Distribution, Prevalence, and Antibiotic Resistance of Non-Tuberculous Mycobacteria on Hainan Island

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ABSTRACT

Background: Non-tuberculous mycobacteria (NTM) represent a diverse group of environmental mycobacteria, with certain species implicated in infections affecting multiple tissues, including the lungs. The incidence of NTM-related diseases has been on the rise globally, particularly in developing countries. This study aimed to systematically investigate the ecological distribution, molecular identification, and antibiotic susceptibility profiles of NTM isolates in Hainan Island, furnishing a scientific basis for local control and prevention strategies. Methods: From 2015 to 2018, 158 suspected NTM samples were collected across 14 cities on Hainan Island. Following standardized sample processing and DNA extraction, molecular identification was conducted through PCR amplification of the rpoB and hsp65 genes, and sequencing, with species confirmation via GenBank database comparison. Phylogenetic analysis was performed to elucidate interspecies relationships and drug susceptibility testing was carried out for five antibiotics on M. abscessus isolates. Results: 118 NTM isolates were successfully identified, encompassing 24 distinct species. M. abscessus and M. intracellulare, accounting for 23.73% and 22.03% respectively, were the most prevalent. Phylogenetic analysis revealed clusters indicative of pathogenic and potentially pathogenic traits among different NTM species, with M. abscessus and M. fortuitum clustering as rapidly growing, pathogenic mycobacteria. Drug sensitivity testing indicated universal resistance of M. abscessus to doxycycline, yet high sensitivity to amikacin (71.4%) and linezolid (50.0%). Additionally, non-NTM species such as Nocardia thailandica, and Tsukamurella paurometabola were detected, suggesting potential misdiagnosis in clinical settings. Conclusion: This study provides a comprehensive dataset on the ecological distribution, molecular characterization, and antibiotic resistance of NTM in Hainan Island, highlighting the dominance of M. abscessus and M. intracellulare in local NTM infections.

Keywords: Non-tuberculous Mycobacteria (NTM); Species Identification; RNA Polymerase Beta Subunit (rpoB); Heat Shock Protein 65 (Hsp65)

INTRODUCTION

Non-tuberculous mycobacteria (NTM), belonging to the Mycobacteriaceae family, ubiquitously inhabit natural environments such as soil, water, and even the human body. With over 200 species documented, approximately 60 of which are known to be pathogenic, these organisms can affect a wide array of tissues including skin, soft tissue, lymphatic system, synovial fluid, joints, bones, and lungs[1]。In immunocompromised individuals, pathogenic NTM can lead to opportunistic NTM pulmonary disease (NTM-PD) and extrapulmonary infections[2]. The global incidence of NTM infections is on the rise, particularly in developing countries [3], with China witnessing a significant increase from 4.9% in 1990 to 22.9% in 2010 for NTM incidence, and the proportion of NTM-caused lung infections soaring from 4.24% to 12.68% by 2021 [4-5], Misdiagnosis of NTM as multidrug-resistant tuberculosis or co-infections with other chronic diseases contributes to escalating mortality rates[6-7]. The AIDS pandemic has further heightened recognition of NTM's clinical significance [8]; however, due to the absence of mandatory reporting systems for NTM infections in many nations, current epidemiological data is limited and potentially underestimates the actual burden[9-10]. An accurate understanding of NTM epidemiology and the implementation of effective surveillance systems are thus crucial for guiding control strategies.

Most regions in China have established comprehensive monitoring systems and research strategies for NTM, especially in developed areas like Shanghai, Beijing, and Guangdong, which feature extensive temporal coverage, diverse sample collection, and a wealth of NTM subspecies, leading to well-developed theoretical frameworks and even rare identifications [11-15]. NTM research of Hainan Island initiated in 2019[16], encountered challenges of geographical limitations and inadequate sample diversity, indicating a significant lag in research compared to other provinces. This study aims to enhance the breadth and depth of NTM investigation in Hainan, providing a robust foundation for more precise and targeted intervention strategies, ensuring their scientific rigor and effectiveness, and advancing the scientific frontiers in NTM prevention and control.

Mycobacterium avium complex (MAC), the most common slowly growing mycobacteria (SGM), is the predominant cause of NTM pulmonary disease globally[17], with strains like M. avium, M. intracellulare, M. chimaera, M. marseillense, M. bouchedurhonense, M. timonense, yongonense, and M. paraintracellulare recognized as pathogens [18]. M.abscessus-complex(MABC) dominated by M. abscessus subspecies , accounts for 65%-80% of Rapidly growing mycobacteria (RGM) pulmonary infections[19] [20], with three key subspecies identified. M. kansasii ranks as the second most frequent NTM pathogen in some European countries[21], whereas in China, MAC and MABC prevail, specifically M. intracellulare and M. abscessus, along with other pathogens such as M. fortuitum, M. gordonae, and M. kansasii. In China, NTM distributions exhibit a southern, coastal, and warm-humid regional predominance over northern, inland, and cold-dry areas[2,5].

Drug susceptibility testing of numerous NTM isolates indicates clarithromycin (CLR) and amikacin (AMK) as highly effective against MAC, serving as first-line agents . MAC shows resistance to linezolid (LZD; 42.86%) and moxifloxacin (MFX; 25.32%), suggesting their use as second-line options. MABC exhibits high resistance to moxifloxacin, ciprofloxacin, and tobramycin (>95%), but retains susceptibility to tigecycline (98.53%) and amikacin (64.71%) [22], highlighting their potential as first-line therapies. M. fortuitum complex displays sensitivity to amikacin and moxifloxacin but is generally resistant to clarithromycin, doxycycline, and trimethoprim/sulfamethoxazole[23]. Given suboptimal NTM treatment outcomes, exploration of alternative antibiotics such as clofazimine, bedaquiline, delamanid, and tedizolid, showing in vitro activity against specific NTM species like M. intracellulare, M. avium (clofazimine-sensitive), M. abscessus, M. fortuitum, and M. chelonae (tedizolid-responsive), underscores their therapeutic potential [24]. This research delves into the distribution and antibiotic resistance profiles of NTM in Hainan, elucidating the current NTM prevalence scenario and augmenting the province's NTM research corpus.

METHODS

Ethics Statement:

This study was conducted with the approval of the Ethics Committee of Hainan Medical University, under the ethics number HYLL-2021-170.

Study Population and Sample Collection:

The experimental samples were sourced from the Hainan Tuberculosis Prevention and Control Institution, covering the period between 2015-2018. A total of 158 sputum and bronchoalveolar lavage fluid specimens, clinically suspected of NTM infection, were collected from 14 cities and counties in Hainan Island, including Baisha, Baoting, Changjiang, Chengmai, Danzhou, Ding'an, Dongfang, Haikou, Ledong, Lingao, Lingshui, Qionghai, Sanya, and Wenchang.

Materials and Equipment:

Bacterial genomic DNA extraction kit (Tiangen Biotech, Beijing), Template DNA, buffer, dNTPs, TaqDNA polymerase (Tiangen Biotech, Beijing), Hsp65 and rpoB gene amplification primers (Tsingke Biotech, Beijing), PCR thermal cycler (Bio-Rad Laboratories, Shanghai), DNA recovery kit (Tiangen Biotech, Beijing). High-speed benchtop centrifuge (Eppendorf, Germany).

Clinical Sample Collection:

Patients are instructed to fast for at least 1-2 hours before sample collection. Before expectoration, patients perform a mouthwash with 0.1% spirulina solution three times, followed by rinsing with clean water, to minimize oral flora contamination. Sputum Production: Deep coughing is encouraged to ensure productive sputum collection into sterile plastic containers with a Spiral mouth, provided by the hospital, emphasizing the importance of collecting deep sputum such as purulent, mucoid, or blood-streaked specimens. Saliva samples may also be accepted if no productive sputum can be produced. Each patient provides three types of sputum samples (morning, night, and on-site) over a day. Morning and night sputa are self-collected at home, while the on-site sample is obtained at the hospital. Samples are transported to the tuberculosis laboratory within 4 hours of collection. If immediate transportation is not feasible, samples are refrigerated at 4℃ until delivery. In the absence of a provincial tuberculosis control institute, samples are sent to designated tuberculosis laboratories in each city for initial testing. Subsequently, positive cultures are forwarded to the Hainan Provincial Center for Disease Control and Prevention for confirmation and further analysis.

PCR-based Species Identification:

DNA was extracted from each sample to serve as a template for subsequent PCR reactions using primers targeting rpoB and hsp65 genes. Primer sequences were as follows: hsp65-F: 5’-ACC AAC GAT GGT GTG TCC AT-3’, hsp65-R: 5’-CTT GTC GAA CCG CAT ACC CT-3‘, rpoB-F: 5’- GAC GAC ATC GAC CAC TTC GG -3’, rpoB-R: 5’-GGG GTC TCG ATC GGG CAC AT-3’. Amplification was conducted in20 μL reaction mixtures containing 2 μL 10×PCR buffer, 200 μM dNTPs, 0.25 μM of each primer, and 1U TransTaqHiFiDNA polymerase (Tiangen Biotech, Beijing). Thermal cycling conditions comprised initial denaturation at 94°C for 5 minutes; 30 cycles of 94°C denaturation for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute; and a final extension at 72°C for 10 minutes. Products were analyzed by agarose gel electrophoresis, and DNA fragments were recovered using a gel extraction kit, and sent to Hainan Nanshan Biotechnology Co., Ltd. for sequencing. Ultimately, nucleotide sequences were aligned with GenBank references via the NCBI BLAST website on 04/22/2024 (https://blast.ncbi.nlm.nih.gov/Blast.cg).

Phylogenetic Tree Construction:

To explore evolutionary relationships between hsp65 and rpoB genes, multiple sequence alignment was performed using the Muscle algorithm in MEGA software (v11.0) with default parameters. Neighbor-joining (NJ) method was applied in MEGA to construct

phylogenetic trees. To enhance tree topology reliability, a bootstrapping process involving 1000 iterations was executed. For intuitive comprehension, the phylogenetic tree was refined and annotated using the Tree Visualization by One Table (TvBOT, https://www.chiplot.online/tvbot.html, version 2.6) online tool.

NTM Susceptibility Testing:

According to Clinical and Laboratory Standards Institute (CLSI) guidelines, broth microdilution assays were employed for in vitro antimicrobial susceptibility testing of selected NTM isolates. Minimum inhibitory concentration (MIC) values require clinical context interpretation and adjustment. MIC was defined as the lowest concentration inhibiting visible growth of the tested strain. The MicroDST™ system (Yinked AUTOBIO Diagnostics Co., Ltd., Zhuhai, China) was utilized for resistance testing of NTM isolates. MIC breakpoints and sensitivity/resistance determination were interpreted per manufacturer instructions. Five antibiotics were tested against M.abscessus isolates: moxifloxacin, amikacin, linezolid, azithromycin, and doxycycline.

RESULTS

NTM Subspecies Characterization Findings

From 158 clinical samples suspected of NTM collected between 2015 and 2018,157 isolates were successfully species-identified, comprising 118 NTM (across 24 species) and 39 non-NTM organisms. Identification primarily relied on rpoB gene sequencing, with hsp65 sequencing serving as a supplementary tool for conclusive determination. The species distribution, as shown in Figure 1, was led by M. abscessus (28/118, 23.73%), M. intracellulare (26/118, 22.03%), M. msimiae (17/118, 14.41%), M. paraintracellare (16/118, 13.56%), and M. colombiense (5/118, 4.24%). MTB(23), Nocardia thailandica(3/16,18.75%), Tsukamurella paurometabola(2/16,12.5%), and Gordonia jinghuaiqii(2/16,12.5%), were also identified. Detailed composition ratios are illustrated in Supplemental Table 1.

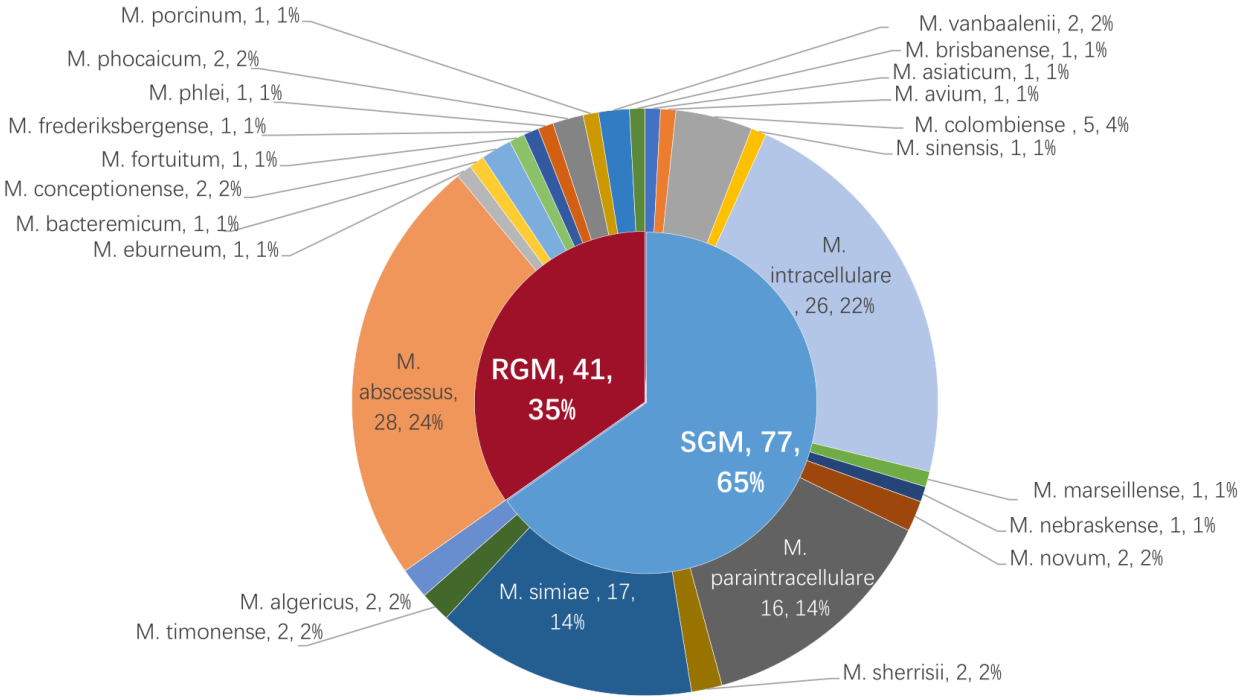

Figure 1 Distribution of non-tuberculous Mycobacteria isolated from patients with suspected pulmonary NTM in Hainan Island, China. Abbreviation： NTM：non-tuberculous Mycobacteria 、RGM: rapidly growing mycobacteria、SGM: slowly growing mycobacteria

rpoB Gene Phylogeny of NTM

A neighbor-joining (NJ) phylogenetic tree was constructed based on NTM rpoB sequences, revealing clear speciation with pathogenic and potentially pathogenic species clustering characteristics. M. fortuitum and M. abscessus grouped into a distinct cluster of rapidly growing, pathogenic mycobacteria. Strong bootstrap support validated the tree's reliability, with M. sherrisii and M. simiae closely related per hsp65 and rpoB analysis, reflecting proximity to MAC, MABC, and M. simiae complexes(Figure 2). HSP 65 Gene Phylogeny of NTM was shown in Supplemental Figure 1.

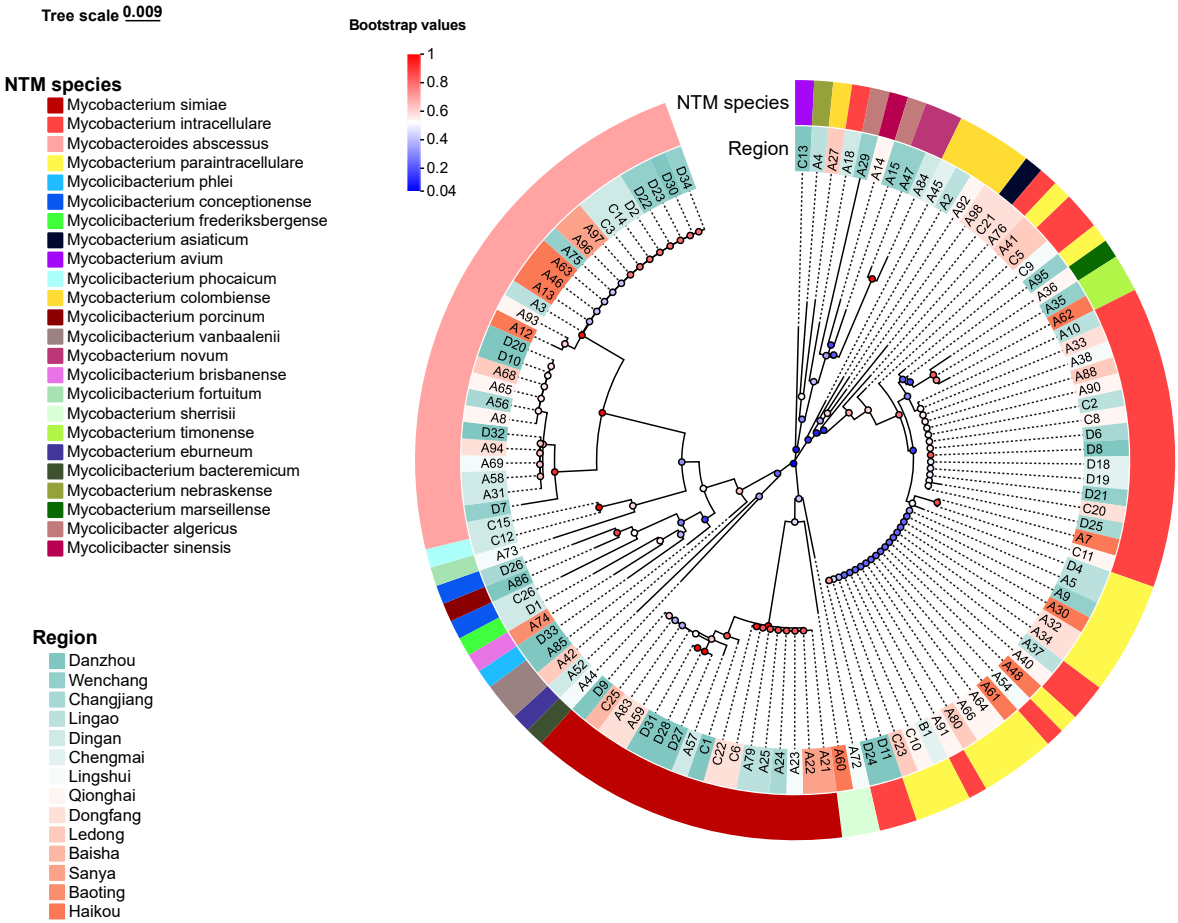

Figure 2 Phylogenetic tree of rpoB gene from 118 NTM using the neighbor-joining method with MEGA11.0 software. Tree-scale 0.007 represents the evolutionary distance unit, and Bootstrap values represents the self-expanding value, which is used to test the calculated evolutionary Tree branch confidence.

Spatial Distribution of NTM in Hainan Island

The 118 NTM isolates were distributed across 14 cities in Hainan, with distinct patterns. Northeastern regions reported higher NTM presence compared to southwestern, and SGMs outnumbered RGMs. MAC and MABC were dominant NTM groups, featuring M. intracellulare and M. abscessus respectively. SGMs were detected in all except Baoting, while RGMs were absent in Chengmai and Baisha. Key distributions included M. intracellulare in Qionghai, Lingshui, Dongfang, and Danzhou, and M. abscessus in Dingan,

Wenchang, and Haikou. Species like M. algericus, M. sinensis, M. bacteremicum, and others showed unique distributions. Refer to Figure 3 for comprehensive mapping.

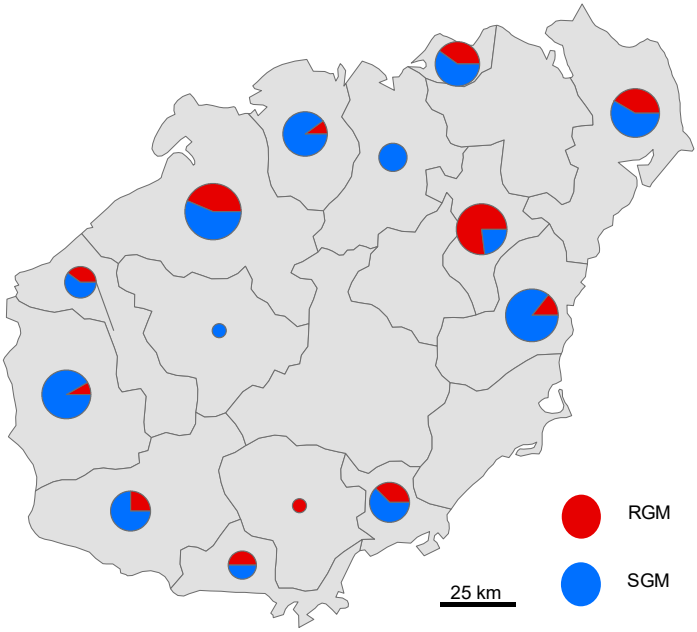

Figure 3 Distribution map of NTM in 14 cities and counties in Hainan Province (Baisha, Baoting, Changjiang, Chengmai, Danzhou, Ding 'an, Dongfang, Haikou, Ledong, Lingao, Lingshui, Qionghai, Sanya, Wenchang）

Susceptibility Test Results of M. abscessus

The MICs and breakpoints for seven antibiotics against 28 M. abscessus isolates are detailed in Table 1. The isolates displayed high levels of antibiotic resistance, with universal resistance to doxycycline but retained sensitivity to amikacin (71.4%) and linezolid (50.0%). Complete resistance and sensitivity profiles are elaborated in Table 1.

Table 1 Drug susceptibility test of M.abscessus（n=28）

Antimicrobial agent	MIC（μg/mL）			Number（%）		
	MIC range	MIC 50	MIC 90	S	I	R
Amikacin	1-64	4	64	(20/28)71.4%	(3/28)10.7%	(5/28)17.9%
Linezolid	2-32	8	32	(14/28)50.0%	(8/28)28.6%	(6/28)21.4%
Moxifloxacin	0.125-16	4	16	(8/28)28.6%	(6/28)21.4%	(14/28)50.0%
Azithromycin	1-32	16	32	(9/28)32.1%	(5/28)17.9%	(14/28)50.0%
Doxycycline	16->128	>128	>128	0	0	(28/28)100%

Abbreviation：S, susceptible; I, intermediary; R, resistant

DISCUSSION

The present study identified five major groups of NTM, including MAC, MABC, M. simiae complex, M. fortuitum complex, and other NTMs. MAC, a common causative agent of human NTM

diseases affecting various organs such as lungs, soft tissues, lymph nodes, and bone marrow, with MAC pulmonary disease being the most prevalent[22]。, was also the primary NTM pathogen detected in our study from pulmonary disease cases in Hainan, with a total of 51 isolates (comprising M. intracellulare, M. paraintracellare, M. colombiense, M. timonense, M. avium). This aligns with previous reports by Zhong [16] and Wang [22] who identified 149 and 154 MAC isolates respectively, reinforcing the widespread distribution of MAC in Hainan. MABC, another dominant NTM group, predominantly consisted of M. abscessus and its subspecies, differing slightly from Zhong and Wang's findings where M. intracellulare was more prevalent. M. simiae complex emerged as the third most frequent NTM in Hainan, further supporting its presence as a significant species in the region, contrasting its less frequent detection in other provinces[2]. The NTM profile in Hainan generally echoed that of previous studies by Zhong [16,25]、Wang[22]and Wu[26], with SGMs outnumbering RGMs. Huang[27] in Beijing also found M. intracellulare as the dominant NTM, followed by M. abscessus and M. avium, mirroring our results where M. abscessus and M. intracellulare dominate. M. abscessus is most prevalent in Hainan, potentially attributed to its hot and humid climate favoring M. abscessus survival over M. intracellulare, which thrives better in Beijing's drier climate.

Comparatively, Northern regions' literature reported M. intracellulare as the predominant species with SGMs more common than RGMs, consistent with our Hainan findings. In central China, M. intracellulare, M. avium, and M. kansasii were the main SGMs, while RGMs included M. abscessus, M. chelonae, and M. fortuitum, similar to Hainan's leading species. However, only one M. avium was isolated in Hainan, without M. kansasii, which is more frequently found in northern regions. Southwest China's NTM composition also showed SGM dominance, led by M. avium, M. intracellulare, and M. kansasii, again echoing Hainan's trend of more SGMs than RGMs.

Notably, M. kansasii and M. smegmatis were not detected in Hainan, despite their prevalence in other parts of China. For instance, M. avium was the dominant NTM in Sichuan [28], unlike in Hainan where M. abscessus dominates. Coastal southeastern regions, such as Fujian[29-30]、Zhejiang[31-34], and Jiangsu[35-36] reported higher M. avium frequencies, contrasting with Hainan's single M. avium isolate, highlighting geographic variation.

Considering Hainan and Taiwan's insular geography and similar latitudes, comparing NTM distributions reveals insights. Huang et al. [37] found M. abscessus and MAC to be the most common in Taiwan's NTM-pulmonary infections, mirroring Hainan's MAC dominance and M. abscessus prevalence. However, the contrast in NTM distribution between northern and southern Taiwan (where M. abscessus and MAC predominate respectively) differs from Hainan's northeast and southwest (both MAC-dominated). Hainan's northeastern NTM abundance contrasts with Taiwan's southern dominance, possibly influenced by its latitude, tropical/subtropical climates, and topography. Hainan's central mountainous terrain might influence coastal NTM prevalence, reflecting the island's central-high and peripheral-low geography. Overall, these findings underscore the impact of geographical and environmental factors on NTM distribution patterns across regions.

The distribution of NTM within Hainan Island exhibits a characteristic pattern, with fewer occurrences in the central mountainous regions compared to the surrounding coastal areas. Factors beyond climate, such as the mountainous terrain of the island's center, should also be considered when examining this phenomenon. Remarkable disparities exist among different regions in Hainan regarding dominant NTM species, with Danshan County dominated by M. simiae, Qionghai, Changjiang, Lingao, Chengmai, and Chengmai featuring M. intracellulare prominently, Dingan and Wenchang showing M. abscessus as the leading species, while Haikou and Ledong highlighted M. paraintracellulare. The top three municipalities in terms of NTM isolation frequency were Danshan, Qionghai, and Dingan, differing from previous research on Hainan's NTM distribution from 2015-2018 [16], which listed Haikou, Dongfang, and Wenchang as the top locations, and the incidence data from2018-2022 [26] placing Haikou, Dongfang, and Wanning as the top three. These changes in regional distribution over time likely relate to economic development, living conditions, lifestyle improvements, and public health awareness. As opportunistic pathogens, NTM typically invades organs such as the lungs, skin, lymph nodes, and bones when host immunity is compromised, with lung infections being most common[38-39]. Although NTM exhibits lower virulence and pathogenicity than MTB, their non-specific clinical symptoms and radiological similarities to MTB lead to diagnostic challenges. Their natural and

acquired drug resistance further complicates clinical management[39] underscoring the importance of NTM species identification and epidemiological investigations.

In Hainan, the hot and humid environment fosters NTM survival and dissemination, making epidemiological studies in this high NTM-endemic region crucial for local control efforts. Given M. abscessus's natural and acquired resistance to many common antibiotics, resistance testing to identify effective treatments is essential; clinical practice often employs anti-tubercular drugs[40]. Guidelines[41] recommend clarithromycin, azithromycin, amikacin, and cefoxitin for M. abscessus. Our susceptibility tests revealed rates of 71.4% for amikacin, 50.0% for linezolid, 32.1% for azithromycin, and 28.6% for moxifloxacin. The high resistance to doxycycline (100%) highlights the need for caution when using this drug clinically. M. abscessus's intrinsic resistance mechanisms involve its waxy, opaque cell wall, drug transport systems, antibiotic modification, and target gene polymorphisms[42]. Subspecies differences in resistance rates [43], particularly with higher clarithromycin resistance in M. abscessus subsp. abscessus than subsp. massiliense, indicating a need for further study to guide treatment.

Accurate and rapid NTM species identification is vital for disease management, reducing iatrogenic harm, and preventing misdiagnosis [25]. Our study used rpoB sequencing to identify 24 NTM species, complementing Hsp65 sequencing that identified 18 species. While Hsp65 is a highly expressed antigen and a powerful genotypic identifier [44], incorporating additional methods like rpoB sequencing provides more precision. Compared to Hsp65, rpoB offers several advantages [45] including sufficient information to distinguish the most recognized mycobacteria and a greater variability for species differentiation. The rpoB-based phylogenetic tree, for example, separates M. intracellulare from M. paraintracellare more accurately.

The study also unexpectedly uncovered numerous non-NTM species, including undetected MTB and species of Corynebacterium, Actinomadura, Gordonia, Tsukamurella, Streptomyces, Nocardia, all belonging to the actinobacteria phylum. Streptomyces tubbatahanensis, not previously reported in China, suggests new perspectives in streptomycete pathogenesis. Tsukamurella paurometabola, rarely pathogenic but previously isolated in Hainan [22] indicates its opportunistic nature. Nocardia species, ubiquitous yet some highly pathogenic especially in immunocompromised individuals, with Nocardia nova known as the second most prevalent [46]. Gordonia species, isolated from various environments, can cause opportunistic infections in immunocompromised hosts. Corynebacterium tuberculostearicum is omnipresent on human skin, yet its health impacts require further investigation. [47]

As an expansion and deepening of previous work in Hainan, this study leverages the province-wide TB control network for multi-dimensional NTM testing, achieving notable progress in geographic coverage and species diversity. By involving hospitals at all levels and standardizing specimen submission, a comprehensive and representative NTM repository was established, surpassing single-center biases and enhancing sample representativeness. This collaborative, cross-regional approach lays a firm foundation for elucidating regional NTM patterns and potential epidemiological rules. It also underscores the direct influence of public health policies on monitoring activities, emphasizing the need for flexibility and adaptability in future surveillance systems to accurately track NTM epidemiology. Collectively, this study enriches Hainan's NTM epidemiological data, informing targeted control strategies, and highlighting the value of interdisciplinary and inter-institutional cooperation in addressing NTM public health challenges.

Conclusion

This study has provided an in-depth exploration into the ecological distribution and drug resistance patterns of NTM in Hainan Island, highlighting M. abscessus and MAC as principal pathogens driving NTM infections in the region, predominantly in Danshan, Qionghai, Dingan, Dongfang, and Wenchang. Novel findings include the documentation of previously unreported NTM species in Hainan, specifically M. novum, M. algericus, M. conceptionense, M. vanbaalenii, M. eburneum, M. nebraskense, M. sinensis, M. bacteremicum, M. frederiksbergense, M. phlei. Moreover, the study underscores the therapeutic potential of amikacin and linezolid against M. abscessus.

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Author Contribution

XX, HW, and TX conducted the experiments; JH was responsible for sample collection; XX, JL, and JH performed the data analysis; XX and LZ wrote and revised the manuscript; LZ designed the study.

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