Current Situations on Identification of Nontuberculous Mycobacteria

Ting-Shu Wu2,3, Chia-Chen Lu1 and Hsin-Chih Lai1

1Department of Medical Biotechnology and Laboratory Science, Chang Gung University;
2Department of Internal Medicine, Chang Gung Memorial Hospital;
3Graduate Institute of Clinical Medical Sciences, Chang Gung University,
Taoyuan, Taiwan.

Non-tuberculous mycobacteria (NTM) cause diseases in both immuno-compromised and immuno-competent patients. Besides sputa, the NTM infections are also identified in nodes, pleura, and osteoarticular. Isolation rate of NTM has been increasing during recent years, ranging between 30-50% among mycobacterial isolates. Although the clinical significance remains to be continuously evaluated, emerging NTM infections are frequently reported. Traditional clinical assessments, acid fast bacilli staining (AFB), and culture methods accompanied with biochemical identifications will gradually not be able to cope with the clinical requirements. Instead, specific in vitro DNA amplification and/or nucleic acid hybridization-based detection systems including (real-time) PCR, chips, arrays, or liquid detection systems, together with new next-generation nucleic acid sequencing technology will gradually replace traditional diagnostic methods in NTM identification. The aim of this article is to provide a general overview on clinical significance, classification, and laboratory diagnostic methods of NTM.

Key words: Non-tuberculous mycobacteria (NTM), identification, diagnosis

Clinical significance of NTM

Nontuberculous mycobacteria (NTM) have been known since the time of Robert Koch, [1] but historically they have been overshadowed by tuberculosis and dismissed as contaminants. NTM generally are free-living organisms that are ubiquitous in the environment. Important reservoirs include water, soil, animals, and dairy products; besides, they can also be found as colonizers of medical equipment such as endoscopes and surgical solutions.[1] Their clinical significance has only been recently appreciated. More and more NTM are becoming recognized as true pathogens and important causes of human infections of different syndromes.[2] The spectrum of clinical infections caused by NTM can be broadly divided into several categories: chronic pulmonary infections, superficial lymphadenitis, soft tissue and osteoarticular infections, disseminated disease, and iatrogenic infections.[2] Infections caused by NTM organisms are identified not only in immuno-compromised patients but also in immuno-competent hosts.[1,2] Even so, person-to-person spread has so far not been reported.[1,2]

General classification

NTM comprise a variety of acid-fast bacilli species. They encompass all mycobacterial species other than Mycobacterium tuberculosis complex (MTBC) and Mycobacterium leprae. More than 125 species of NTM have been identified,[3] approximately 60 of which are suspected or known to be pathogenic.[1,2] The slow growth of many NTM hinders cultures, which require special medium and prolonged incubation. Traditionally, NTM have been grouped into 4 broad categories according to the Runyon system.[2] In this classification, NTM are divided by growth rates and pigment production. Groups I to III are slow-growing NTM. They are subdivided into group I photochromogens (pigment
producers in the presence of light, such as *Mycobacterium kansasii* and *Mycobacterium marinum*, group II scotochromogens (pigment producers in the absence of light, including *Mycobacterium gordonae* and *Mycobacterium scrofulaceum*), and group III nonchromogens, including *Mycobacterium avium-intracellulare* (MAI) and *Mycobacterium ulcerans*. Group IV NTM are fast growers (i.e., can be detected in culture within 7 days), including *Mycobacterium fortuitum*, *Mycobacterium chelonae*, and *Mycobacterium abscessus*). Although roughly grouped, this classification system provides physicians with a clinically relevant, presumptive speciation.[3]

**Laboratory Safety, Specimen Collection and Processing**

Care must be taken in treatment of clinical samples containing potential NTM. For hospital-based laboratories dealing with NTM specimens, the Biosafety Level 2 protocols [4] must be followed. Besides, all specimens processing should be performed in a Class I or Class II biosafety cabinet, attempting to avoid aerosol formation during any liquid manipulations. Screening of laboratory personnel by annual PPD skin testing and appropriate postexposure clinical evaluation and testing are essential. [4]

All samples should be collected in sterile, properly labeled containers and immediately transported to the laboratory.[5,6] Specimens that cannot be sent within about an hour should be refrigerated.[7,8] Although the presence of MTB in any specimen is considered clinically significant, oral contamination from NTM is a possible source of false positivity.[9] For initial diagnostic purposes, specimen should be collected on 3 to 5 consecutive days; for evaluating therapeutic efficacy, specimens should be collected weekly starting 3 weeks after beginning treatment.[5,10,11,12] Also, to optimize culture sensitivity, concentration of the specimen by centrifugation prior to inoculation is recommended. [13]

**Staining, Culture and Biochemical Identification of NTM**

Traditional diagnosis of mycobacterial infections from sputum samples in the mycobacteria laboratory is primarily based on demonstrating the presence of the acid-fast bacilli (AFB) in the smear, followed by a positive culture and physiological/biochemical identification testing of the isolate.[1,2,3] Due to the presence of mycolic acid in the lipid-rich bacterial cell wall, all mycobacteria are acid-fast bacilli, and thus do not decolorize with acidified alcohol after staining with carbol fuchsin. At least 300 fields of a carbol fuchsin-stained smear should be thoroughly searched at high power (1000x) before declaring it to be negative. [6,12] Fluorescently stained smears, using auramine-rhodamine, highlight the organisms as orange-yellow rods against a black background and can be screened at lower magnification (250x) for correspondingly fewer fields (at least 30); these have become the preferred method of smear examination. [3,5]

NTM cultures are time-consuming. Plate and broth media are used for NTM isolation. Lowenstein-Jensen media, an egg-based medium containing malachite green dye to inhibit growth of contaminating organisms, is the traditional solid media for culture of mycobacteria. Alternatively, the use of agar-based Middlebrook medium can facilitate early detection of colony growth. Visible colony growth can take up to 6 weeks using either method. With liquid media and modern culture systems (such as the BACTEC AFB or Mycobacteria Growth Indicator Tubes),[14,15] growth can typically be seen in approximately 2 weeks. However, contamination has to be taken into consideration [3,16]. Although broth-based cultures are more sensitive and can yield quicker growth, solid-phase cultures allow assessment of colony morphology and longer-term storage. While most mycobacteria grow optimally between 35 and 37°C in 5% to 10% CO2, a subset including *M. marinum*, *M. ulcerans*, *M. chelonae*, and *M. haemophilum* grow better between 25 and 33°C.

The current diagnosis of mycobacterial disease is based on a combination of clinical features, microbiologic data, radiographic findings, and histopathologic studies. Mycobacterial isolates can be preliminarily and roughly classified by pigmentation and growth characteristics. Further identification of mycobacterial species is time consuming and involves biochemical tests that could require weeks of subcultures. Alternatively, examining mycolic acid ester patterns via high-performance liquid chromatography is used for NTM identification.[17]

**Molecular Diagnosis**

Recently, the development of specific *in vitro* DNA amplification and detection-based methods for rapid identification and differentiation of mycobacterial organisms...
has significantly improved the diagnosis efficiency in terms of both sensitivity and specificity.[18-27] Identification of NTM organisms can be achieved using aliquots of broth culture, picked colonies or directly from clinical specimens. Basically the molecular methods comprised mainly two steps. The first one involves amplification of target DNA fragment(s). The other is to detect the amplified fragments. Both steps can be performed either independently or in a combined real-time detection manner. Polymerase chain reaction (PCR) is most frequently used for DNA amplification, although there are many other different commercially available DNA amplification methods developed, including Loop-mediated isothermal PCR (LAMP), Strand-displacement amplification (SDA), and ligase chain reaction (LCR). etc.[28,29] Target DNAs including hsp65, 16S rRNA gene, 23S rRNA gene, the internal transcribed spacer sequence (ITS) DNA, and some transposable elements were used for NTM diagnosis.[28,29] Among these, 16S rRNA gene was most frequently used. Amplified DNA fragments can be detected by a variety of different probe hybridization-based methods [18-22], including polymerase chain reaction restriction fragment length polymorphism analysis (PRA),[30] immuno-chromatography (ICT),[31] and the commercially available kits including Mycobacterium Tuberculosis Direct Test (GenProbe, USA), and INNO-LiPA Mycobacteria (LiPA; Innogenetics, Belgium). INNO-LiPA Mycobacteria assay targets the 16S-23S rRNA spacer region and simultaneously detects and identifies Mycobacterium species in culture and identifies the Mycobacterium tuberculosis complex, the M. avium complex (MAC), and the following Mycobacterium species: M. kansasii, M. avium, M. intracellulare, M. scrofulaceum, M. gordonae, M. xenopi, and the M. chelonae-M. abscessus complex.[32] Real-time PCR significantly improved the detection efficacy. The recently developed real-time PCR assays included the commercial AMPLICOR MTC assay (Roche, Switzerland), the SDA assay (Beckton-Dickinson, USA), and the LCR assay (Nestle, Switzerland). Many other “homebrew” tests have also been created to detect M tuberculosis rapidly and directly from specimens.[26–31] Depending on the particular assay, and specimen type and volume, reported sensitivities vary from 50% to 100% and specificity is usually greater than 95%.[23-27]

We had previously developed a flow cytometer-based assay for rapid identification of mycobacteria from clinical isolates.[33] However, lack of specific antibodies was a major hindrance for application of this assay. Using IS6110 and rv3618 as the DNA targets, we also developed a PCR-ICT assay for differentiation of M. tuberculosis from other members of the M. tuberculosis complex and NTM directly from sputum samples.[31] Recently, another immuno-gold probe assay was also developed for a similar purpose [unpublished data]. The principle of this assay is based on color shift after probes and thus nanogold aggregation in the presence of target DNA. Even though these assays showed practical clinical applicability, their main purpose is to detect M. tuberculosis (complex) and thus can not differentiate the NTM species. To circumvent this problem, a nested PCR-restriction fragment length polymorphism assay focusing on analysis of the hsp65 gene was recently developed.[30] This assay identified up to 54 NTM species directly from clinical sputum samples, with high sensitivity for acid-fast bacilli (AFB) staining 3+ (100%) and 2+ (95%) samples. Another multiplex PCR assay aiming at a similar purpose was also tested in parallel [unpublished data]. To further improve the detection efficacy, currently a pyrosequencing based assay [34] was under testing. This assay can directly identify almost all NTM species by direct sequencing around 30 nucleotides in the 16S, 23S and the internal transcribed spacer sequence (ITS) DNA regions after PCR [35]. It is expected that the direct DNA sequencing approach will gradually replace chip or array assay for rapid identification of NTM species.

**NTM prevalence**

While the true prevalence of infection with NTM remains to be further clarified, Mycobacterium avium-intracellulare is the most commonly encountered NTM world-widely, followed by M. fortuitum complex, and M. kansasii [36,37]. This rise in NTM infections has been attributed to an increased recognition of NTM clinical syndromes and the emergence of not only the immuno-compromised patient, particularly patients with human immunodeficiency virus/acquired immunodeficieny syndrome (HIV/AIDS), but also those immuno-suppressed for other reasons such as organ transplantation [38].

**Conclusion**

NTM are a diverse group of mycobacterial species that cause a wide range of human diseases. NTM cause diseases in both immuno-compromised and immuno-competent individuals. Main diseases caused by NTM
mainly include pulmonary infections, lymphadenitis, disseminated disease, and indolent processes in both immuno-competent and immuno-compromised patients. The diagnosis of NTM disease is not straightforward. A combination of clinical features, radiographic findings, microbiologic data, and histopathologic studies will be required. Molecular diagnostic methods will gradually replace traditional methods for NTM identification. Among these, direct DNA sequencing approach might further dominate among these methods.

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非結核性分枝桿菌之鑑定現況

吳丁樹
陸嘉真
賴信志

1桃園縣龜山鄉長庚大學醫學生物技術暨檢驗學系
2長庚紀念醫院內科部
3長庚大學臨床醫學研究所

非結核性分枝桿菌可引起免疫低下及正常人之疾病。除了痰檢體，其亦可於淋巴液、肋膜液、及骨關節液中被鑑別出來。近年來非結核性分枝桿菌被分離出來的機率有增高的趨勢，在所有分離出之分枝桿菌中約佔30到50個百分比。雖然其臨床重要性還需持續地評估，但目前非結核性分枝桿菌感染的報告仍十分常見。在傳統的臨床評估中，抗酸性染色及痰液培養合併生物化學的方法鑑別已逐漸無法應付臨床的需求。取而代之的是以特有的DNA複製及/或核酸雜交-為基礎的偵測系統，包括即時PCR，chips，arrays或液體監測系統合併成新的未來核酸序列診斷技術，以逐漸取代傳統對於非結核性分枝桿菌之鑑別診斷。此篇文章的目的是提供一個全面性的總論針對非結核性分枝桿菌之重要性，分類及實驗室鑑別診斷的方法來介紹。

關鍵詞：非結核性分枝桿菌、鑑別、診斷