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TITLE: Non-tuberculous Mycobacteria: Identification and Susceptibility Testing

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Slide 1:
Hello, my name is Nira Pollock. I am the Associate Medical Director of the Infectious Diseases Diagnostic Laboratory at Boston Children's Hospital. Welcome to this Pearl of Laboratory Medicine on "Non-tuberculous Mycobacteria: Identification and Susceptibility Testing."

Slide 2:
Non-tuberculous mycobacteria, also known as "NTM," are defined as all species of mycobacteria other than those in the *M. tuberculosis* complex. More than 160 species and subspecies of NTM have been identified and catalogued. The increase over the past 15 years in both the total number of NTM species and the number of NTM species considered clinically significant is likely due to both improved methods of NTM isolation from clinical specimens and to increased usage of sequencing-based methods for species definition. NTM are typically opportunistic pathogens in humans. In parallel with the increased capability for isolation and speciation of NTM, there has been increased recognition of the role of NTM as potential pathogens in immunocompromised individuals, including those with HIV. NTM are widely distributed in the environment, including in soil and in both natural and municipal water sources. Notably, there is no definitive evidence of either human-to-human or animal-to-human transmission of NTM; rather, human disease is assumed to be acquired from environmental exposure.

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NTM can cause both asymptomatic infection, also called "colonization," and symptomatic disease in humans. By far, the most common clinical manifestation of NTM disease is pulmonary disease. Pulmonary disease due to NTM is often associated with structural lung disease such as bronchiectasis, chronic obstructive pulmonary disease, or cystic fibrosis, but can also occur in people without any recognized predisposing factors. Interpretation of respiratory cultures can be particularly complex, however, given that finding NTM in this context can potentially represent either colonization or disease. Other forms of NTM disease include lymphadenitis; skin, soft tissue, and bone disease; and disseminated disease.

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Mycobacterium avium complex, or "MAC," which includes *M. avium* and *M. intracellulare*, is by far the most common NTM-causing disease. However, many other species have been implicated in disease. After MAC, commonly isolated potentially pathogenic species of NTM include *M. kansasii. M. abscessus*, *M. chelonae*, and *M. fortuitum*, followed by less common isolates such as *M. haemophilum, M. marinum*, *M. malmoense*, and others. Even less commonly isolated NTM include *M. genavense, M. smegmatis*, and others shown.

Slide 5:
It is helpful to note the most common associations between certain NTM species and specific clinical presentations, particularly pulmonary disease, lymphadenitis, disseminated disease, and skin, soft tissue and bone disease, as shown in this slide adapted from the 2007 guidelines of the American Thoracic Society and the Infectious Diseases Society of America. Note that these are merely the most common associations; other NTM than those listed here can also cause each clinical presentation. NTM can also be recovered as specimen contaminants. The NTM most commonly isolated as a specimen contaminant is *M. gordonae*, which can be easily cultured from tap water samples, pipelines, and lab faucets.

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When collecting clinical specimens for isolation of NTM, it is important to avoid contamination with tap water, which often contains environmental mycobacteria. For collection of sputum specimens for diagnosis of NTM pulmonary disease, collection of three early-morning specimens on three different days is suggested, as it is for diagnosis of pulmonary tuberculosis. Collection of bronchoalveolar lavage, lung biopsy, body or abscess fluid, or tissue should be by standard methods. Commercial mycobacterial blood culture systems are suitable for growth of NTM.

It is recommended that laboratory manipulations of specimens suspected to contain mycobacteria be performed either in a biosafety level III laboratory or in a biosafety level II laboratory with biosafety level III practices. Specimens from non-sterile body sites should be digested and decontaminated before inoculation of culture media to prevent overgrowth of mycobacterial cultures with bacteria and fungi. Typically, digestion and decontamination methods utilize N-acetyl-L-cysteine-sodium hydroxide. The concentration of sodium hydroxide can be adjusted based on the observed contamination rate for the laboratory; having 2-5% of specimens overgrown by normal flora is considered an appropriate rate, indicating that the decontamination technique is neither inadequate nor too harsh.

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AFB smears for detection of NTM in primary specimens can be done using either fluorochrome, Kinyoun, or Ziehl-Neelsen methods. Fluorochrome is most commonly used and is recommended in the 2007 guidelines from the American Thoracic Society and the Infectious Diseases Society of America. However, it should be noted that false-negative results can occasionally occur with rapidly-growing mycobacteria; this may be a specimen-dependent phenomenon. Gram stains are not useful for detection of mycobacteria. For confirmation of suspected mycobacteria grown in culture, carbol-fuchsin-based stains are recommended. Semi-quantitative smear results should be reported.

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Cultures for mycobacteria should include both liquid and solid media. Liquid cultures have the advantages of higher yield and more rapid growth of mycobacteria, but are more frequently contaminated or overgrown by bacteria. Thus, liquid media typically contains antibiotics to inhibit non-mycobacterial growth. Solid cultures allow formal evaluation of growth rates, observation of colony morphology, distinction of multiple mycobacterial species within one culture, and quantification of growth. Typical solid media for NTM cultures include egg-based media such as Lowenstein-Jensen ("LJ") and agar-based media such as Middlebrook 7H10 and 7H11. Depending on the specimen source, one can choose to plant specimens to these media with or without added antimicrobials to inhibit non-mycobacterial growth. The CLSI guidelines recommend semiquantitative colony counts using a 0-4+ scale, but not all labs perform this. Some NTM are fastidious and require special supplements for recovery in culture. For example, growth of *M. haemophilum* requires that media be supplemented with iron-containing compounds.

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While most clinically significant NTM grow well at 35-37°C, some NTM, specifically *M. chelonae, M. haemophilum, M. ulcerans*, and *M. marinum*, grow preferentially at 28-30°C .To be sure to recover these organisms, it is recommended to culture skin, joint fluid, and bone specimens at both temperatures. *M. xenopi* uniquely requires higher temperature, specifically 40-42°C, for growth.

Slide 10:
NTM have a range of growth rates, and growth rates of mycobacteria on subculture are fairly consistent by species. Most "slowly-growing" NTM, including MAC, *M. kansasii*, and *M. marinum*, grow in 2-3 weeks on subculture, though *M. ulcerans* and *M. genovense* can take up to 8-12 weeks to grow. In contrast, the "rapidly-growing mycobacteria," including *M. abscessus, M. chelonae*, and *M. fortuitum*, are capable of growing out within 7 days on subculture.

In contrast to the consistent growth rates obtained on subculture, growth from primary specimens is dependent in part on the burden of the organism in that specimen. Liquid media is typically held for 6 weeks and solid media for 8 weeks before being considered culture-negative.

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Because different NTM have different antimicrobial susceptibility profiles, identification of clinical NTM isolates to the species level is typically important for clinical decision-making. Note that a current exception to this rule is MAC, as differentiation between *M. avium* and *M. intracellulare* is not thought to be clinically significant. As part of the NTM identification process, it is important to assess whether an NTM isolate is actually clinically significant. Clinical significance depends on context, and factors to consider include the clinical setting, testing indication, and host risk factors, the pathogenic potential of the isolated organism, recovery of the organism from multiple specimens or sites, whether the specimen came from a normally sterile site, and the quantity of organism in a given specimen (for example, environmental contamination with NTM would be very unlikely to result in a positive AFB smear on a primary specimen). For the first isolate of an NTM from a given patient, species identification will typically be required. Communication with the patient's clinician can guide decisions about the need for speciation and subsequent susceptibility testing.

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Classic phenotypic tests utilized for identification of NTM include growth rate (as previously discussed), pigmentation, and colony morphology, followed by conventional biochemical analysis. Pigmentation and colony morphology can be particularly useful to rule out that isolate as being a member of the *M. tuberculosis* complex, or MTBC, as MTBC colonies are non-pigmented and rough. Pigmented species can be photochromogens, meaning that colonies develop pigment when the colony is exposed to light, or scotochromogens, meaning that colonies are pigmented even when grown in the dark. Conventional biochemical testing can be initiated based on the results of these phenotypic tests. However, conventional biochemical testing is less and less desirable as a sole method of NTM identification, given that it is time-consuming, labor-intensive, and potentially unable to identify many newly-described NTM.

Slide 13:
Various methods for rapid identification of NTM now exist, and the field continues to evolve. For a small number of organisms, including MAC, *M. kansasii*, and *M. gordonae*, specific DNA probes are available for direct identification of isolates grown in solid or liquid culture. High Performance Liquid Chromatography, or HPLC, which is based on analysis of fatty acids in the mycobacterial cell wall, can be used either in combination with or in place of biochemical and molecular methodology. HPLC can be performed using colonies grown out on solid media, AFB-positive broth cultures, or even sediments from smear-positive specimens. However, HPLC is not definitive for identification of some species of slowly-growing NTM, and is not an optimal method for distinguishing all of the many different species of rapidly growing mycobacteria.

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Sequence analysis of genomic DNA targets is an increasingly common method for identification of NTM as well as for many other bacterial genera. This method involves PCR-based amplification of the region to be sequenced, followed by sequencing of that amplicon. The ribosomal RNA, or "rRNA," genes are commonly used as targets for bacterial identification and taxonomy. The 16S rRNA gene is the most widely used region and conveniently contains sequence stretches that range from highly conserved to variable. However, for separation of closely related mycobacterial species, 16S rRNA sequencing can sometimes be insufficiently discriminatory, and in such cases must be supplemented by additional DNA targets. Commonly used alternative targets include the rpoB and hsp65 genes, followed by the internal transcribed spacer region (ITS), 23S rRNA gene, gyrB, dnaA, recA, and secA1 genes.

Another method used for identification in some labs is PCR Restriction Enzyme Analysis, or PRA, which involves PCR amplification of a given target sequence, digestion of that amplicon with restriction enzymes, and gel electrophoresis of the digested fragments, followed by pattern analysis. Finally, some labs are starting to use matrix-assisted laser desorption/ionization time of flight mass spectrometry, also known as MALDI-TOF MS, for NTM identification.

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The 2007 guidelines on treatment of NTM disease from the American Thoracic Society and Infectious Diseases Society of America, which remain the most recent such guidelines, note the ongoing debate about the role that in vitro antimicrobial susceptibility testing of NTM should or should not play in management of NTM disease. Importantly, the correlation between in vitro susceptibility results and

clinical response can vary significantly depending on the NTM species and the drugs tested. CLSI guidelines recommend that susceptibility testing of NTM be performed only on "clinically significant" isolates, requiring an assessment of the context from which the NTM was recovered (as discussed previously). CLSI further recommends that testing be focused on isolates which exhibit variability in susceptibility to clinically useful antimicrobial agents AND/OR significant risk of acquired mutational resistance to one or more of these agents. Even after choosing the bug/drug combinations to test, the laboratory must contend with controversies regarding optimal susceptibility breakpoints and testing methods. Overall, clinicians need to be aware of these limitations as they request and evaluate susceptibility testing results for NTM.

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The 2011 CLSI guidelines recommend broth microdilution as the standard for antimicrobial susceptibility testing for NTM. Detailed guidelines are provided only for MAC, *M. kansasii, M. marinum*, and the rapidly growing mycobacteria. For MAC, the primary drugs for testing and treatment are the macrolides, because these are the only antimicrobial agents for which a correlation between in vitro susceptibility testing results and clinical response has been demonstrated in controlled clinical trials. The mechanism of acquired mutational macrolide resistance in MAC isolates is a 23S rRNA gene mutation and is the same for both clarithromycin and azithromycin, so it is only necessary to test one of these two drugs. Tentative breakpoints exist for moxifloxacin and linezolid for secondary testing. For ethambutol, rifampin, and rifabutin, in vitro MIC data have shown poor correlation with clinical response. Therefore, optimal MIC breakpoints for these drugs are unclear, despite the drugs' clinical utility. Limited data exist for amikacin and streptomycin, though emerging data suggests that amikacin MICs may correlate with clinical response.

For *M. kansasii,* primary testing for susceptibility to rifampin and clarithromycin is recommended. If rifampin-resistant, testing a group of secondary agents is recommended; however, not all of these secondary agents have established MIC breakpoints.

For *M. marinum*, routine susceptibility testing is not recommended because untreated strains typically are susceptible to agents used for empiric therapy and acquired mutational resistance is rare. If the patient fails therapy, however, a panel of agents can be tested.

For the rapidly-growing mycobacteria, it is recommended to test a broad panel of drugs as listed here, and breakpoints exist for each drug. Additionally, to ensure detection of inducible macrolide resistance in rapidly growing mycobacteria, which would be due to the presence of the inducible *erm* gene and consequent methylase production, it is recommended that a second clarithromycin susceptibility reading be performed at day 14, unless resistance is recognized earlier.

Slide 17: Acknowledgments

Slide 18: References

Slide 19: Disclosures

Slide 20: Thank You from www.TraineeCouncil.org

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