derived cfDNA in patients with tuberculosis. However, the methodologies in some of these studies do not seem suitable for cfDNA isolation and detection [26]. In addition, the results of sensitivity and

Table 3: Classification of non-human reads

Count	MCRPM	Annotation
MAC-1		
252	48.75	*S. aureus*
119	23.02	*R. solanacearum*
99	19.15	*P. multocida*
27	5.22	*Hydrogenophaga* sp.
27	5.22	*N. gonorrhoeae*
13	2.51	*A. baumannii*
8	1.55	*K. pneumoniae*
7	1.35	*B. multivorans*
5	0.97	*C. acnes*
MAC-2		
508	55.52	*R. solanacearum*
356	38.91	Human endogenous retrovirus K113
336	36.72	*S. aureus*
204	22.30	*P. multocida*
79	8.63	*R. pickettii*
57	6.23	*Hydrogenophaga* sp. NH-16
41	4.48	*N. gonorrhoeae*
30	3.28	Proteus phage VB_PmiS-Isfahan
35	3.83	*K. pneumoniae*
37	4.04	*R. pickettii*
18	1.97	*A. baumannii*
14	1.53	*M. restricta*
13	1.42	*C. acnes*
1	0.11	*M. chimaera*
1	0.11	*M. yongonense*
MAC-3		
2562	146.4	*R. solanacearum*
2238	127.8857	*S. aureus*
1797	102.6857	Human endogenous
1239	70.8	*P. multocida*
609	34.8	Proteus phage
372	21.25714	*K. pneumoniae*
327	18.68571	*N. gonorrhoeae*
312	17.82857	*Hydrogenophaga sp.*
186	10.62857	*B. thuringiensis*
93	5.314286	*R. pickettii*
84	4.8	*M. restricta*
69	3.942857	*A. baumannii*
54	3.085714	BeAn 58058
51	2.914286	*B. cereus*
48	2.742857	*S. paucimobilis*
36	2.057143	*E. coli*
30	1.714286	*S. haemolyticus*
27	1.542857	*B. pseudomallei*
27	1.542857	*Clostridioides difficile*
27	1.542857	*P. mutanolyticus*
18	1.028571	Alcanivorax sp.
15	0.857143	*T. phaffii*
12	0.685714	*C. acnes*
12	0.685714	*F. oxysporum*
12	0.685714	*M. osloensis*
12	0.685714	*P. melaninogenica*
12	0.685714	*P. putida*
9	0.514286	*C. higginsianum*
9	0.514286	*G. vaginalis*
9	0.514286	*M. aquaticum*

Contd...

Table 3: Contd...

Count	MCRPM	Annotation
MAC-3		
6	0.342857	*B. vesicularis*
6	0.342857	*R. gummiphilus*
6	0.342857	Torque teno
3	0.171429	*B. naejangsanensis*
3	0.171429	*C. aquatic*
3	0.171429	*D. radiodurans*
3	0.171429	*D. tsuruhatensis*
3	0.171429	*D. soudanensis*
3	0.171429	*Formosa* sp.
3	0.171429	Hepatitis B
3	0.171429	*L. lactis*
3	0.171429	*Melaminivora sp.*
3	0.171429	*M. radiotolerans*
3	0.171429	*M. terrae*
3	0.171429	*M. chimaera*
3	0.171429	*M. yeatsii*
3	0.171429	*Pseudomonas* sp.
3	0.171429	*R. erythropolis*
3	0.171429	*S. enterica*
3	0.171429	Sequence 187
3	0.171429	Simbu virus
3	0.171429	*S. natans*
3	0.171429	*S. macrogoltabida*
3	0.171429	*S. thermophiles*
3	0.171429	*Y. lipolytica*
MAC-4		
273	22.75	*S. aureus*
259	21.58	*R. solanacearum*
229	19.08	Human endogenous retrovirus
143	11.92	*P. multocida*
34	2.83	*N. gonorrhoeae*
32	2.67	*Hydrogenophaga* sp.
25	2.08	*K. pneumoniae*
14	1.17	*C. dublinensis*
12	1.00	*C. acnes*
12	1.00	*A. baumannii*
10	0.83	*Clostridioides difficile*
8	0.67	*Alcanivorax* sp.
6	0.50	*B. pseudomallei*
6	0.50	*F. verticillioides*
5	0.42	Proteus phage
4	0.33	*P. mutanolyticus*
4	0.33	*Roseomonas* sp.
3	0.25	*F. fujikuroi*
3	0.25	*A. fumigatus*
2	0.17	*N. castellii*
2	0.17	*P. sihuiensis*
2	0.17	*P. chlamydosporia*
2	0.17	*D. tsuruhatensis*
2	0.17	*Rhizobium* sp.
2	0.17	*R. mucosa*
2	0.17	*Chryseobacterium* sp.
2	0.17	*F. oxysporum*
2	0.17	*C. segmentosum*
2	0.17	*E. coli*
2	0.17	*L. mirabilis*

Contd...

Table 3: Contd...

Count	MCRPM	Annotation
MAC-4		
2	0.17	*B. velezensis*
2	0.17	*B. thuringiensis*
2	0.17	*B. thuringiensis*
2	0.17	*C. taklimakanense*
2	0.17	BeAn 58058 virus
MAC-5		
56	37.76	Coliphage phi-X174
34	22.93	*S. aureus*
25	16.86	Human endogenous
21	14.16	*C. dublinensis*
18	12.14	*R. solanacearum*
16	10.79	*K. pneumoniae*
16	10.79	*E. hormaechei*
16	10.79	*C. taiwanensis*
11	7.42	*P. multocida*
11	7.42	*K. pneumoniae*
10	6.74	*C. perfringens*
10	6.74	*B. hansenii*
9	6.07	*A. hadrus*
8	5.39	*A. hallii*
8	5.39	*L. mucosae*
8	5.39	Enterobacter sp.
8	5.39	Proteus phage
8	5.39	*Blautia sp.*
7	4.72	*S. equinus*
7	4.72	*S. enterica*
7	4.72	*B. multivorans*
7	4.72	*B. dorei*
7	4.72	*C. malonaticus*
6	4.05	*F. prausnitzi*
5	3.37	*B. adolescentis*
5	3.37	*E. rectale*
4	2.70	*B. cepacia*
4	2.70	*A. baumannii*
4	2.70	*N. meningitides*
4	2.70	*R. pickettii*
4	2.70	*Hydrogenophaga* sp.
3	2.02	*Mitsuaria sp.*
2	1.35	*N. gonorrhoeae*
2	1.35	*R. pickettii*
2	1.35	*Clostridioides difficile*
2	1.35	*B. bifidum*
2	1.35	*R. gelatinosus*
2	1.35	*S. multivorans*
2	1.35	*Alcanivorax* sp.
1	0.67	*M. abscessus*
1	0.67	BeAn 58058
1	0.67	*E. cylindroides*
PTB-1		
141	28.2	*S. aureus*
84	16.8	Human endogenous retrovirus
65	13	*R. solanacearum*
49	9.8	*P. multocida*
33	6.6	*Hydrogenophaga* sp.
4	0.8	*N. gonorrhoeae*
4	0.8	*M. restricta*

Contd...

Table 3: Contd...

Count	MCRPM	Annotation
PTB-1		
2	0.4	*C. acnes*
2	0.4	*Alcanivorax* sp.
2	0.4	*M. hominis*
2	0.4	*Pseudomonas* sp.
2	0.4	*C. segmentosum*
2	0.4	*A. baumannii*
2	0.4	*Clostridioides difficile*
2	0.4	BeAn 58058
2	0.4	*K. pneumoniae*
2	0.4	*P. sputorum*
1	0.2	*Pseudomonas* sp.
1	0.2	*Pseudomonas* sp.
1	0.2	*B. cereus*

cfDNA: Cell-free DNA, MCPRM: Microbial cfDNA reads per million, *M. avium: Mycobacterium avium*, MAC: *M. avium* complex, *S. aureus: Staphylococcus aureus, R. solanacearum: Ralstonia solanacearum, P. multocida: Pasteurella multocida, N. gonorrhoeae: Neisseria gonorrhoeae, A. baumannii: Acinetobacter baumannii, K. pneumoniae: Klebsiella pneumonia, B. multivorans: Burkholderia multivorans, C. acnes: Cutibacterium acnes, R. pickettii: Ralstonia pickettii, M. restricta: Malassezia restricta, M. chimaera: Mycobacterium chimaera, M. yongonense: Mycobacterium yongonense, B. thuringiensis: Bacillus thuringiensis, B. cereus: Bacillus cereus, S. paucimobilis: Sphingomonas paucimobilis, E. coli: Escherichia coli, S. haemolyticus: Staphylococcus haemolyticus, B. pseudomallei: Burkholderia pseudomallei, P. mutanolyticus: Paracoccus mutanolyticus, T. phaffii: Tetrapisispora phaffii, F. oxysporum: Fusarium oxysporum, M. osloensis: Moraxella osloensis, P. melaninogenica: Prevotella melaninogenica, P. putida: Pseudomonas putida, C. higginsianum: Colletotrichum higginsianum, G. vaginalis: Gardnerella vaginalis, M. aquaticum: Methylobacterium aquaticum, B. vesicularis: Brevundimonas vesicularis, R. gummiphilus: Rhizobacter gummiphilus, B. naejangsanensis: Brevundimonas naejangsanensis, C. aquatic: Comamonas aquatic, D. radiodurans: Deinococcus radiodurans, D. tsuruhatensis: Delftia tsuruhatensis, D. soudanensis: Desulfuromonas soudanensis, L. lactis: Lactococcus lactis, M. radiotolerans: Methylobacterium radiotolerans, M. terrae: Methylobacterium terrae, M. yeatsii: Mycoplasma yeatsii, R. erythropolis: Rhodococcus erythropolis, S. enterica: Salmonella enterica, S. natans: Sphaerotilus natans, S. macrogoltabida: Sphingopyxis macrogoltabida, S. thermophiles: Streptococcus thermophiles, Y. lipolytica: Yarrowia lipolytica, C. dublinensis: Cronobacter dublinensis, F. verticillioides: Fusarium verticillioides, F. fujikuroi: Fusarium fujikuroi, A. fumigatus: Aspergillus fumigatus, N. castellii: Naumovozyma castellii, P. sihuiensis: Pseudomonas sihuiensis, P. chlamydosporia: Pochonia chlamydosporia, R. mucosa: Roseomonas mucosa, C. segmentosum: Corynebacterium segmentosum, L. mirabilis: Lautropia mirabilis, B. velezensis: Bacillus velezensis, C. taklimakanense: Chryseobacterium taklimakanense, E. hormaechei: Enterobacter hormaechei, C. taiwanensis: Cupriavidus taiwanensis, C. perfringens: Clostridium perfringens, B. hansenii: Blautia hansenii, A. hadrus: Anaerostipes hadrus, A. hallii: Anaerobutyricum hallii, L. mucosae: Lactobacillus mucosae, S. equinus: Streptococcus equinus, B. dorei: Bacteroides dorei, C. malonaticus: Cronobacter malonaticus, F. prausnitzii: Faecalibacterium prausnitzii, B. adolescentis: Bifidobacterium adolescentis, E. rectale: Eubacterium rectale, B. cepacia: Burkholderia cepacia, N. meningitides: Neisseria meningitides, B. bifidum: Bifidobacterium bifidum, R. gelatinosus: Rubrivivax gelatinosus, S. multivorans: Sulfurospirillum multivorans, M. abscessus: Mycobacteroides abscessus, E. cylindroides: Eubacterium cylindroides, M. hominis: Mycoplasma hominis, P. sputorum: Pandoraea sputorum, C. difficile: Clostridioides difficile*

specificity testing in these studies were highly variable [26]. Pan *et al.*, used MTB-cfDNA to identify PTB in participants and reported a sensitivity of 54.2%, and the sensitivity

Table 4: Clinical characteristics in enrolled patients with and without detectable *Mycobacterium avium* complex-cell-free DNA in next-generation sequencing analysis

	MAC cfDNA positive (*n*=2), *n* (%)	MAC cfDNA negative (*n*=3), *n* (%)	*P*
Age (years)	79.5±10.6	73.33±14.47	0.564
Sex (male)	1 (50)	0	0.400
BMI	17.62±1.72	20.25±2.17	0.248
Cavitary lesion	0	1 (33.3)	1.000
Underlying disease			
DM	1 (50)	1 (33.3)	1.000
Malignancy	0	0	
CKD	0	0	
Bronchiectasis	1 (50)	2 (66.7)	1.000
Interstitial lung disease	1 (50)	0	0.400
Sputum smear positive	2 (100)	1 (33.3)	0.400
Sputum smear grade	1.50±0.71	1.0±1.73	0.554
Radiographic score	10.0±0	7.33±1.53	0.076
Nonhuman read	2794.0±2084.0	518.7±225.6	0.083

BMI: Body mass index, DM: Diabetes mellitus, CKD: Chronic kidney disease, *M. avium: Mycobacterium avium,* MAC: *M. avium* complex, cfDNA: Cell-free DNA

increased to 79.2% after incorporating a blood monocyte to lymphocyte ratio ≥0.42 into the analysis [15]. According to Amy Oreskovic *et al*.'s study that yielded a higher cfDNA detection rate for active tuberculosis, MTB-cfDNA would more likely be detected in patients with a smear-positive disease or a disease with greater lung involvement [14]. However, whether NTM-derived cfDNA can be detected in patients with active NTM-LD remains unclear.

In this study, we confirmed NTM-derived cfDNA can be detected in patients with NTM-LD. The DNA fragments were DNA polymerase III alpha subunit and ATP-binding cassette transporters permease, which were found to have similar sequences to those of standard strains of MAC. The alpha subunit is a component of DNA polymerase III, and its functions include catalytic polymerase activity and DNA synthesis. The alpha subunit is encoded by the DNAE gene [27]. DNA polymerase III has been reported to be related to DNA replication in MTB [28]. ATP-binding cassette transporters are a transport system unique to bacteria, and the transport system is known to influence the lengthy survival of MTB within a host [29]. Although the MCRPM of 0.11 and 0.67 for MAC spp. were relatively low compared with the MCRPM for other bacteria in this study, this result is in line with a study of patients with early breast cancer that had MCRPMs ranging from 0.12 to 0.38 for *Mycobacterium spp.* [21]. Although MCRPM results of microbial cfDNA are reliable [30], additional studies with a larger number of NTM patients are warranted to evaluate definitive cutoff values. To improve the sensitivity of using the microbial cfDNA method to identify patients with MAC-LD, in the future, it would be worthwhile to design a more specific quantitative PCR/droplet digital PCR primer/probe for MAC-cfDNA.

Using an NGS-based method to detect microbial cfDNA, the most common bacteria in our MAC and PTB patients were *R. solanacearum*, *S. aureus*, and *P. multocida*. *R. solanacearum* is a Gram-negative bacillus. It is a soil-borne microbe and is known as a plant pathogenic bacteremia [31]. It has been reported to be associated with ventilator use, as the risk of *Ralstonia spp*. colonization and infection increases for patients receiving mechanical ventilation [32]. *S. aureus* is a Gram-positive coccus. Nearly 30% of healthy people are colonized by *S. aureus* without exhibiting any symptoms. *S. aureus* can cause a variety of diseases, such as cellulitis, pneumonia, and infectious endocarditis. It is worth noting the resistance of *S. aureus* to oxacillin is a health threat [33]. *P. multocida* is a Gram-negative bacillus that causes a wide range of diseases in a variety of hosts such as cattle, pigs, sheep, and fowl, and human infections have also been reported [34]. Each of these referenced studies has demonstrated these bacteria can exist in humans without causing an infectious disease. In our study, none of the patients received a blood culture at that time, which indicated that there is no infection sign clinically. Since the origins of these pathogen-derived cfDNA are unknown, more data are needed to evaluate the clinical significance of the bacterial cfDNA detected in MAC and TB patients.

In addition, we could also make a diagnosis faster. In routine clinical procedures, the diagnosis of NTM-LD is often delayed due to nonspecific clinical symptoms, image findings, and several times of sputum collection [6]. In routine clinical procedures, TB culture, sputum NTM real-time PCR test, which included *M. avium*, *M. intracellulare,* or other pathogens of sputum samples will be prescribed directly for symptomatic patients. However, using the sputum NTM real-time PCR test to confirm whether it is MAC can take up to 3 days from the collection to the issuance of the PCR report. In terms of time series, this experiment is designed to wait for sputum culture positive for MAC without mixed infection with other NTM species for about 30 days, and it takes just 30 h to get the analysis results of many pathogenic bacteria except MAC after receiving the case of blood NGS. Previous studies showed NGS methods could help the diagnosis of tuberculosis [35,36]. However, the data of NGS for MAC-LD is still lacking.

There are some limitations of this study. First, as a pilot study, the sample size is relatively small. Using cfDNA to detect MAC was possible in our trial, but more data from a large-scale study are needed for further confirmation. Second, there were cfDNA from other bacteria found in our study. Whether these bacteria correlate with mycobacterial infection requires additional investigation. Third, in this study, we enrolled neither healthy people nor patients with pulmonary NTM colonization. Finally, due to the limitations of experimental design and case selection, the use of NGS to design this experiment is not superior to the current clinical routine test results. A healthy control group and a nondiseased control group with MAC colonization should be included in the future to assess the specificity of using MAC-cfDNA to detect MAC-LD.

Conclusion

Our study demonstrated 40% of patients with MAC-LD

had MAC-specific cfDNA as detected by NGS tests. Our proof-of-concept study suggests future PCR-based cfDNA studies should consider using MAC-specific cfDNA, including polymerase III alpha subunit and ATP-binding cassette transporters permease, as targets to assist in evaluating MAC-LD patients. Since MAC-LD has become an emergent pulmonary disease, future studies exploring the clinical value of using NGS tests to detect MAC-specific cfDNA in patients are warranted.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of interest

There are no conflicts of interest.

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Supplementary Material

A C C T C G G T G G G T G A C G A C A A G G A C A A G G C C G C C G T C T A C C T G G C C G A C T G C A G C A A G C T G G G C A T C A C G G T G T T G C C G C C C G A C G T C A A C G A A T C G C T G G T G A A C T T C G C C T C G GTCGGAACGGACATCCGTTTCGGGCTGGGTGCGGTG.

C G T T C C G A T T G A T T G C G C A T A G A A C A A A G T T G T C A C C A G C T G G G C G G A A A A G A A CGAGAGGAGGACCGCGACCGAATATAGTGGGATAGTGACGATGAAGGCGCCAATAACTCGGGTAGATACCAAATACG AAACTGATTTGATTCCCAGA.