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Original Article

Investigation of non-tuberculous mycobacteria in a primary hospital from southeastern China

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Abstract

Introduction: Non-tuberculous mycobacterium (NTM) can colonize the human body, leading to opportunistic infection. This study was conducted to analyze the NTM species composition in a primary hospital and investigate the potential features of the patients with different NTM species.

Methodology: Mycobacterial strains were collected from the patients admitted at the hospital from January 2016 to May 2019. MPB64 assay was used to screen NTM strains and confirmed by Rv0577 amplification. The species were identified by hsp65 sequencing. The clinical records of patients with NTM were retrospectively reviewed.

Results: Among the 122 identified NTM isolates, the most common strains were *Mycobacterium avium* complex (MAC, n = 102, 83.6%), *Mycobacterium abscessus* (n = 9, 7.4%) and *Mycobacterium lentiflavum* (n = 5, 4.1%). The predominant species among MAC were *Mycobacterium chimaera* (n = 57, 46.7%), followed by *Mycobacterium intracellulare* (n = 25, 20.5%) and *Mycobacterium colombiense* (n = 17, 13.9%). A significantly lower percentage of positive acid-fast assay was observed in *Mycobacterium colombiense* positive patients than in those with *Mycobacterium intracellulare* and *Mycobacterium chimaera*. *Mycobacterium intracellulare* was more frequently isolated in patients from the infectious department than in other MAC members.

Conclusions: A predominant prevalence of *Mycobacterium chimaera* in Dongyang of Zhejiang Province was different from other regions in China, indicating that its prevalence has been likely underestimated. The heterogeneity in clinical features, caused by different MAC members, required an accurate species identification of the NTM isolated in the primary hospitals.

Key words: non-tuberculous mycobacterium; *Mycobacterium avium* complex; *Mycobacterium chimaera*; Mycobacterium colombiense; Mycobacterium intracellulare.

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Introduction

As ubiquitous bacteria, non-tuberculous mycobacteria (NTM) can be present in many environments such as water and soil, thus potentially colonizing the human body, leading to infectious diseases in humans, especially in the immunocompromised ones [1]. The isolation of NTM in clinical specimens are increasing over time and more patients are diagnosed as infected by NTM according to an official statement by (Infectious Diseases Society of America/American Thoracic Society Consensus Guidelines, ATS/IDSA) [1-3]. Since clinical symptoms and radiographical characteristics of patients affected by NTM are indistinguishable from patients affected by *Mycobacterium tuberculosis*, culture and species identification are essential for the accurate diagnosis of NTM disease [2,4,5].

So far, more than 200 species of NTM have been identified, with a few number causing infections in humans (http://www.bacterio.net/mycobacterium.html) [6]. Once infected by NTM strains, the therapy should be applied based on the risks and benefits for the patient, but the corresponding regimens vary across the different species of NTM [2]. Although *Mycobacterium avium complex* (MAC) is one of the most commonly isolated bacteria from clinical specimens, a significant

geographic variability was observed among these species [1]. Furthermore, among MAC, *Mycobacterium intracelluare (M. intracellulare)*, *Mycobacterium avium (M. avium), Mycobacterium colombiense (M. colombiense)* and *Mycobacterium chimaera (M. chimaera)* with a heterogeneity in pathogenicity and treatment response, cannot be identified by traditional tests [7, 8].

Currently, several genetic regions have been used to identify the species of NTM, including *hsp65* [9], 16S rRNA [10], 16S–23S rRNA internal transcribed spacer (ITS) sequence [11] and *rpoB* [12]. However, 16S rRNA possesses a low discriminatory power. Based on the single gene sequencing, *hsp65* sequencing shows a higher discriminatory power for species identification than 16S rRNA and *rpoB* [13, 14]. Moreover, *hsp65* is ideal for identifying highly homogenous species within MAC with a high specificity and sensitivity [14].

Although accurate species identification of NTM has been conducted in big hospitals in some regions, there are rare data from primary hospitals where patients are firstly visited [15,16]. Due to insufficient attention to NTM related diseases, the diagnosis and therapy was commonly ignored in primary hospitals when compared to MTBC infection. Therefore, in this study, mycobacterial strains were isolated from patients who visited a primary hospital located at the central part of Zhejiang Province in China. The NTM species were screened by MBP64 proteins detection and identified by *hsp65* sequencing. The results presented in this work will help the primary doctor to evaluate the significance of NTM in TB-epidemic primary hospital and to be aware of a variety in clinical features infected by different NTM species.

Methodology

Patients and sample collection

Clinical samples including urine, sputum and cerebrospinal fluid from patients who were suspected of having mycobacterial infection based on clinical symptoms and medical image, were collected and cultured using the BACTEC MGIT 320 System (BD Bioscience, New Jersey, USA) or on the Lowenstein-Jenden medium. Samples were collected from January 2016 to May 2019 in a primary hospital located at the central part of Zhejiang Province in China.

Positive culture samples were confirmed by Zieh1-Neelsen staining (Baso, Zhuhai, China) and identified as suspicious NTM or MTBC based on the MPB64 protein assay kit (Hangzhou Genesis Biodetection & Biocontrol Co., Ltd, Hangzhou, China) according to the manufacturer's instructions.

The laboratory tests including fast-acid staining of sputum smear and T. spot assay were analyzed. The corresponding clinical information of patients with NTM strains were also retrospectively reviewed anonymously. This study was approved by the Ethics Committee of Dongyang People's Hospital Ethics Committee and Institutional Review Board.

DNA preparation

The DNA was prepared from strains asreported previously with minor modifications [17]. Briefly, the bacterial samples were treated with 75% alcohol at room temperature for 2 hours. Then, the pellet was collected by centrifugation at 10,000g for 5 minutes at room temperature, and washed by Phosphate-Buffered Saline buffer. Finally, the collected pellet was resuspended in Tris-EDTA buffer and lysed at 95 ℃ for 10 minutes. After centrifugation, the supernatant containing the DNA was collected and stored at -20 ℃.

NTM species identification

Rv0577 gene was amplified and analyzed as positive for MTBC and negative for NTM [18]. *Hsp65* sequences were obtained by amplification and sequencing by the corresponding primers (Table 1). All the amplifications were performed in 20 μL PCR mixtures, such as 2 μL of 10× PCR buffer, 200 μM of each dNTP, 0.25 μM of each primer and 1 U of TransTaq HiFi DNA Polymerase (TransGen Biotech Co., Ltd, Beijing, China). Amplification reaction conditions were the followings: initial denaturation at 94 °C for 5 minutes; 30 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 30 seconds, extension at 72 °C for 1 minute; a final extension at 72 °C for 10 minutes. The PCR products were confirmed by electrophoresis and then sent to GENEWIZ Company (China) for sequencing. The exact species

Table 1. The primers involved in this study.

Primers	Sequences	Length of product (bp)
Rv0577-F	5-ATGCCCAAGAGAAGCGAATACAGGCAA-3	786
Rv0577-R	5-CTATTGCTGCGGTGCGGGCTTCAA-3	
HSPF3 #	5-ATCGCCAAGGAGATCGAGCT-3	644
HSPR4	5-AAGGTGCCGCGGATCTTGTT-3	

The primers used in the sequencing process.

were determined based on the *hsp65* alignment results in the database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the identity > 99%. The species of submitted sequence was identified as the one in database with top scores in alignments results.

Results

Characteristics of the species in NTM

A total of 155 available suspected NTM strains were collected based on positive results of mycobacterial culture and negative response in MPB64 assay. They were isolated from 183 clinical samples (including sputum, urine and cerebrospinal fluid) from 155 patients. As a result of *Rv0577* amplification, 28 samples were identified as MTBC. Based on the *hsp65* gene sequencing results, 28 strains with positive amplification of *Rv0577* were confirmed as MTBC and 5 suspected NTM strains with *Rv0577* negative belonged to Nocardia.

In the 122 NTM strains, MAC strains accounted for 83.6% (102/122). *M. abscess* (n = 9, 7.4%) and *Mycobacterium lentiflavum* (n = 5, 4.1%) were also identified. Other species included *Mycobacterium gastri* (n = 1, 0.8%), *Mycobacterium paraense* (n = 2, 1.6%), *Mycobacterium paragordonae* (n = 1, 0.8%) and *Mycobacterium smegmatis* (n = 1, 0.8). Among MAC strains, *M. chimaera* (n = 57, 46.7%), *M. intracellulare* (n = 25, 20.5%), *M. colombiense* (n = 17, 13.9%), *M. avium* (n = 2, 1.6%), *M. marseillense* (n = 1, 0.8) and *M. yongonense* (n = 1, 0.8%) were identified.

Clinical characteristics of patients with MAC and non-MAC

One hundred and twenty NTM strains were from the sputum samples, and the remaining two, which belonged to M. colombiense, were isolated from urine and cerebrospinal fluid, respectively. After excluding eight outpatients due to unavailable information, the clinical information of patients with NTM positive was further analyzed. In general, only three patients showed no symptoms related to isolated sites, two of who had cavities in lungs confirmed by computed tomography.

Table 2. Characteristics of inpatients with MAC vs non-MAC strains (n = 114) a.

Features	MAC	Non-MAC	P[a]
Gender			
male	46 (48.4)	11 (57.9)	0.451
female	49 (51.6)	8 (42.1)	
Age (mean ± SD)	72.6 ± 8.6	70.1± 16.5	0.537[b]
T. spot assay			
positive	7 (20.6)	1 (33.3)	0.530 [c]
negative	27 (79.4)	2 (66.7)	
Acid-fasting staining			
Positive	20 (21.1)	2 (21.1)	0.517 [c]
Negative	75 (78.9)	15 (78.9)	
TB history			
Yes	27 (28.4)	4 (21.1)	0.510
No	68 (71.6)	15(78.9)	
COPD			
Yes	41 (43.2)	7 (36.8)	0.611
No	54 (56.8)	12 (63.2)	
Bronchiectasis			
Yes	26 (27.4)	6 (31.6)	0.709
No	69 (72.6)	13 (68.4)	
Departments			
Infectious	16 (16.8)	0 (0)	0.127[c]
Respiratory	66 (69.5)	15 (78.9)	
Others	13 (13.7)	4 (21.1)	
Tumor			
Yes	8 (8.4)	3 (15.8)	0.389 [c]
No	87 (91.6)	16 (84.2)	
Diabetes			
Yes	2 (2.1)	4 (21.1)	0.007 [c]
No	93 (97.9)	15 (78.9)	

[a]: The significance was analyzed by Pearson Chi-Square Test except for the data marked by symbols; [b]: Student *t* test; [c]: Fisher exact test.

Considering the dominant prevalence of MAC in this region, it is of great significance to investigate the clinical and laboratory features of patients with MAC strains. Therefore, the clinical features of the patients with MAC and non-MAC were compared, but there was no significant difference except for the incidence of the diabetes (Table 2). In general, the patients with MAC owned a slightly higher percentage of chronic obstructive pulmonary diseases (COPD) than those with non-MAC strains. 20.6% of patients with MAC strains showed positive responses in T. spot assay. None of the patients with non-MAC strains was isolated from the patient in the infectious department while 16.8% of patients with MAC were found in infectious department. In addition, all the patients with non-MAC strains who were accompanied by bronchiectasis were positive for *M. abscess* while the remaining three patients with *M. abscess* were not with bronchiectasis.

Clinical characteristics of patients with different MAC strains

To clarify the differences among the clinical features of the patients caused by different members of MAC, the patients with *M. chimaera*, *M. intracellulare* and *M. colombiense* were analyzed respectively (Table 3). *M. chimaera* strains were more likely isolated in females than in males, although without significance. Moreover, none of the patients with *M. colombiense* had positive acid-fasting responses, while patients with *M. chimaera* and *M. intracellulare* owned the percentages of positive results ranging from 24% to 27% (P = 0.049). In addition, 36.4% (8/22) of *M. intracellulare* strains were isolated from the patients in the infectious department, more frequently than other members of MAC (P = 0.024).

Discussion

Since patients infected by NTM and MTBC should be subjected to different therapeutic regimens due to the different transmission ability, it was of utmost importance to achieve the precise identification of the pathogens in a short time after sample collection. However, in China, acid -fast staining, mycobacterial culture and radiographic analysis are routinely used for diagnosis of MTBC, despite NTM infection displays similar results, causing a delay or missed diagnosis of NTM infection [19]. Although there were significant differences between laboratory and clinical features of patients with NTM and MTBC in our previous study [20], the detection of NTM strain isolated from clinical samples is important and necessary for a confirmed NTM diagnosis. MPB64, as a specific gene for MTBC, which is applied for a rapid discrimination between NTM and MTBC following routine mycobacterial culture [21], was also supported in this study. However, there were still some MTBC strains identified as NTM. Therefore, we used a PCR amplification targeting the MTBC specific gene *Rv0577* for confirming the results

Table 3. Characteristics of inpatients infected by different members of MAC strains a.

Features	*M. chimaera* N. (%)	*M. intracellulare* N. (%)	*M. colombiense* N. (%)	P[a]
Gender				
male	24 (44.4)	12 (54.5)	10 (62.5)	0.397
female	30 (55.6)	10 (45.5)	6 (37.5)	
Mean age (mean ± SD)	73.1 ± 7.9	71.5 ± 11.6	70.3 ± 8.5	0.56[b]/0.25[c]
Acid-fasting staining				
positive	13 (24.1)	6 (27.3)	0 (0)	0.049[d]
Negative	41 (75.9)	16 (72.7)	16 (100)	
TB history				
Yes	15 (27.8)	8 (36.4)	3 (18.5)	0.557[d]
No	39 (72.2)	14 (63.6)	13 (81.5)	
COPD				
Yes	25 (46.3)	7 (31.8)	9 (56.3)	0.302
No	29 (53.7)	15 (68.2)	7 (43.7)	
Bronchiectasis				
Yes	14 (22.0)	6 (28.6)	4 (31.3)	1.0[d]
No	40 (78.0)	16 (71.4)	12 (68.8)	
Departments				
Infectious	7 (13.0)	1 (8. 8 (36.4)	1 (6.25)	0.024[d]
Respiratory	42 (77.8)	10 (45.5)	11 (68.8)	
Others	5 (9.3)	4 (18.2)	4 (25.0)	

[a]: The significance was analyzed by Pearson Chi-Square Test except for the data marked by symbols; [b]: Student *t* test between the individuals with *M. chimaera* and the ones with *M. intracellulare;* [c]: Student *t* test between the individuals with *M. chimaera* and the ones with *M. colombiense;* [d]: Fisher exact test.

[18]. Our results showed that *Rv0577* could discriminate NTM from MTBC with a high efficience, suggesting that this gene detection after MPB64 assay was necessary for discrminate MTBC from NTM.

Considering that the treatment and prognosis of NTM infection varied among the NTM species, further identification at species level might facilitate the individual management and treatment of NTM infected patients. In this study, sequencing the *hsp65* gene could not only identify MAC in NTM, but also resulted in a powerful discrimination between *M. chimaera* and *M. intracellulare* [22]. Our results showed that the most common NTM was represented by MAC strains, similar to the epidemiology of NTM in China and in many regions in the world. The predominant species among MAC in this study was *M. chimaera*, while *M. intracellulare* was the main one in other regions of China [15,16,23]. In addition, the *M. colombiense* and *M. lentiflavum* found in this study were hardly reported in China, indicating that some species of NTM were underestimated in clinical samples [23]. The reasons for the different predominant species between this study and others were largely due to the differences in the identification methods used. Indeed, *M. chimaera* could not be easily distinguished from the *M. intracellulare* based on 16S rRNA and *rpoB* sequencing, causing incorrect species identification by commonly used laboratory techniques [24,25]. Therefore, the common identification methods based on 16S rRNA in China may lead to an underestimated prevalence of *M. chimaera* [15,16,19]. In addition, the species varies across the different regions as reported, which may be explained by the heterogeneities in exposure routes and susceptibility of individuals [26,27].

In China, the patients were arranged in different departments based on the diagnosed diseases for convenient management. For example, the patients who were diagnosed as MTB infection should be sent to infectious departments for further treatment. We found that all the NTM strains, which were isolated from the infectious departments, belonged to the MAC strains. Moreover, *M. intracellulare* were more frequently found in infectious department than other MAC members, resulting a possibility that the patients with *M. intracellulare* were diagnosed as TB and received TB therapy before the species identification. The incorrect diagnosis may lead to emergence of drug resistance or over-therapy in NTM disease.

The limitations of this study included the insufficient associations between the isolated strain and diseases, though most of them were suspected as mycobacterial infection based on the symptoms and radiological examination. This partly resulted from not only the underestimated importance of NTM infection in China but also the fact that two repeatable sputum samples were time and labor-costing for the most primary hospitals. This study shed some light on the prevalence and species composition of NTM in clinical samples, but further invstigations are needed in order to understand how much they really contributed to the corresponding infection.

Conclusion

In summary, NTM identification is urgently needed in China where the diagnosis of NTM infection is probably underestimated. Combination of MPB64 assay and *Rv0577* amplification could distinguish NTM from MTBC. Based on the *hsp65* gene sequencing, we found a unique predominant prevalence of *Mycobacterium chimaera* in clinical specimens, indicating its significant role in prevalence. The differences in clinical features caused by different MAC species indicated the need of accurate species identification and may require species-level specific management in NTM diseases.

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