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Development of a Diagnostic Algorithm for Identification of Non-Tuberculous Mycobacteria Species and Drug Sensitivity

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Keywords: *NTM, MTB, drug resistance, LPA, NTM, DST, sequencing.*

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Strictly as per the compliance and regulations of:

Development of a Diagnostic Algorithm for Identification of Non-Tuberculous Mycobacteria Species and Drug Sensitivity

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Abstract- **Diagnosis and management of non-tuberculous mycobacterial (NTM) infections remain formidable due to their nonspecific clinical presentation, environmental persistence and adaptability, and intrinsic drug resistance to existing treatments. The diverse species exhibiting variable drug susceptibility and lacking standardised treatment regimens make the precise identification of NTM species more critical. Therefore, precise and prompt species-level identification is paramount to confirm diagnosis, suggest appropriate therapeutics to improve patient outcomes. NTM infections often mimic tuberculosis, leading to frequent misdiagnosis, inappropriate therapy, and increased morbidity. Compounding this, advanced molecular tools required for precise identification are limited inresource-constrained setups. Moreover, no robust diagnostic algorithm exists for accurately identifying NTM at the species level. This study aims to develop a rapid, reliable, and reasonable diagnostic algorithm for clinically relevant NTMs. By evaluating the non-complexity of molecular techniques, accuracy of the diagnosis, the turnaround time, a robust algorithm is to be proposed. Standardization of Drug Susceptibility Test (DST) in the study will guide the prompt and targeted therapy.**

Keywords: NTM, MTB, drug resistance, LPA, NTM, DST, sequencing.

I. Background

NTM refer to the mycobacteria other than *M. tuberculosis* complex and *M. leprae*[1]. They are ubiquitously present in the environment, specifically in soil and water, and are capable of causing a range of diseases in humans and animals[2]. They affect both immunocompromised and healthy individuals, and such infections are now increasing globally[3], [4], [5]. Similar to *M. tuberculosis*, NTM infections have the potential to disseminate systemically, mimicking *M. tuberculosis* infections, and can resultin skin and soft tissue infections, lymphadenitis, and pulmonary infections[6]. Again, due to the varied clinical representation of NTM infection, it results in delayed diagnosis and is often misdiagnosed as TB, increasing the burden on the National TB elimination programme[7]. There are 150 species identified in NTM, but a few, such as *Mycobacterium avium complex* (*MAC*), *Mycobacterium abscessus*, and *M. kansasii,* are capable of causing pulmonary infections [8]. *MAC* is well associated with nosocomial infections, while *M. abscessus* is found to bea notorious pathogen that causes high mortality because of its innate resistance to multiple medications[9].

Diagnosis and disease management in NTM infections are challenging. As NTM is an environmental pathogen, it is difficult to distinguish colonisation frominfection[10]. Management of the disease is also toilsome as all NTM species except *M. kansasii* are intrinsically or partially resistant to standard anti-tubercular drugs[11]. Antimicrobial susceptibility testing for all drugs of interest and each species has notbeen standardised yet[12]. Moreover, the clinical application of such AST available drugs with optimal doses is also debatable. Due to their wide availability in the environment and hydrophobic nature, these organisms are adaptable to low pH, high temperature and capable of producing biofilm, thus creating antimicrobial resistance[13], [14].

Treatment of NTM infection involves a protracted regimen of combined antibiotics, and the outcome can vary depending on the species causing the particular infection[15]. In addition, relapse of infection due to the same organism or new recurrent infection due to other mycobacteria is relatively common[16].

The isolation and rapid identification of NTM from clinical specimens areessential due to the differences in treatment strategies for tuberculosis and other mycobacterial infections [17]. This will save the government resources as misdiagnosis of NTM infection leads to unnecessary intake of tuberculosis drugs by the patients[18]. The delay in treatment for non-tubercular infection also leads to increased mortality[19]. The intake of tuberculosis drugs increases the risk of drug-related side effects in patients, leading to increased morbidity[20]. Identification at the species level is necessary for making prompt clinical decisions, as treatment of infections varies by species. But species-specific diagnosis demands advanced molecular techniques, which are not readily available everywhere. Currently, there is no existing robust algorithm for identification of non-tuberculous mycobacteria upto the species level[21], [22], [23], [24]. Therefore, a

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properand early diagnostic algorithm is crucial for the effective management of the disease.

II. Methods/Study Design

Primary Objectives: 1: Identification and characterisation of non-tuberculous mycobacteria in clinically suspected cases.

Objective 2: Development of a diagnostic algorithm for identification of non-tuberculous mycobacteria upto species level by comparing different methods.

Objective 3: Standardisation of drug susceptibility testing for each species of nontuberculous mycobacteria.

a) Study Design

This study will be conducted as a cross-sectional analysis over a 2-year time period.

Study site: The study will be conducted in tertiary TB diagnosis and care centres. i. National Reference Laboratory for TB, RMRC, Bhubaneswar (10 districts) ii. IRL for TB, Cuttack (20 districts) iii. SCB Medical College and Hospital, Cuttack, which caters for most of the tuberculosis cases of the state and central Odisha. iv. MKCG Medical College and Hospital, Berhampur,serves as the referral centre for TB cases from southern Odisha. VSS Medical College and Hospital, Burla, is the referral centre for Western Odisha.

i. *Sample Size*

This study aims to develop a diagnostic algorithm for identifying non-tuberculous mycobacteria atthe species level. All the samples suspected of non-tuberculous mycobacteria infection will be included. Sample size is calculated using Open EPI software. Assuming non-tuberculous mycobacterium prevalence in suspected tuberculosis samples to be 10%, 20% relative error, 95% CI and 80% power, the minimum sample size required is estimated to be 864. Considering a 10% loss to follow-up, the final sample size is calculated to be 950 (864 + 86).

Inclusion criteria for NTM infection (pulmonary and extrapulmonary):

1. Patients with AFB smear-positive results and not responding to anti-tubercular therapy (ATT) for a minimum of 3 months.
2. Sample with AFB smear positive but negative findings both on CBNAAT and LPA
3. Clinically suspected cases of Mycobacteriosis.

Exclusion Criteria:

- CBNAAT Positive
- LPA positive
- Confirmed TB cases

ii. *Sample Selection and Segregation*

The study site has both clinicians and microbiologists as co-investigators, who will be responsible for diagnosing and referring samples for NTM. Samples suspected of NTM infection will be divided into sterile and non-sterile samples. Sterile samples will be directly cultured into solid and liquid media, and then microscopy will be done. Similarly, non-sterile samples will be inoculated in the liquid and solid culture media after a proper decontamination procedure. The inoculated media will be incubated for 6 weeks before being reported as culture negative. The samples with positive culture reports will be identified by growth pattern, pigment production and other characteristics. Further kit-based tests will be used for species identification and DST of the isolates. A panel for drug sensitivity testing will be designed for the treatment of NTM species isolated from patients' samples. Clinical data, including treatment history, will be collected for all the subjects included in the study.

Pulmonary Disease

Suspected NTM lung infection should be evaluated for: (1) a chest radiograph or a high-resolution computed tomography (HRCT) scan in the absence of cavitary lesions; (2) At least two sputum specimens for acid-fast bacilli (AFB) analysis should be collected; and (3) exclusion of other diseases, specifically tuberculosis. An accurate diagnosis of NTM lung disease requiresthe simultaneous fulfilment of clinical, radiographic, and microbiologic criteria.All demographic parameters of the suspected cases will be recorded in a pre-tested questionnaire, along with the Revised National Tuberculosis Control Program (RNTCP) form.

Clinical

Pulmonary: In pulmonary disease or infection, nodular or cavitary opacities are seen on chest radiograph, and HRCT scan may show multifocal bronchiectasis with multiple small nodules.

Extra pulmonary: a. Detailed clinical history of chronic lesions with discharge, which are smear positive and not responding to standard ATT, will be taken. b. Clinically suspected mycobacteriosis, which is smear negative and CBNAAT negative, but pathologically suspicious, will also be taken into consideration.

Microbiologic

Staining: After collection of suspected clinical specimens will be subjected to smear microscopy using the Ziehl-Neelsen (ZN) staining method.

Culture: Specimens will be cultured on solid (LJ) and liquid (MGIT) media.

1. A culture result for sputum is taken as positive from at least two separate expectorated sputum samples (If the initial one is non-conclusive, and repetition of culture and AFB analysis will occur).
2. A Positive culture findingfrom at least one bronchial wash or lavage.

3. Transbronchial or other lung biopsy demonstrating histopathologic features with mycobacteriosis (granulomatous inflammation or AFB)

Extra Pulmonary Disease

Samples that will be included are pleural fluid, lymph node aspirate for cold abscesses, pus, synovial fluid, cerebrospinal fluid, ascitic fluid, urine, gastric aspirate, bone marrow aspirate, biopsy material. Tissue samples or fluids from sterile sites do not require decontamination. Tissues should be ground aseptically in sterile physiological saline or bovine albumin and then directly inoculated onto the media. The samples will be further processed for microscopy, culture isolation in liquid and solid media.

b) Identification of NTM by Different Methods

Identifying Mycobacteria at the species level, particularly non-tuberculous mycobacteria (NTM), using conventional biochemical or microbiological approaches is sometimes non-specific, labour-intensive, and time-consuming. To address these constraints, numerous improved approaches for accurately identifying NTM at the species level have been developed. In this work, we will analyse and compare three diagnostic approaches for NTM species identification. Each approach will be evaluated using critical metrics, including time to completion, cost-effectiveness, diagnostic accuracy, and overall effectiveness. This comparative study will allow us to identify the most efficient and reliable technique, which can subsequently be integrated into a simplified diagnostic procedure for NTM in clinical and research settings.

i. *Line Probe Assay*

This approach uses nucleic acid amplification and reverse hybridisation techniques to identify different Mycobacterial species[25]. Primarily, the target sequence is amplified by PCR using biotinylated primers[26]. Then, amplified productsare reverse-hybridised on a nitrocellulose strip containing immobilised species-specific fragments[27]. Finally, a colour will be developed in an enzyme-mediated method. Then those strips were visually compared to an NTM speciation chart, provided by a commercially available kit.

The Genotype Mycobacterium CM/AS kit & the GenoType NTM DR have been developed for subspecies identification and detection of resistance to macrolides and aminoglycosides in clinical isolates, which will be used for species identification[28]. The performance of this test is comparable to that of DNA, which will be done from culture-positive samples[29]. The GenoType Mycobacterium assay (Hain Lifescience, Germany) is a reverse hybridisation procedure after a multiplex PCR assay [30]. There are three kits: GenoType MTBC, GenoType Mycobacterium CM, and GenoType Mycobacterium AS. GenoType MTBC is used for the differential identification of M. tuberculosis complex, based on the gyrB gene polymorphism[31]. GenoType Mycobacterium CM identifies MTB complex and 24 most common NTM species, while GenoType Mycobacterium AS simultaneously identifies 19 additional NTM species[32] bytargeting 23SrDNA as its target [33].

ii. *PCR Restriction Fragment Length Polymorphism Analysis (PRA) with Hsp65 As Target Gene*

The hsp65 gene from relevant Mycobacterium species will be retrieved from the Gen Bank database for PCR-restriction fragment length polymorphism (RFLP) assay [34]. Primers targeting the *hsp65* gene will be designed for the digestion of amplicon with Sau96I and CfoIto yield a distinct and specific restriction pattern for NTM species and tuberculosis. The expected fragment sizes resulting from Sau96I and CfoI digestion will be analysed for each species. There are eight distinct restriction patterns predicted for Sau96I and ten for CfoI. Based on these patterns, an identification algorithm for PRA will be developed.

iii. *Next Generation Sequencing*

DNA will be extracted by culturing the isolated strains in LJ media. Then, DNA library preparation will be employed for both WGS and tNGS procedures. Extracted DNA will be cleaned using the Quantabio Spar Qmagnetic solution, and for library preparation, [35] the rapid PCR barcoding kit from Oxford Nanopore will be used. Then libraries will be sequenced using the MinION sequencer (Minion1KC; ONT)[36] with flow cells R9.4.1 for 72 h and base calling using guppy (v4.3.4)[37]. The samples will be identified, and antimicrobial resistance (AMR) analysis will be completed by using EPI2ME software[38]. In case of tNGS, DNA sequences will be PCR amplified using DEEPLEX MYC-TB by Genoscreenkit (Illumina)[39]. Then all the procedures will be followed as in WGS. All bioinformatic analyses will be analysed by different algorithms from Oxford Nanopore platforms. For the NTM species, we are also developing a pipeline for their identification and AMR analyses with highaccuracy.

III. Standardization of DST for Ntm

Drug susceptibility testing of NTM will be performed usingthe broth microdilution resazurin method, as outlined in the 2018 guidelines fromthe Clinical and Laboratory Standards Institute (CLSI) and Sharma et al. (2021)[40]. For the initial culture of rapidly growing mycobacteria and slowly growing mycobacteria, Cation-Adjusted Muller-Hinton Agar (CAMHB) and Middlebrook 7H9 media will be used. After copious growth, a bacterial suspension will be prepared with McFarland Standard1. Further, the suspension will be diluted to 1:20 in respective media. Drugs with different concentrations will be prepared and

added to each well in a gradient manner, except in the positive control wells. Then the prepared bacterial suspension will be added to each well except in the wells assigned for negative controls. Plates will be incubated at 37°C for 3 to 5 days for RGM and 7 to 14 days for *SGM*. Then, resazurin dye will be added to assess the viability of the NTM species and determine MIC cutoffs. A change in colour from blue to pink indicated bacterial viability. The minimum inhibitory concentration (MIC) will be determined as the lowest concentration of the drug that maintains the blue colour, indicating inhibition of bacterial growth[41][42].

IV. Implementation Strategy

Al the samples referred for presumptive TB/suspected NTM infection diagnosis will be included in the study. It will be strictly seen that the algorithm is followed till the process is completed. A pretested questionnaire will be used to capture all relevant data.

V. Statistical Analysis

The statistical analysis will be done using STATA software. We will report descriptive summary statistics, comparative analyses, and interpretations of the findings.

VI. Discussion

The occurrence of NTM infections is increasing globally and poses a significant public health threat[43],[44],[45]. They are emerging pathogens capable of affecting both the immunocompromised and the competent population[44],[45]. NTM group comprises multiple species with a non-specific clinical presentation and intrinsic or partial resistance to existing antimicrobial therapy[48]. Due to these characteristics, NTM diagnosis at the species level is quite challenging, and no pathogen-specific assay is available to date. Confirmation requires Clinical correlation, radiological interpretation and microbiological elucidation. As they are ubiquitously present in the environment, repeated sample collections and tests are required to confirm the infection. Different molecular methods are developed from time to time for the identification of the infectious agent[47],[48],[49]. However, no precise algorithmic strategy has been recommended for identifying theseinfecting agents. No confirmed DST has been prescribed for each NTM species, which is important for the development of a precise treatment policy. This study has been designed to develop an algorithm for NTM identification and DST analyses, providing a solid treatment strategy. This is a two-year, cross-sectional, hospital-based study covering different regions of Odisha to understand the incidence of the disease. In addition to this, the study aimsto developan algorithm tool for identifying non-tuberculous mycobacteria tothe species level. All the samples suspected of non-tuberculous mycobacteria infection will be included in the study. The sample size will be calculated based on the prevalence shown in the scientific literature, using the openEpi software[52]. Assuming non-tuberculosis mycobacterium prevalence in suspected tuberculosis samples to be 10 % [53][54], 20% relative error, 95% CI and 80% power, the minimum sample size required will be estimated to be 864. Considering a 10% loss to follow-up, thefinal sample size is calculated to be 950 (864 + 86). Then, samples will be collected according to the American Thoracic Society guidelines(2007)[55]. Identification of non-tuberculous mycobacteria (NTM) will be carried out through a combination of clinical presentation, microbiological assessment, and molecular diagnostic tools. Following initial clinical and microbiological characterisation, species-level identification will be confirmed using three molecular platforms, such as Line Probe Assay (LPA), PCR-Restriction Analysis (PRA), and Next-Generation Sequencing (NGS). These tools will enable both species identification and detection of antimicrobial resistance (AMR) at the genetic level. Subsequently, the study will compare all the molecular diagnostic tools based ontheir diagnostic performance, technological complexity, turnaround time, and cost-effectiveness. Based on these comparative analyses,a diagnostic algorithm for the identification of NTM at the species level and corresponding AMR profiling will be established. Furthermore, the resistance profiles generated through the NGS platform will be validated by phenotypic drug susceptibility testing (DST) using all recommended antimycobacterial agents. Taken together, the study aims to standardise AMR profiling for individual NTM species and propose empirically driven treatment regimens tailored to prevalent specific NTM pathogens.

a) Declarations

- Ethics Approval and Consent to Participate

The study will not be initiated before the protocol, consent form, and information sheet have received approval/favourable opinion from the Institutional Ethics Committee, RMRC-ICMR, Bhubaneswar. All research tools will also be translated into the local languages, Hindi and English, for review by the RMRC Institutional Ethics Committee prior to the commencement of the research. Minor protocol amendments only for logistical or administrative changes may be implemented immediately, and the Institutional Ethics Committees will be informed.

List of Abbreviations

- AFB - Acid-Fast Bacilli
- AMR - Antimicrobial Resistance
- AST - Antimicrobial Susceptibility Testing
- ATT - Anti-Tubercular Therapy

- CAMHB - Cation-Adjusted Mueller-Hinton Broth
- CBNAAT - Cartridge-Based Nucleic Acid Amplification Test
- CFU - Colony-Forming Unit
- CI - Confidence Interval
- CLSI - Clinical and Laboratory Standards Institute
- DNA - Deoxyribonucleic Acid
- DST - Drug Susceptibility Testing
- EPI2ME - EPI2ME Bioinformatics Platform (Oxford Nanopore Technologies)
- GMIT - Growth Mycobacterial Indicator Tube (if applicable)
- HRCT - High-Resolution Computed Tomography
- LJ - Löwenstein-Jensen Medium
- LPA - Line Probe Assay
- MAC - Mycobacterium avium Complex
- MGIT - Mycobacterial Growth Indicator Tube
- MIC - Minimum Inhibitory Concentration
- MTB - Mycobacterium tuberculosis
- Mtb - Mycobacterium tuberculosis (used synonymously with MTB)
- NGS - Next-Generation Sequencing
- NTM - Non-Tuberculous Mycobacteria
- ONT - Oxford Nanopore Technologies
- PCR Polymerase Chain Reaction
- PRA - PCR - Restriction Fragment Length Polymorphism Analysis
- RGM - Rapidly Growing Mycobacteria
- RNTCP - Revised National Tuberculosis Control Programme
- RFLP - Restriction Fragment Length Polymorphism
- SGM - Slowly Growing Mycobacteria
- TB - Tuberculosis
- tNGS - Targeted Next-Generation Sequencing
- WGS - Whole-Genome Sequencing
- ZN - Ziehl-Neelsen (Staining Method)

Consent for Publication:

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Authors' Contributions:

JT: study conception and review, SP and MR: writing the original draft.

References Références Referencias

1. "What Are Nontuberculous Mycobacteria (NTM) Infections?," Cleveland Clinic. Accessed: Mar. 27, 2025. [Online]. Available: https://my.clevelandclinic.org/health/diseases/21200-nontuberculous-mycobacteria-infections
2. I. Pavlik, V. Ulmann, and J. O. Falkinham, "Nontuberculous Mycobacteria: Ecology and Impact on Animal and Human Health," *Microorganisms*, vol. 10, no. 8, p. 1516, Jul. 2022, doi: 10.3390/microorganisms10081516.
3. A. Bethencourt Mirabal, A. D. Nguyen, and G. Ferrer, "Lung Nontuberculous Mycobacterial Infections," in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2025. Accessed: Mar. 27, 2025. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK551583/
4. W.-J. Koh, "Nontuberculous Mycobacteria—Overview," *Microbiol. Spectr.*, vol. 5, no. 1, p. 10.1128/microbiolspec.tnmi7-0024–2016, doi: 10.1128/microbiolspec.tnmi7-0024-2016.
5. A. A. Velayati, S. Rahideh, Z. Derakhshani Nezhad, P. Farnia, and M. Mirsaeidi, "Nontuberculous mycobacteria in Middle East: Current situation and future challenges," *Int. J. Mycobacteriology*, vol. 4, no. 1, pp. 7–17, Mar. 2015, doi: 10.1016/j.ijmyco.2014.12.005.
6. M. M. Johnson and J. A. Odell, "Nontuberculous mycobacterial pulmonary infections," *J. Thorac. Dis.*, vol. 6, no. 3, pp. 210–220, Mar. 2014, doi: 10.3978/j.issn.2072-1439.2013.12.24.
7. K. M. Pennington *et al.*, "Approach to the diagnosis and treatment of non-tuberculous mycobacterial disease," *J. Clin. Tuberc. Mycobact. Dis.*, vol. 24, p. 100244, May 2021, doi: 10.1016/j.jctube.2021.100244.
8. M. M. Johnson and J. A. Odell, "Nontuberculous mycobacterial pulmonary infections," *J. Thorac. Dis.*, vol. 6, no. 3, pp. 210–220, Mar. 2014, doi: 10.3978/j.issn.2072-1439.2013.12.24.
9. K. To, R. Cao, A. Yegiazaryan, J. Owens, and V. Venketaraman, "General Overview of Nontuberculous Mycobacteria Opportunistic Pathogens: Mycobacterium avium and Mycobacterium abscessus," *J. Clin. Med.*, vol. 9, no. 8, p. 2541, Aug. 2020, doi: 10.3390/jcm9082541.
10. J.-Y. Feng, W.-C. Chen, Y.-Y. Chen, and W.-J. Su, "Clinical relevance and diagnosis of nontuberculous mycobacterial pulmonary disease in populations at risk," *J. Formos. Med. Assoc.*, vol. 119, pp. S23–S31, Jun. 2020, doi: 10.1016/j.jfma.2020.05.012.
11. J. V. Philley *et al.*, "Treatment of Non-Tuberculous Mycobacterial Lung Disease," *Curr. Treat. Options Infect. Dis.*, vol. 8, no. 4, pp. 275–296, Dec. 2016, doi: 10.1007/s40506-016-0086-4.
12. B. A. Brown-Elliott, K. A. Nash, and R. J. Wallace, "Antimicrobial Susceptibility Testing, Drug Resistance Mechanisms, and Therapy of Infections with Nontuberculous Mycobacteria," *Clin. Microbiol. Rev.*, vol. 25, no. 3, pp. 545–582, Jul. 2012, doi: 10.1128/CMR.05030-11.
13. S. Saxena, H. P. Spaink, and G. Forn-Cuní, "Drug Resistance in Nontuberculous Mycobacteria:

Mechanisms and Models," *Biology*, vol. 10, no. 2, p. 96, Jan. 2021, doi: 10.3390/biology10020096.
14. V. Jarlier and H. Nikaido, "Mycobacterial cell wall: structure and role in natural resistance to antibiotics," *FEMS Microbiol. Lett.*, vol. 123, no. 1–2, pp. 11–18, Oct. 1994, doi: 10.1111/j.1574-6968.1994.tb07194.x.
15. T. M. Johnson *et al.*, "Contemporary Pharmacotherapies for Nontuberculosis Mycobac-terial Infections: A Narrative Review," *Infect. Dis. Ther.*, vol. 12, no. 2, pp. 343–365, Feb. 2023, doi: 10.1007/s40121-022-00750-5.
16. M. M. Johnson and J. A. Odell, "Nontuberculous mycobacterial pulmonary infections," *J. Thorac. Dis.*, vol. 6, no. 3, pp. 210–220, Mar. 2014, doi: 10.3978/j.issn.2072-1439.2013.12.24.
17. K. M. Pennington *et al.*, "Approach to the diagnosis and treatment of non-tuberculous mycobacterial disease," *J. Clin. Tuberc. Mycobact. Dis.*, vol. 24, p. 100244, May 2021, doi: 10.1016/j.jctube.2021.100244.
18. V. Dartois and T. Dick, "Therapeutic developments for tuberculosis and nontuberculous mycobacterial lung disease," *Nat. Rev. Drug Discov.*, vol. 23, no. 5, pp. 381–403, May 2024, doi: 10.1038/ s41573-024-00897-5.
19. C. T. Sreeramareddy, K. V. Panduru, J. Menten, and J. Van den Ende, "Time delays in diagnosis of pulmonary tuberculosis: a systematic review of literature," *BMC Infect. Dis.*, vol. 9, p. 91, Jun. 2009, doi: 10.1186/1471-2334-9-91.
20. B. E. Gülbay *et al.*, "Side effects due to primary antituberculosis drugs during the initial phase of therapy in 1149 hospitalized patients for tuberculosis," *Respir. Med.*, vol. 100, no. 10, pp. 1834–1842, Oct. 2006, doi: 10.1016/j.rmed.2006.01.014.
21. M. Yazdanmanesh, K. Tadayon, D. Bagherian Koshkghazi, and N. Mosavari, "Isolation and identification of non-tuberculous mycobacteria from aquarium fish in Ilam, Iran," *J. Clin. Tuberc. Mycobact. Dis.*, vol. 37, p. 100478, Dec. 2024, doi: 10.1016/j.jctube.2024.100478.
22. L. Rindi, V. Puglisi, I. Franconi, R. Fais, and A. Lupetti, "Rapid and Accurate Identification of Nontuberculous Mycobacteria Directly from Positive Primary MGIT Cultures by MALDI-TOF MS," *Microorganisms*, vol. 10, no. 7, p. 1447, Jul. 2022, doi: 10.3390/microorganisms10071447.
23. R. N. Yadav, Y. Y. Chowdary, M. Bhalla, and A. K. Verma, "Identification of Nontuberculous Mycobacterium Species by Polymerase Chain Reaction - Restriction Enzyme Analysis (PCR-REA) of rpoB gene in Clinical Isolates," *Int. J. Mycobacteriology*, vol. 13, no. 3, p. 307, Sep. 2024, doi: 10.4103/ijmy.ijmy_134_24.
24. C. J. Opperman, S. Singh, W. Goosen, H. Cox, R. Warren, and A. Esmail, "Incorporating direct molecular diagnostics in management algorithms for nontuberculous mycobacteria: Is it high time?," *IJID Reg.*, vol. 10, pp. 140–145, Mar. 2024, doi: 10.1016/j.ijregi.2023.12.003.
25. R. kamali Kakhki, E. Aryan, Z. Meshkat, and M. Sankian, "Development of a Cost-Effective Line Probe Assay for Rapid Detection and Differentiation of Mycobacterium Species: A Pilot Study," *Rep. Biochem. Mol. Biol.*, vol. 8, no. 4, pp. 383–393, Jan. 2020.
26. P. Desikan *et al.*, "Line probe assay for detection of Mycobacterium tuberculosis complex: An experience from Central India," *Indian J. Med. Res.*, vol. 145, no. 1, pp. 70–73, Jan. 2017, doi: 10.4103/ijmr.IJMR_831_14.
27. E. Richter, S. Rüsch-Gerdes, and D. Hillemann, "Evaluation of the GenoType Mycobacterium Assay for Identification of Mycobacterial Species from Cultures," *J. Clin. Microbiol.*, vol. 44, no. 5, pp. 1769–1775, May 2006, doi: 10.1128/JCM.44.5.1769-1775.2006.
28. H. J. Huh *et al.*, "GenoType NTM-DR Performance Evaluation for Identification of Mycobacterium avium Complex and Mycobacterium abscessus and Determination of Clarithromycin and Amikacin Resistance," *J. Clin. Microbiol.*, vol. 57, no. 8, pp. e00516-19, Jul. 2019, doi: 10.1128/JCM.00516-19.
29. A. Stary, W. Kopp, B. Zahel, S. Nerad, L. Teodorowicz, and I. Hörting-Müller, "Comparison of DNA-probe test and culture for the detection of Neisseria gonorrhoeae in genital samples," *Sex. Transm. Dis.*, vol. 20, no. 5, pp. 243–247, 1993, doi: 10.1097/00007435-199309000-00001.
30. E. Richter, S. Rüsch-Gerdes, and D. Hillemann, "Evaluation of the GenoType Mycobacterium Assay for Identification of Mycobacterial Species from Cultures," *J. Clin. Microbiol.*, vol. 44, no. 5, pp. 1769–1775, May 2006, doi: 10.1128/JCM.44.5.1769-1775.2006.
31. E. Richter, M. Weizenegger, S. Rüsch-Gerdes, and S. Niemann, "Evaluation of Genotype MTBC Assay for Differentiation of Clinical Mycobacterium tuberculosis Complex Isolates," *J. Clin. Microbiol.*, vol. 41, no. 6, pp. 2672–2675, Jun. 2003, doi: 10.1128/JCM.41.6.2672-2675.2003.
32. "GenoType Mycobacterium CM / AS." Accessed: Mar. 27, 2025. [Online]. Available: https://www.bruker.com/en/products-and-solutions/moleculardiagnostics/assays/mycobacteria/genotypemycobacterium-cm-as.html
33. Z. Gitti, I. Neonakis, G. Fanti, F. Kontos, S. Maraki, and Y. Tselentis, "Use of the GenoType Mycobacterium CM and AS Assays To Analyze 76 Nontuberculous Mycobacterial Isolates from

Greece," *J. Clin. Microbiol.*, vol. 44, no. 6, pp. 2244–2246, Jun. 2006, doi: 10.1128/JCM.02088-05.
34. F. Brunello, M. Ligozzi, E. Cristelli, S. Bonora, E. Tortoli, and R. Fontana, "Identification of 54 Mycobacterial Species by PCR-Restriction Fragment Length Polymorphism Analysis of the hsp65 Gene," *J. Clin. Microbiol.*, vol. 39, no. 8, pp. 2799–2806, Aug. 2001, doi: 10.1128/JCM.39.8.2799-2806.2001.
35. "Leading DNA & RNA Amplification Reagent Provider - Quantabio." Accessed: Mar. 27, 2025. [Online]. Available: https://www.quantabio.com/
36. "MinION portable nanopore sequencing device." Accessed: Mar. 27, 2025. [Online]. Available: https://nanoporetech.com/products/sequence/minion
37. "Basecalling and demultiplexing of raw reads," microbepore. Accessed: Mar. 27, 2025. [Online]. Available: https://felixgrunberger.github.io/microbepore/data_analysis/Basecalling/
38. "Transition from EPI2ME Agent to EPI2ME Desktop," EPI2ME. Accessed: Mar. 27, 2025. [Online]. Available: https://epi2me.nanoporetech.com/agent-transition/
39. "Illumina GenoScreen Deeplex® Myc-TB Assay with LP and Indexes." Accessed: Mar. 27, 2025. [Online]. Available: https://sapac.illumina.com/prod ucts/by-type/sequencing-kits/library-prepkits/deeplex-myctb-combo-kit.html
40. M. Sharma, B. Malhotra, and S. Khandelwal, "Drug susceptibiity testing of nontuberculous mycobacteria by broth microdilution method," *Indian J. Med. Microbiol.*, vol. 39, no. 3, pp. 306–310, Jul. 2021, doi: 10.1016/j.ijmmb.2021.03.015.
41. A. N. Umubyeyi, A. Martin, G. Zissis, M. Struelens, E. Karita, and F. Portaels, "Evaluation of the resazurin microtiter assay for rapid detection of ofloxacin resistance in M. tuberculosis," *Int. J. Tuberc. Lung Dis. Off. J. Int. Union Tuberc. Lung Dis.*, vol. 10, no. 7, pp. 808–811, Jul. 2006.
42. J.-C. Palomino, A. Martin, M. Camacho, H. Guerra, J. Swings, and F. Portaels, "Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis," *Antimicrob. Agents Chemother.*, vol. 46, no. 8, pp. 2720–2722, Aug. 2002, doi: 10.1128/AAC.46.8.2720-2722.2002.
43. "Nontuberculous Mycobacterial Disease Mortality in the United States, 1999–2010: A Population-Based Comparative Study | PLOS One." Accessed: Mar. 27, 2025. [Online]. Available: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0091879
44. "Frontiers | The Rise of Non-Tuberculosis Mycobacterial Lung Disease." Accessed: Mar. 27, 2025. [Online]. Available: https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2020.00303/full
45. E. Yıldız, "Beyond Tuberculosis: The Growing Threat of Nontuberculous Mycobacteria," *Balk. Med. J.*, vol. 40, no. 6, pp. 384–385, Oct. 2023, doi: 10.4274/balkanmedj.galenos.2023.2023-28092023.
46. "Non-Tuberculous Mycobacteria: Molecular and Physiological Bases of Virulence and Adaptation to Ecological Niches." Accessed: Mar. 27, 2025. [Online]. Available: https://www.mdpi.com/2076-2607/8/9/1380
47. "Epidemiology and Clinical Significance of Nontuberculous Mycobacteria in Patients Negative for Human Immunodeficiency Virus in Switzerland | Clinical Infectious Diseases | Oxford Academic." Accessed: Mar. 27, 2025. [Online]. Available: https://academic.oup.com/cid/article-abstract/15/2/330/374519?login=true
48. S. Tarashi, S. D. Siadat, and A. Fateh, "Nontuberculous Mycobacterial Resistance to Antibiotics and Disinfectants: Challenges Still Ahead," *BioMed Res. Int.*, vol. 2022, p. 8168750, Feb. 2022, doi: 10.1155/2022/8168750.
49. M. Hashemzadeh *et al.*, "Molecular identification of non-tuberculous mycobacterial species isolated from extrapulmonary samples using real-time PCR and rpoB sequence analysis," *AMB Express*, vol. 13, p. 43, May 2023, doi: 10.1186/s13568-023-01553-8.
50. W.-C. Huang, M.-C. Yu, and Y.-W. Huang, "Identification and drug susceptibility testing for nontuberculous mycobacteria," *J. Formos. Med. Assoc.*, vol. 119, pp. S32–S41, Jun. 2020, doi: 10.1016/j.jfma.2020.05.002.
51. K. M. Pennington *et al.*, "Approach to the diagnosis and treatment of non-tuberculous mycobacterial disease," *J. Clin. Tuberc. Mycobact. Dis.*, vol. 24, p. 100244, May 2021, doi: 10.1016/j.jctube.2021.100244.
52. "OpenEpi Menu." Accessed: Mar. 27, 2025. [Online]. Available: https://www.openepi.com/Menu/OE_Menu.htm
53. V. M. Katoch, "Diagnosis & management of infections due to non-tuberculous mycobacteria in developing countries: Looking ahead," *Indian J. Med. Res.*, vol. 150, no. 5, pp. 429–431, Nov. 2019, doi: 10.4103/ijmr.IJMR_2096_19.
54. A. K. Maurya *et al.*, "Prevalence of nontuberculous mycobacteria among extrapulmonary tuberculosis cases in tertiary care centers in Northern India," *BioMed Res. Int.*, vol. 2015, p. 465403, 2015, doi: 10.1155/2015/465403.
55. S. M. Brown, B. E. Jones, A. R. Jephson, and N. C. Dean, "Validation of the Infectious Disease Society of America/American Thoracic Society 2007 guidelines for severe community-acquired pneumonia*," *Crit. Care Med.*, vol. 37, no. 12, p. 3010, Dec. 2009, doi: 10.1097/CCM.0b013e3181b030d9.