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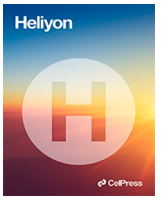

Review article

Epidemiology and laboratory detection of non-tuberculous mycobacteria

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ABSTRACT

The global incidence of non-tuberculous mycobacteria (NTM) infections is on the rise. This study systematically searched several databases, including PubMed, Web of Science, Google Scholar, and two Chinese libraries (Chinese National Knowledge Infrastructure and Wanfang) to identify relevant published between 2013 and 2023 related to the isolation of NTM in clinical specimens from various countries and provinces of China. Furthermore, a comprehensive literature review was conducted in PubMed and Google Scholar to identify randomized clinical trials, meta-analyses, systematic reviews, and observational studies that evaluated the diagnostic accuracy and impact of laboratory detection methods on clinical outcomes. This review presented the most recent epidemiological data and species distributions of NTM isolates in several countries and provinces of China. Moreover, it provided insights into laboratory bacteriological detection, including the identified strains, advantages and disadvantages, recent advancements, and the commercial *Mycobacterium* identification kits available for clinical use. This review aimed to aid healthcare workers in understanding this aspect, enhance the standards of clinical diagnosis and treatment, and enlighten them on the existing gaps and future research priorities.

1. Introduction

Non-tuberculous mycobacteria (NTM), also known as atypical mycobacteria, refer to mycobacterial species other than those belonging to the *Mycobacterium tuberculosis* complex (MTBC, including *M. africanum*, *M. bovis*, *M. caprae*, *M. canetti*, *M. microti*, and *M. pinnipedii*) and *M. leprae* [1,2]. NTM are environmental and opportunistic pathogens that are found primarily in water and soil, predominantly infecting individuals with risk factors [3,4]. With over 200 species/subspecies identified (http://www.bacterio.net/mycobacterium.html), approximately 60 are responsible for the disease [5]. The incidence of NTM infections has been steadily rising, posing significant public health challenge and threatening human health [6], probably owing to the increased number of patients with underlying respiratory diseases or immunosuppression coupled with advancements in diagnostic techniques [7]. NTM infections can cause four different clinical symptoms: chronic lung disease, lymphadenitis, skin disease, and disseminated disease, with non-tuberculous mycobacterial pulmonary disease (NTM-PD) being the most prevalent. The diagnosis of NTM-PD necessitates fulfillment of all clinical, radiographic, and microbiological criteria [8]. Moreover, extrapulmonary mycobacterial infections can affect a plethora of anatomical sites including the brain, eye, mouth, tongue, lymph nodes, bones, muscles, skin, pleura, pericardium, gastrointestinal tract, peritoneum, and genitourinary system [9]. The differential diagnosis between *M. tuberculosis* (MTB) and NTM is

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intricate due to their similar smear acid-fast staining positivity, often leading to diagnostic confusion [10]. In many epidemiological investigations, NTM has demonstrated resistance to anti-tuberculosis (TB) drugs, and antimicrobial susceptibilities vary among species, further complicating clinical management [11,12]. Therefore, improving understanding of NTM and accurately identifying the species is imperative for prompt and precise treatment of NTM-related diseases. This review presented the most recent epidemiological data and species distributions of NTM isolates in various countries and provinces of China. Moreover, it elucidated advancements in laboratory bacteriological detection and catalogs commercial *Mycobacterium* identification kits. This review aimed to aid healthcare workers in understanding the epidemiology and laboratory detection of NTM, enhance the standards of clinical diagnosis and treatment, and enlighten them of the existing gaps and future research priorities.

2. Methods

Several databases, such as PubMed, Web of Science, Google Scholar, and two Chinese libraries (Chinese National Knowledge Infrastructure and Wanfang), were searched for studies published between 2013 and 2023 that investigated the isolation of NTM in clinical specimens from various countries and provinces of China, especially in high-TB burden and developing countries. Additionally, a comprehensive literature search was conducted in PubMed and Google Scholar to identify randomized clinical trials, meta-analyses, systematic reviews, and observational studies to evaluate the diagnostic accuracy and impact of laboratory detection methods on clinical outcomes. The search terms used were “non-tuberculous mycobacteria,” “NTM,” “China,” “India,” “Africa,” “epidemiology,” “prevalence,” “isolation,” “molecular diagnostics,” “multiplex polymerase chain reaction (PCR),” “real-time PCR,” and “detection.” Epidemiological studies were excluded from the analysis if they only focused on TB; involved specific patient groups, such as patients with HIV-positive test results; and involved animal/nonhuman studies. In cases where multiple articles used epidemiological data from the same geographic area, preference was given to the most recent, comprehensive, and representative study.

3. Epidemiology

The incidence and prevalence of NTM are increasing at the national level in many countries [7,13,14]. In a study spanning 30 countries across 6 continents, the most common NTM species isolated from pulmonary specimens were the *M. avium* complex (MAC, including *M. avium* and *M. intracellulare*), followed by *M. gordonae*, *M. xenopi*, *M. fortuitum*, *M. abscessus* complex (MABC), and *M. kansasii* [15]. The common NTM species found in extrapulmonary specimens included MABC, *M. fortuitum*, and *M. scrofulaceum* [9]. The distribution of NTM species isolated from clinical samples exhibits geographic specificity, and the species of NTM can vary within the same country [16]. Upon examining Table 1, in China, the most commonly isolated NTM species were *M. intracellulare*, *M. abscessus*, and *M. kansasii*. In India, the prevalent NTM species included *M. abscessus*, *M. fortuitum*, and *M. porcinum*. Meanwhile, in Iran, the frequently isolated NTM species were *M. simiae*, *M. fortuitum*, *M. kansasii*, and *M. marinum*.

Table 1
Three most prevalent non-tuberculous mycobacteria species found in various clinical specimens in several countries.

Region	Study period	NTM isolates (n)	Ranking of most commonly isolated NTM species (% of isolates)			Ref.
			1st	2nd	3rd	
Singapore	2012–2016	2026	*M. abscessus* complex (49.9 %)	*M. fortuitum* group (17.0 %)	*M. avium* complex (15.4 %)	[17]
Korea	2016–2020	2521	*M. intracellulare* (45.8 %)	*M. avium* (21.4 %)	*M. abscessus* (7.9 %)	[18]
China	2017–2019	513	*M. intracellulare* (46.5 %)	*M. abscessus* (28.6 %)	*M. kansasii* (17.4 %)	[19]
Japan	2012–2013	26,059	*M. avium* (61.8 %)	*M. intracellulare* (31.1 %)	*M. kansasii* (2.1 %)	[20]
South India	2018–2020	45	*M. intracellulare* (25.5 %)	*M. abscessus* (15.3 %)	*M. scrofulaceum* (12.2 %)	[21]
North India	2014–2016	42	*M. abscessus* (35.7 %)	*M. intracellulare* (28.6 %)	*M. simiae* (11.9 %)	[22]
India	2015–2020	69	*M. abscessus* (33.3 %)	*M. fortuitum* (24.6 %)	*M. porcinum* (5.8 %)	[23]
Cambodia	2012–2014	123	*M. fortuitum* (22.3 %)	*M. intracellulare* (18.7 %)	*M. abscessus* (11.3 %)	[24]
Pakistan	2016–2019	191	*M. avium* complex (61 %)	*M. abscessus* (24 %)	*M. fortuitum* (5.5 %), *M. kansasii* (5.5 %)	[25]
Saudi Arabia	2006–2012	380	*M. avium* complex (35 %)	*M. fortuitum* (24 %)	*M. abscessus* complex (17 %)	[26]
Iran	2020–2021	62	*M. simiae* (29.0 %)	*M. fortuitum* (21.1 %)	*M. kansasii* (14.5 %), *M. marinum* (14.5 %)	[27]
Indonesia	2020–2021	94	*M. fortuitum* (51 %)	*M. abscessus* (38.3 %)	*M. intracellulare* (3.1 %)	[28]
American	2019–2020	231	*M. avium* (41.6 %)	*M. intracellulare* (18.6 %)	*M. abscessus* complex (8.7 %)	[29]
Brazil	2003–2013	100	*M. avium* complex (35 %)	*M. kansasii* (17 %)	*M. abscessus* (12 %)	[30]
UK	2007–2014	853	*M. intracellulare* (31.3 %)	*M. avium* (21.2 %)	*M. gordonae* (15.2 %)	[31]
French	2002–2013	170	*M. avium* (31.8 %)	*M. intracellulare* (20 %)	*M. marinum* (13.5 %)	[32]
Poland	2013–2022	395	*M. kansasii* (34 %)	*M. avium* (30 %)	*M. gordonae* (10 %)	[33]
Belgium	2010–2017	384	*M. avium* (25 %)	*M. intracellulare* (16.7 %)	*M. gordonae* (14.6 %)	[34]
Tunisia	2002–2016	27	*M. kansasii* (23.3 %)	*M. fortuitum* (16.6 %), *M. novocastrense* (16.6 %)	*M. chelonae* (10.0 %)	[35]
South Africa	2010	133	*M. intracellulare* (45.9 %)	*M. avium* (11.3 %)	*M. gordonae* (6 %)	[36]
Nigeria	2010–2011	69	*M. intracellulare* (30.4 %)	*M. abscessus* (11.6 %)	*M. fortuitum* (5.8 %)	[37]

MAC was the dominant species in most location, as shown in Table 1. However, the prevalence of MAC ranged from 0 % in Iran [27] to 93.3 % in Japan [20]. Upon examining Table 1 and it was evident that *M. abscessus* was the most common isolated NTM species in Singapore and India, *M. kansasii* in Poland and Tunisia, *M. simiae* in Iran, *M. fortuitum* in Indonesia.

In the United States, MAC was more common in the South, while MABC and *M. chelonae* were more common in the West [38]. In Japan, the isolation rate of MABC was higher in the northern region [39]. In India, *M. intracellulare* was the most common NTM species in South India, and *M. abscessus* in North India (Table 1). Furthermore, differences within China were also noted, as outlined in Table 2.

According to Table 2 and Fig. 1, notable differences in NTM isolation rates were observed among provinces, especially between the southern and northern regions. Coastal areas such as Taiwan, Guangdong, Zhejiang, and Jiangsu provinces exhibited the highest NTM isolation rates. Moreover, the isolation rate of NTM in coastal areas was higher than that in inland areas. Overall, *M. intracellulare* and *M. avium/intracellulare* emerged as the most common NTM species in China, with isolation rates reaching as high as 70 % in some provinces. Conversely, *M. chelonae/abscessus* was more prevalent in the south than in the north, while *M. kansasii* exhibited greater prevalence in the north.

NTM can be detected not only in respiratory tract specimens but also in extrapulmonary specimens such as ascites, hydrothorax, urine, cerebrospinal fluid, skin/soft tissue, and pericardial effusion [9]. This indicates that NTM extrapulmonary infections include meningitis, pleurisy, pericarditis, and skin diseases. During the epidemiological investigation, sputum was used as the primary NTM sample. Colonized NTM may be present in sputum, such as *M. gordonae.* Therefore, the calculation of isolation rates often includes contaminating bacteria.

The average age of patients with NTM infections reportedly falls between 50 and 70 years [64]. Table 2 demonstrates that individuals aged >60 years constitute the largest age group among patients with NTM-PD. Hypoimmunity serves as a risk factor for NTM, and certain underlying diseases, such as bronchiectasis, chronic obstructive pulmonary disease (COPD), acquired immunodeficiency syndrome (AIDS), and diabetes, are observed in a proportion of patients listed in Table 2. The clinical symptoms of patients with NTM-PD include cough, expectoration, hemoptysis, and extrapulmonary NTM infections, characterized by myalgia, ulcers, diarrhea, necrotic abscesses, and edematous lesions [9,10]. These symptoms are nonspecific and resemble those of TB [10].

Understanding the epidemiology of NTM aids in the clinical diagnosis of NTM and its subspecies. However, recent data shows compositional differences compared with those reported in previous years [65]. The epidemiological patterns may vary depending on the study period. Therefore, we conducted searches to obtain the most recent data available from various countries and provinces.

4. Detection methods

The detection methods include smears, cultures and molecular methods. Molecular diagnostic techniques include polymerase chain reaction (PCR)-based molecular biology, chips, probes and sequencing technologies. Compared with traditional phenotypic and biochemical methods, molecular diagnostic techniques exhibit superior sensitivity, accuracy, and efficiency. Each method has its advantages and disadvantages and is constantly evolving. The following section provides an overview of the identified strains, advantages and disadvantages, the recent advancements, and the commercial *Mycobacterium* identification kits (Table 3) available for clinical use. The discussion of these aspects offers a more comprehensive understanding of the NTM identification technology used, facilitates the application of appropriate identification methods in clinical settings, and inspires the development of a more rapid, accurate, and efficient identification method that can be applied in clinical and laboratory settings in the future.

4.1. Acid-fast bacilli smear

Mycobacteria contain mycolic acids in their cell walls and acid-fast bacilli (AFB) can be visualized under a light microscope as reddish-pink bacilli [81]. Although, smears can identify mycobacteria, they cannot distinguish between MTB and NTM. AFB smears offer rapid and cost-effective identification but exhibit poor specificity [81]. A previous study demonstrated the sensitivities of AFB smears for MTB and NTM were 51.6 % and 53.1 %, respectively [82].

4.2. NTM culture

Various methods are available for pathogen culture, with common clinical approaches including the BACTEC MGIT 960 method and the Lowenstein-Jensen (L-J) culture medium, which can be used for preliminary identification. Mature colonies of rapidly growing mycobacteria (RGM) typically appear on solid media within 7 days, while those of slowly growing mycobacteria (SGM) may take longer than 7 days to manifest [83]. MGIT instruments detect growth by reading the fluorescent indicator in MGIT tubes, which fluoresce as bacterial respiration occurs and O_2 is depleted. The culture time of liquid medium was shorter than that in the solid medium; however, the liquid medium could not distinguish between mixed infections [81]. Some studies involved incubating both solid and liquid cultures for 6–8 weeks after inoculation [84]. In addition, specimens should be centrifuged, digested, or decontaminated prior to inoculation into the medium. This process serves to minimize the contaminating bacteria and reduce NTM recovery [85]. Ditommaso et al. reported a new culture method utilizing an NTM Elite agar, which does not require a decontamination step. This novel method successfully detected NTM in 27.7 % (30/108) of water samples analyzed [86].

4.3. Multiplex PCR

Multiplex PCR (mPCR) involves adding two or more pairs of primers to the same mPCR system to amplify multiple nucleic acid

Table 2
Overview of studies on non-tuberculous mycobacteria in China.

Province	Study period	NTM isolation rate (%)	Predominant species	Age ratio of NTM-PD patients	Complications of NTM-PD patients	Clinical symptoms of NTM-PD patients	Ref.
Beijing (north region)	2019	6.5	*M. intracellulare* (24.69 %) *M. abscessus* (24.69 %) *M. kansasii* (19.75 %)	0–40:32.10 % 40–60:13.58 % ≥60:54.32 %	NA	NA	[40]
Tianjin (north region)	2018 ~ 2019	NA	*M. intracellulare* (29.08 %) *M. chelonae/abscessus* (26.60 %) *M. kansasii* (23.41 %)	15–40:26.60 % 40–60:29.79 % ≥60:43.62 %	NA	NA	[41]
Jilin (north region)	2017 ~ 2018	2.68	*M. intracellulare* (40.59 %) *M. kansasii* (26.73 %) *M. avium* (4.95 %)	≤40:5.94 % 40–60:21.78 % ≥60:72.28 %	NA	NA	[42]
Liaoning (north region)	2016 ~ 2021	3.95	*M. intracellulare* (55.91 %) *M. abscessus* (25.20 %) *M. kansasii* (8.66 %)	NA	TB:4.85 % COPD:4.85 % Diabetes:10.68 % Cancer:5.83 %	NA	[43]
Shandong (north region)	2015 ~ 2019	11.6	*M. intracellulare* (69.77 %) *M. kansasii* (12.71 %) *M. abscessus* (9.89 %)	< 45:15.25 % 45–60:35.88 % 60–90:48.87 %	NA	NA	[44]
Henan (north region)	2018 ~ 2020	3.93	*M. intracellulare* (74.93 %) *M. kansasii* (9.37 %) *M. abscessus* (5.79 %)	<50:22.87 % ≥50:77.13 %	NA	NA	[45]
Shaanxi (north region)	2019 ~ 2021	3.5	*M. intracellulare* (36.7 %) *M. chelonae/abscessus* (26.6 %) *M. kansasii* (25.0 %)	14–39:32.58 % 40–65:44.94 % >65:19.10 %	NA	Cough/ Expectoration:82.56 % Hemoptysis:27.91 % Fever:36.05 % Asthma:48.84 %	[46]
Gansu (north region)	2012 ~ 2014	4.91	*M. intracellulare* (72.09 %) *M. kansasii* (6.97 %)	NA	NA	NA	[47]
Xinjiang (north region)	2009 ~ 2011	4.98	*M. avium/ intracellulare* (43.72 %) *M. chelonae/abscessus* (13.12 %)	NA	NA	NA	[48]
Chongqing (south region)	2016 ~ 2017	NA	*M. abscessus* (38.75 %) *M. intracellulare* (31.25 %) *M. fortuitum* (17.50 %)	< 30:23.18 % 30–40:10.53 % 40–50:22.11 % 50–60:15.79 % 60–80:25.28 %	NA	NA	[49]
Sichuan (south region)	2016 ~ 2021	2.75	*M. chelonae/abscessus* (31.06 %) *M. avium* (26.52 %) *M. intracellulare* (26.52 %)	< 20:3.4 % 20–40:27.3 % 40–60:40.2 % ≥60:29.3 %	AIDS:14.8 % Bronchiectasis:18.2 % COPD:3.8 % Diabetes:6.1 %	NA	[50]
Hubei (south region)	2016 ~ 2020	6.66	*M. intracellulare* (37.88 %) *M. abscessus* (13.63 %) *M. gordonae* (5.01 %)	<35:10.22 % 35–54:23.65 % 55–64:24.05 % >64:42.09 %	Bronchiectasis:11.22 % COPD:4.21 % Cancer:1.20 % AIDS:0.40 %	NA	[51]
Anhui (south region)	2021 ~ 2022	8.2	*M. intracellulare* (74.5 %) *M. abscessus* (13.6 %) *M. kansasii* (7.6 %)	20–39:5.5 % 40–59:36.7 % ≥60:57.8 %	Bronchiectasis:33.76 % COPD:13.92 % AIDS: 13.92 % Cancer:2.53 % Diabetes:5.06 %	Cough/ Expectoration:71.73 % Hemoptysis:21.94 % Fever:10.97 % Asthma:16.88 %	[52]
Jiangsu (south region)	2017 ~ 2020	22.3	*M. intracellulare* (60.7 %) *M. chelonae/abscessus* (16.1 %) *M. avium* (10.7 %)	NA	NA	NA	[53]

(*continued on next page*)

Table 2 (*continued*)

Province	Study period	NTM isolation rate (%)	Predominant species	Age ratio of NTM-PD patients	Complications of NTM-PD patients	Clinical symptoms of NTM-PD patients	Ref.
Shanghai (south region)	2017 ~ 2018	NA	*M. intracellulare* (54.4 %) *M. abscessus* (22.2 %) *M. kansasii* (7.8 %)	NA	NA	Cough:83.58 % Expectoration:80.60 % Hemoptysis:25.37 % Fever:47.76 % Dyspnea:22.39 %	[54]
Zhejiang (south region)	2009 ~ 2014	25.8	*M. intracellulare* (60.74 %) *M. kansasii* (13.33 %) *M. avium* (4.44 %)	15–35:16.1 % 35–45:13.6 % 45–55:23.5 % 55–65:19.8 % ≥65:31.5 %	NA	NA	[55]
Fujian (south region)	2016 ~ 2019	6.35	*M. intracellulare* (65.73 %) *M. chelonae/abscessus* (18.78 %) *M. kansasii* (4.23 %)	NA	NA	NA	[56]
Jiangxi (south region)	2017 ~ 2020	NA	*M. avium* (50 %) *M. intracellulare* (21.15 %), *M. chelonae/abscessus* (14.23 %)	NA	Bronchiectasis:21.15 % COPD:19.23 % AIDS: 3.85 % Cancer:28.85 % Diabetes:6.73 %	Cough:53.85 % Expectoration:40.38 % Hemoptysis:18.27 % Fever:32.69 %	[57]
Hunan (south region)	2019 ~ 2020	8.06	*M. abscessus* (38.29 %), *M. intracellulare* (33.14 %) *M. avium* (12.19 %)	NA	NA	NA	[58]
Guizhou (south region)	2012 ~ 2013	2.64	*M. intracellulare* (32.26 %), *M. chelonae/abscessus* (29.03 %) *M. avium* (25.81 %)	NA	NA	NA	[59]
Yunnan (south region)	2013 ~ 2015	1.61	*M. intracellulare* (44.83 %) *M. abscessus* (27.59 %) *M. avium* (11.49 %)	< 20:1.15 % 20–39:18.39 % ≥40:80.46 %	NA	NA	[60]
Guangdong (south region)	2018 ~ 2019	30.3	*M. chelonae/abscessus* (41.2 %) *M. avium/ intracellulare* (40.5 %)	< 30: 19.35 % 30–40:10.99 % 40–50:14.09 % 50–60:21.98 % 60–70:24.43 % > 70:12.09 %	NA	NA	[61]
Hainan (south region)	2016 ~ 2021	13.4	*M. intracellulare* (39.49 %) *M. chelonae/abscessus* (32.91 %) *M. avium* (7.59 %)	≤40:8.9 % > 40:91.1 %	NA	NA	[62]
Taiwan (south region)	2000 ~ 2012	56.9	*M. avium/ intracellulare* (41.51 %) *M. abscessus* (30.81 %) *M. fortuitum* (13.36 %)	NA	NA	NA	[63]

fragments simultaneously. Singh et al. investigated a one-step mPCR method for detecting *Mycobacterium*, including MAC, *M. kansaii*, *M. abscessus* based on 16SrRNA, and *ITS* [87]. Recently, Kim et al. developed a two-step mPCR method to identify the causative agents of major mycobacterial infections. MTB, *M. intracellulare*, and *M. avium* were distinguished in the first mPCR step, while *M. kansasii*, *M. fortuitum*, *M. abscessus*, and *M. massiliense* were distinguished in the second step [88]. Shin et al. conducted a study employing a five-target mPCR to effectively distinguish *M. avium* complex species and subspecies. This assay relied on the amplification of specific genetic markers such as 16SrRNA, *IS900*, *IS901*, *IS1311*, and DT1 [89]. Chae et al. developed a one-step mPCR assay for the differential detection of *Mycobacterium* species, MTBC, MTB, the MTB Beijing family, *M. avium*, *M. intracellulare*, *M. abscessus*, *M. massiliense*, and *M. kansaii*. This assay relied on the amplification of specific genetic markers such as 16SrRNA, *rv0577*, RD9, *mtbk_20680*, *IS1311*, DT1, *mass_3210*, and *mkan_rs12360* [90]. Multiplex PCR is a simple, rapid, convenient, and reliable technique for identifying NTM at a relatively low cost. It has the advantage of being able to detect mixed infection cases of MTB and NTM. Many commercial kits utilize multiplex PCR amplification, with the PCR product combined with a probe, thereby significantly reducing gene fragment amplification

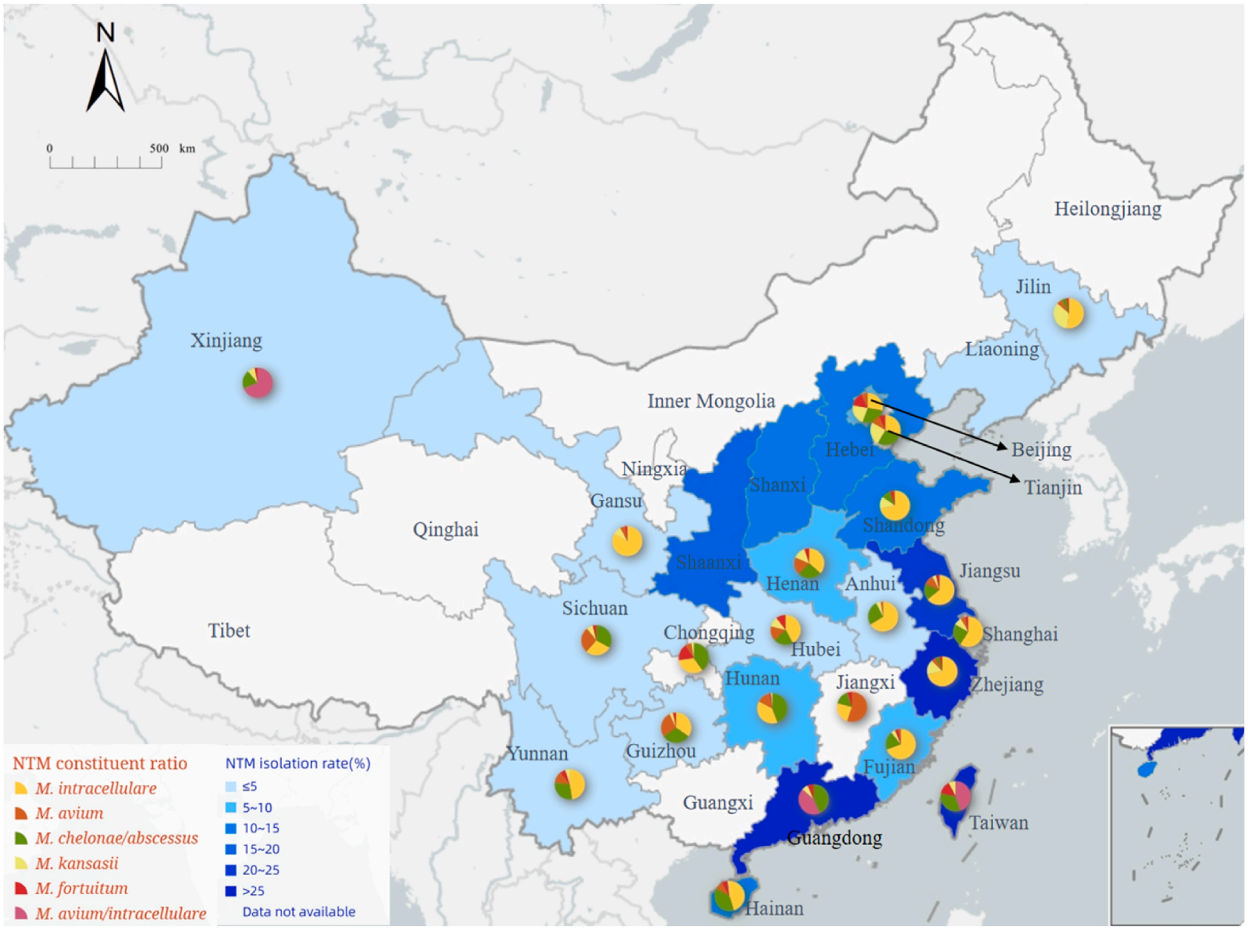

Fig. 1. Isolation rate and composition of NTM in parts of China.

time. This method can be used to identify the most common NTM in pulmonary specimens. However, multiplex PCR does not simply mix multiple pairs of specific primers into a single reaction system. Owing to the incompatibility of amplification conditions among multiple targets, the formation of dimers between primers, and mutual inhibition, the practical application of multiplex PCR is subject to many limitations.

4.4. Real-time PCR

Real-time PCR was employed to monitor the entire PCR amplification process using a fluorescence signal, enabling real-time observation of the PCR dynamics. In a study by Franciele Costa Leite Morais, the utilization of quantitative PCR to detect NTM species using 16S rRNA as a target exhibited the potential to swiftly and effectively differentiate NTMs from *M. tuberculosis* [91]. An Australian laboratory developed a real-time PCR method targeting *IS2404* for the direct detection of *M. ulcerans* [92]. Kim et al. developed a multiplex real-time PCR assay capable of directly detecting 20 mycobacterial species in clinical specimens, with a sensitivity and a specificity for detecting NTM isolates of 53.3 % and 99.9 %, respectively [93]. Numerous commercial kits based on real-time PCR, such as GENEDIA® MTB/NTM Detection Kit, AdvanSure TB/NTM Detection Kit, and Real-Q MTB/NTM Detection Kit, are used to distinguish between MTB and NTM [66]. In addition, Real Myco-ID® (Optipharm) was developed for the rapid and accurate detection and identification of 17 *Mycobacterium* species (Table 3) [67]. Real-time PCR is a highly sensitive, specific, and rapid molecular assay. A closed PCR system reduces the risk of contamination and the probability of false-positive results [66]. However, the presence of PCR inhibitors in samples may reduce the sensitivity of real-time PCR.

4.5. Melting curve analysis

The principle of melting curve analysis involves heating the PCR products to generate a fluorescence signal curve, from which the melting curve is obtained. The melting temperature corresponding to the peak value of the melting curve is the melting temperature value of double-stranded DNA. This method is primarily based on real-time PCR. Keerthirathne et al. conducted a real-time mPCR

Table 3
Examples of commercial *Mycobacterium* identification kit.

Technique	Product	Target gene	Manufacturer	Pathogen(s) detected	Detection time	Detection type	Sensitivity, specificity	Concordance	Ref.
Real-time PCR	AdvanSure™ TB/ NTM Detection Kit	MTBC: *IS6110* NTM: *ITS*	LG Life Sciences	MTB and NTM	140 min	cultures	NA	100 %	[66]
	GENEDIA® MTB/ NTM Detection Kit	MTBC: *IS6110* NTM: *ITS* and *rpoB*	Green Cross Medical Science Corp	MTB and NTM	140 min	cultures	NA	98.8 %	[66]
	Real-Q MTB & NTM kit	MTBC: *IS6110* NTM: *ITS*	BioSewoom, Seoul	MTB and NTM	135 min	cultures	NA	98.8 %	[66]
	Real Myco-ID®	*rpoB*	Optipharm	MTBC, *M. avium/intracellulare*, *M. abscessus/massiliense*, *M. chelonae*, *M. fortuitum* complex, *M. ulcerans/marinum*, *M. kansasii/gastri*, *M. celatum*, *M. terrae/nonchromogenicum*, *M. gordonae/szulgai*, and *M. mucogenicum*	4 h	cultures	NA	100 %	[67]
Melting curve analysis	LightCycler® *Mycobacterium* Detection Kit	16SrRNA	Roche	*M. tuberculosis*, *M. avium* and *M. kansasii*	90 min	clinical samples	NA	NA	[68]
	MeltPro Myco assay (MMCA®)	*ITS*	Zeesan Biotech	MTBC, *M. avium*, *M. intracellulare*, *M. absessus*, *M. chelonae*, *M. kansasii*, *M. fortuitum*, *M. gordonae*, *M. scrofulaceum/intracellulare*, *M. malmoense*, *M. interjectum*, *M. szulgai*, *M. marinum/ ulcerans*, and *M. xenopi*, *M. simiae*, *M. terrae*, *M. nonchromogenicum*, *M. malmoense*, *M. lentiflavum*, *M. bovis*/BCG	4 h	clinical samples	NA	NA	[69]
Biochip assay	Mycobacterial Species Identification Kit	16SrRNA	CapitalBio	*M. tuberculosis*, *M. intracellulare*, *M. chelonae/abscessus*, *M. kansasii*, *M. avium*, *M. gordonae*, *M. fortuitum*, *M. scrofulaceum*, *M. gilvum*, *M. terrae*, *M. phlei*, *M. nonchromogenicum*, *M. marinum/ulcerans*, *M. aurum*, *M. szulgai/malmoense*, *M. xenopi*, and *M. smegmatis*	6 h	clinical samples	82 %, 99.6 %	NA	[70]
Line Probe assay	INNO-LiPA Mycobacteria V2 assay	*ITS*	Innogenetics	MTBC, *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. kansasii*, *M. xenopi*, *M. chelonae*, *M. gordonae*, *M. fortuitum* complex, *M. malmoense*, *M. genavense*, *M. simiae*, *M. smegmatis*, *M. haemophilum*, *M. marinum/ulcerans* and *M. celatum*	2 h	cultures	100 %, 94.4 %	NA	[71]
	GenoType Mycobacterium CM/ AS assay	23SrRNA	Hain Lifescience GmbH	CM: MTBC, *M. avium*, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. scrofulaceum*, *M. interjectum*, *M. kansasii*, *M. malmoense*, *M. marinum/ ulcerans*, *M. peregrinum*, *M. xenopi* AS: *M. simiae*, *M. mucogrnicum*, *M. goodii*, *M. celatum*, *M. smegmatis*, *M. genavense*, *M. lentiflavum*, *M. szulgai*, *M. phlei*, *M. heckeshornense*, *M. haemophilum*, *M. kansasii*, *M. ulcerans*, *M. gastri*, *M. asiaticum*, *M. shimoidei*	CM:6 h AS:5 h	cultures	93.7 ~ 100 %, 100 %.	NA	[72, 73]
	GenoType Mycobacterium NTM-DR assay	23SrRNA	Hain Lifescience GmbH	*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp.*massiliense*, *M. abscessus* subsp. *Bolletii*, *M. chelonae*, *M. avium*, *M. intercelleulare*, *M. chimaera*	5 h	cultures	100 %, 100 %	100 %	[74]
	Speed-oligo Mycobacteria assay	16SrRNA and *ITS*	Vircell	MTBC, *M. abscessus*, *M. marinum/ulcerans* complex, *M. kansasii*, *M. xenopi*, *M. intracellulare*,	2 h30 min	cultures	NA	93.5 %	[75]

(*continued on next page*)

Table 3 (*continued*)

Technique	Product	Target gene	Manufacturer	Pathogen(s) detected	Detection time	Detection type	Sensitivity, specificity	Concordance	Ref.
				M. avium, *M. scrofulaceum*, *M. malmoense*, *M. gordonae*, *M. interjectum*, *M. chelonae*, *M. fortuitum* and *M. peregrinum*					
	AdvanSure Mycobacteria GenoBlot assay	*ITS*	LG Life Sciences Inc.	MTBC, *M. avium*, *M. intracellulare*, *M. scroflaceum*, *M. abscessus*, *M. chelonae*, *M. kansasii*, *M. szulgai*, *M. gordonae*, *M. celatum*, *M. marinum/ulcerans*, *M. simiae*, *M. lentiflavum/genavense*, *M. xenopi*, *M. smegmatis*, *M. malmoense*, *M. gastri*, *M. flavescennse*, *M. vaccae*, *M. fortuitum* complex	NA	clinical samples	100 %, 98.5 %	89.7 %	[76]
PCR-reverse blot hybridization assay	MolecuTech REBA Myco-ID®	*rpoB*	YD Diagnostics	MTBC, *M. avium*, *M. intracellulare*, *M. scroflaceum*, *M. abscessus*, *M. massilience*, *M. chelonae*, *M. fortuitum* complex, *M. marinum/ulcerans*, *M. kansasii*, *M. genavense/simiae*, *M. celatum*, *M. terrae/nonchromogenicum*, *M. gordonae*, *M. szulgai*, *M. mucogenicum*, and *M. aubagnense*	NA	cultures	NA	94.3 %	[67, 77]
	PCR-REBA Myco-ID	16S rRNA	Yaneng BioSciences	MTB, *M. smegmatis*, *M. intracellulare*, *M. kansasii*, *M. chelonae*, *M. marinum*, *M. fortuitum*, *M. terrae*, *M. nonchromogenicum*, *M. avium*, *M. scrofulaceum*, *M. abscessus*, *M. xenopi*, *M. gilvum*, *M. phlei*, *M. triviale*, *M. gordonae*, *M. gastri*, *M. vaccae*, *M. szulgai*, *M. diernhoferi*, *M. simiae*	4 h	clinical samples	99 %, 100 %	NA	[78]
	Myco-Panel	NA	Medical & Biological Laboratories Co., Ltd.	*M. tuberculosis* var. *tuberculosis*, *M. tuberculosis* var. *BCG*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, *M. abscessus* subsp. *massiliense*, *M. chelonae*, *M. gordonae*, *M. xenopi*, *M. fortuitum*, *M. szulgai*, *M. marinum/ulcerans*, *M. scrofulaceum*, *M. simiae*, *M. asiaticum*, *M. lentiflavum*, *M. nonchromogenicum*, *M. shimoidei*, *M. terrae*, *M. shinjukuense*, *M. mucogenicum*, *M. peregrinum*, *M. triviale*, *M. malmoense*, *M. chimaera*, *and M. heckeshornense*	NA	clinical samples and cultures	NA	83.1 %	[79]
DNA-DNA hybridization test	Genotype CM Direct® (GTCMD)	NA	Bruker, Billerica	MTBC, *M. avium*, *M. intracellulare*, *M. absessus* complex, *M. chelonae*, *M. kansasii, M. fortuitum* group, *M. gordonae*, *M. scrofulaceum/intracellulare*, *M. malmoense*, *M. interjectum*, *M. szulgai*, *M. marinum/ulcerans*, and *M. xenopi*	NA	clinical samples	89.2 %, 81.5 %	NA	[80]
	VisionArray MyCo® (VAM) 2.0	NA	ZytoVision,	MTBC, *M. avium* complex, *M. chimaera*, *M. absessus* complex, *M. chelonae*, *M. kansasii*, *M. fortuitum*, *M. gordonae*, *M. malmoense*, *M. scrofulaceum/parascrofulaceum*, *M. haemophilum, M. genavense*, *M. marinum/ulcerans*, *M. smegmatis*, *M. simiae*, and *M. xenopi*	NA	clinical samples	73.0 %, 96.3 %	NA	[80]

assay in two separate reactions. Reaction I utilized primers specific for MTB and MAC, while reaction II employed primers specific for the *M. chelonae/abscessus* group and *M. fortuitum*, aiding in the identification of RGM and SGM, respectively, by obtaining the melting curve [94]. The LightCycler® *Mycobacterium* Detection Kit can detect *M. tuberculosis*, *M. avium*, and *M. kansasii* by performing a melting curve analysis on the LightCycler® 2.0 (Roche Diagnostics, Germany) [68]. Recently, a system known as the MeltPro Myco assay has been developed, capable of identifying 19 species of mycobacteria (Table 3). The test sample can be a liquid medium stored in the laboratory or institute, or sputum samples with positive acid-fast staining [69]. This method offers advantages such as rapidity, high accuracy, sensitivity, and high throughput; reduces the risk of contamination; and detects mixed infections [69]. However, in clinical detection, various influencing factors such as primers, probes, and Taq enzymes can affect the curve.

4.6. Biochip assay (DNA microarray chip)

The principle of a biochip assay involves arranging various DNA probes on a carrier, such as a glass sheet or fiber membrane, using microarray technology. These probes are then hybridized with labeled samples to obtain the hybridization signal intensity of each probe molecule. The *Mycobacterium* identification array kit (CapitalBio, Beijing, China) is a gene chip product designed for *Mycobacterium* species identification in China capable of identifying 17 common species or groups (Table 3) [70]. Compared with traditional methods, DNA microarray chips have the characteristics of rapidity, reliable results, good repeatability, and high throughput. This method enables strain identification within 6–8 h, providing a timely and accurate laboratory basis for patients to receive appropriate treatment [82]. However, the reagent costs approximately US$9.8 for the identification of a single strain, and it cannot distinguish between *M. abscessus* and *M. chelonae*, *M. marinum* and *M. ulcerans* [82].

4.7. Line probe assay

Line probe assay (LPA) operates on the principle of reverse hybridization of PCR products with complementary probes, with the results displayed using enzyme-linked immunocolorimetry. The different line probe assays utilized the INNO-LiPA Mycobacteria v2 assay (Innogenetics, Ghent, Belgium), GenoType Mycobacterium CM/AS and NTM-DR (Hain Lifescience GmbH, Nehren, Germany), Speed-oligo Mycobacteria assay (Vircell, Spain), and AdvanSure Mycobacteria GenoBlot assay (LG Life Sciences Inc., Hour, Korea). Only GenoType Mycobacterium NTM-DR could identify the *M. abscessus* subspecies [95]. LPA offers several advantages such as rapidity, high accuracy, a wide range of identification, and low technical requirements [96]. In addition, multiple strains can be identified using a single probe, and NTM in mixed cultures can be detected simultaneously. However, due to the heterogeneity of the probe-binding region resulting in unsuccessful hybridization, each detection method may yield false-positive or false-negative results. LPA also carries the risk of contamination of amplified products, has limited types of identification, involves relatively complex operations, and is greatly influenced by the proficiency of the operator.

4.8. PCR-reverse blot hybridization assay

The principle of PCR-reverse blot hybridization assay (PCR-REBA) involves spotting the probe on the nylon membrane, hybridizing it with the PCR amplification products, and determining whether the probe hybridizes with the DNA fragment based on the color of the specific position of the membrane strip. The common kits used for this purpose are REBA Myco-ID® (YD Diagnostics, Yongin, South Korea) and PCR-REBA Myco-ID (Yaneng BIOsciences, Shenzhen). This method is rapid, inexpensive, and user-friendly, and suitable for use with clinical specimens [78]. However, it could not further classify *M. abscessus* subspecies. The PCR-REBA Myco-ID has not been widely adopted into the workflow of many mycobacterial laboratories [78]. Recently, a PCR-reverse sequence-specific oligonucleotide probe method based on the mycobacterial detection panel test was developed for the rapid identification of 28 mycobacterial species and subspecies (Table 3). This method can be utilized with clinical respiratory samples and mycobacterial suspensions to distinguish the *M. abscessus* subspecies [79].

4.9. DNA-DNA hybridization test

The basic principle of the DNA-DNA hybridization test involves the denaturation and renaturation of nucleic acid molecules to form hybrid double-stranded DNA fragments from different sources according to complementary base pairing. The common kits used for this test include Genotype CM Direct® (GTCMD) (Bruker, Billerica, Ma, USA) and VisionArray MyCo® (VAM) (ZytoVision, Bremerhaven, Germany). In a study by Hans-Ulrich Schildhaus [80], GTCMD showed sensitivity and specificity rates of 89.2 % and 81.5 %, respectively, while VAM demonstrated rates of 73.0 % and 96.3 %. These kits can be used to identify mycobacteria directly from clinical specimens, but cannot identify *M. abscessus* subspecies. However, only a few published studies have explored these two products.

4.10. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) operates by comparing spectral data from extracted proteins to a database. Mycobacterial species were identified by MALDI-TOF MS according to the reference spectra contained in the database. This method is fast and reliable, making it suitable for routine and high-throughput use in clinical laboratories. A previous study showed that MALDI-TOF MS accurately identified 97 % and 77 % of the strains from L-J and MGIT media,

respectively [97]. However, mycobacteria require additional processing before analysis by MALDI-TOF MS due to biosafety concerns and their structural characteristics [98]. Identifying NTM strains using MALDI-TOF MS poses challenges due to their slow growth rate, limited number of ribosomal proteins, thick cell walls, and hydrophobicity. In addition, mass spectrometers are expensive. Although MALDI-TOF MS can distinguish between *M. abscessus* and *M. chelonae*, it cannot distinguish *M. abscessus* subspecies [99]. Upgrading the database can significantly improve the ability to identify NTM strains. The accuracy of the MALDI-TOF MS using the upgraded My-coDB v2.0-beta database was notably superior to that reported in previous studies [84].

Rindi et al. showed that MALDI-TOF MS can be used as a routine diagnostic tool to identify mycobacteria directly from positive primary MGIT cultures in only 30 min [100]; however, the study has some limitations, including the small sample size. Previous studies have shown that liquid chromatography-mass spectrometry can be used to identify clinically relevant *M. abscessus* complex organisms, with higher analytical performance compared with MALDI-TOF [99]. Nucleic MALDI-TOF MS detection integrates the high sensitivity of PCR technology, high throughput of chip technology, and high precision of MALDI-TOF MS, enabling the amplification of multiple genes used for analyzing single nucleotide polymorphisms, gene mutations, DNA methylation, and copy number identification. Li et al. demonstrated that the sensitivity, specificity, and accuracy of nucleotide MALDI-TOF MS for mycobacterial identification were 96.91 %, 100 %, and 97.22 %, respectively [101].

4.11. Sanger sequencing

This method can identify bacteria at the species level by analyzing the differences in homologous DNA sequence composition. The sequences commonly used for the identification of *Mycobacterium* species include 16SrRNA, 16S-23SrRNA (*ITS*), *rpoB*, and *hsp65*. Among these, *RpoB* is widely utilized [102], while *Hsp65* demonstrates superior discriminatory ability compared with *rpoB* and *ITS*. However, 16S rRNA is not suitable for distinguishing closely related strains, such as pathogenic *M. kansasii* and non-pathogenic *M. gastri*, *M. chelonae* and *M. abscessus*, and cannot distinguish between MTB and *M. avium* [103]. Currently, *ERM (41)* [104], *argH*, and *cya* gene markers [105] are used to identify the *M. abscessus* subspecies (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*). Multilocus sequence typing (MLST) defines different strains of bacteria by analyzing the sequence differences in several conserved internal segments that encode host genes. Diricks et al. developed a robust cgMLST scheme for the emerging pathogen *M. abscessus*, which delineates the MAB population structure, outbreaks, and within-patient diversity. Using open-source software or web-based tools, the whole-genome sequencing-based cgMLST approach has proven to be a powerful tool for high-resolution molecular epidemiological investigation of MAB strains [106]. Kolb et al. applied MLST technology to type *M. avium* strains of human, animal, and environmental origins and examined the degree of genetic diversity [107]. An important advantage of this methodology is its high level of reproducibility and the possibility of digitalizing the data for easy worldwide comparison [107].

Some researchers have developed an open-source software for the Sanger sequencing-based identification of NTM species, named SnackNTM. SnackNTM can analyze multiple samples simultaneously. In the processing time comparison test, the analysis and reporting of 30 samples, which required 150 min manually, were completed in just 40 min using SnackNTM [108].

4.12. Whole-genome sequencing

Whole-genome sequencing (WGS) is a method that uses enzymatic hydrolysis or mechanical force to fragment gene sequences, and then sequences these fragments using bioinformatic methods to assemble the sequenced sequences from scratch into a complete genome. WGS of pathogenic microorganisms based on next-generation sequencing technology has been widely used in the detection of infectious diseases. It aids in diagnosing infections with two or more mixed NTM, and is also commonly used to identify rarely isolated species and understand resistance mechanisms by obtaining complete genome sequences [109]. WGS also allows the identification of NTMs at the clone level to determine the nature of the ongoing disease (relapse/reinfection) [110]. However, due to its high cost and complexity, it is not suitable for large-scale applications [111].

4.13. Mycobacterial interspersed repetitive unit-variable number tandem repeat typing

This technique relies on analyzing the number of tandem repeats within a specific region of the genome. These specific regions, called mycobacterial interspersed repetitive units (MIRUs), contain a variable number of repeat sequences (variable number of tandem repeats, VNTRs) that may differ between strains. Owing to its high throughput, good repeatability, and easy standardization, the MIRU-VNTR classification method is widely used in molecular epidemiological studies of TB, especially for tracking transmission chains and exploring the spread and evolution of strains [112]. Zsuzsanna Rónai applied eight loci, MIRU-VNTR, contributing greatly to our knowledge of the genetic diversity of *M. avium* subspecies [113]. In a study by Kalvisa, MIRU-VNTR genotyping revealed 13 distinct genotypes, allowing the efficient discrimination of *M. avium* subspecies [114]. Understanding the impact of homoplasy among widely used repeating unit genotyping markers, such as MIRU-VNTR, is important for reducing the probability of misidentification of *M. avium* and other monomorphic and slowly growing mycobacteria.

5. Limitations and prospects

To the best of our knowledge, this was the only review of epidemiology and identification technology that presents the most recent epidemiological data and species distribution of NTM isolates in several countries and provinces of China, along with a comprehensive list of commercial *Mycobacterium* identification kits. In comparison to other similar studies, our research offered a more extensive

examination of the current landscape. Currently, there are persistent challenges in epidemiological research on NTM. Only limited epidemiological studies have been conducted in the Philippines, Bangladesh, and other countries as well as in certain areas of northern and western China, resulting in a lack of up-to-date statistical data. Maintaining consistency in study periods across different regions posed challenges, potentially leading to errors in the comparative analyses. Moreover, some studies have overlooked the crucial clinical features of NTM diseases, contributing to gaps in our understanding of the epidemiology. NTM identification involves two to three steps: strain culture, nucleic acid extraction, and technical testing. Some studies involved incubating both solid and liquid cultures for 6–8 weeks after inoculation [84]. Standardized nucleic acid extraction procedures are crucial to ensure accuracy in testing. In the biochip assay, a matching nucleic acid extraction solution (Capital Bio, Beijing, China) was used to extract nucleic acids [70]. Similarly, the Real Myco-ID® kit has the matched DNA extraction solution (Optipharm) [67]. The conventional method for extracting DNA directly from cultures typically involves the use of conventional cetyltrimethylammonium bromide [87,89,91]. The direct extraction of nucleic acids from sputum specimens requires two steps: cell lysis and DNA purification. The methods include phenol-chloroform based methods, freeze-thaw-boiling, detergent-based procedures, Chelex 100 resins (Bio-Rad Laboratories, Hercules, CA), QIAmp DNA Mini Kit (Qiagen, Hilden, Germany), Roche Cobas TNAI-AMPLI-Prep, and the Promega DNA IQ Casework sample kit with Maxwell 16 robot [115]. The characteristics of various technical tests are described above. For example, MS is the fastest method for strain identification, but requires strain culture; therefore, the entire process is time-consuming. The Mycobacteria Identification Array Kit, MeltPro Myco assay, GTCMD and VAM enable direct testing on specimens, significantly reducing detection time. In the future, identification techniques will be developed to directly identify samples without the need for culture, with fewer influencing factors, higher identification accuracy and sensitivity, and a wider range of species/subspecies.

6. Conclusions

This review discussed the epidemic status and laboratory identification methods of NTM. It underscored the variability of NTM epidemiology across regions, emphasizing the importance of timely regional epidemiological investigation to understand and address evolving trends. Updates and advancements in identification methods are ongoing, and faster, more convenient, and more accurate methods will be developed in the future to enhance NTM detection.

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Data availability statement

Data associated with the study were obtained from the relevant literature, the references have been marked. And no additional data was used for the research described in the review article. Chinese libraries: Chinese National Knowledge Infrastructure and Wanfang.

Ethics statement

Not applicable.

Abbreviations list:

NTM	Non-tuberculous Mycobacteria
MTB	*Mycobacterium tuberculosis*
TB	Tuberculosis
MTBC	*Mycobacterium tuberculosis* complex
NTM-PD	Non-tuberculous mycobacterial pulmonary disease
MAC	*M. avium* complex
MABC	*M. abscessus* complex
COPD	Chronic obstructive pulmonary disease
AIDS	Acquired immunodeficiency syndrome
PCR	Polymerase chain reaction
AFB	Acid-fast bacilli
RGM	Rapidly growing mycobacteria
SGM	Slowly growing mycobacteria
LPA	Line probe assay
mPCR	Multiplex PCR
PCR-REBA	PCR-reverse blot hybridization assay
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MLST	Multilocus sequence typing
WGS	Whole genome sequencing
MIRU-VNTR	Mycobacterial interspersed repetitive unit-variable number tandem repeat typing
L-J	Lowenstein-Jensen
NA	Data not available in article
Ref.	Reference

CRediT authorship contribution statement

Nuo Xu: Writing – original draft, Methodology, Investigation. **Lihong Li:** Data curation. **Shenghai Wu:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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