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Advances and Challenges in Laboratory Diagnosis of *Nocardia* Infections: Traditional and Emerging Approaches

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ABSTRACT

Nocardia spp. are filamentous bacteria that may cause nocardiosis; however, the disease's clinical symptoms often mimic tuberculosis, fungal infections and malignancies, complicating diagnosis. Several factors limit the effectiveness of traditional cultures and biochemical methods, including slow bacterial growth, challenges in interpreting the variable partial acid-fast staining, and suboptimal sensitivity and specificity. Molecular techniques such as real-time polymerase chain reaction (real-time PCR), multilocus sequence analysis (MLSA) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) enhance species identification but are costly, technically complex, and, in the case of real-time PCR, prone to false positives due to airway colonization. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) accelerates identification, but its accuracy depends on robust reference spectra and optimized extraction protocols, particularly for Gram-positive actinomycetes. Advanced genomic tools, including whole genome sequencing (WGS) and metagenomic next-generation sequencing (mNGS), offer high resolution strain differentiation and antimicrobial resistance profiling; however, their widespread clinical use is limited by high costs and complex data interpretation. To address these limitations, emerging technologies such as Raman spectroscopy and electrochemical sensors promise rapid and sensitive detection but are still largely experimental. Current challenges include a lack of standardized protocols, limited accessibility in resource-poor settings, and gaps in standardized antimicrobial susceptibility testing. Integrating genomic and proteomic approaches may improve diagnostic accuracy and support targeted therapeutic strategies. Despite technological advances, the development of rapid, affordable diagnostic tools is essential to improve detection of nocardiosis. In this review, advances and challenges in laboratory diagnosis of *Nocardia* infections are discussed.

INTRODUCTION

Nocardia spp. are ubiquitous bacteria with ecological significance and clinical implications, characterized by their unique morphologies and pathogenicity. These microorganisms are primarily distinguished by their partial acid-fast staining properties, which are attributed to the mycolic acids present in their cell walls [1]. *Nocardia* is a genus of aerobic, Gram-positive, high-GC-content bacteria commonly detected in soil and water, where it plays vital roles in the decomposition of organic matter [2]. *Nocardia* spp. exhibit branching structures that fragment into coccoid and rod-shaped forms and produce aerial hyphae. They belong to the order Corynebacteriales within the phylum Actinomycetota (also referred to as Actinobacteria) and are closely linked to other mycolic acid-producing genera such as *Mycobacterium* and *Corynebacterium* [3]. Molecular phylogenetic studies have refined their taxonomy, with species reassigned to associated genera. Taxonomically, the genus *Nocardia* now includes over 100 validly published species, of which more than 50 are recognized as opportunistic human

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pathogens [4]. The global incidence of *Nocardia* infections is poorly defined, largely because nocardiosis is not a reportable disease in most public health systems and is frequently underdiagnosed due to its nonspecific presentation and specialized laboratory requirements; however, evidence indicates increasing rates in regions such as the USA and South Africa. This increase is largely attributed to a growing population of immunocompromised individuals and enhanced diagnostic capabilities. Variability in incidence may be influenced by geographic and climatic factors that affect the distribution and infection rates of *Nocardia* spp. [5, 6]. As opportunistic pathogens, *Nocardia* species can cause a range of infections, most commonly in immunocompromised patients. However, approximately one-third of cases occur in immunocompetent individuals [4, 6]. The most common disease caused by these bacteria, nocardiosis, frequently involves the lungs, skin and brain but has the potential to affect almost any other organ. The most clinically significant species include *N. asteroides* complex, *N. farcinica* and *N. brasiliensis*, with their prevalence varying by geographic regions [7]. Due to their non-specific presentation, clinical manifestations of nocardiosis often resemble those of other pulmonary diseases, particularly tuberculosis, and may mimic malignancies, *Rhodococcus equi* infections in HIV-positive patients, and fungal infections such as *Aspergillus* pneumonia [8]. Typically, nocardiosis presents as pulmonary disease followed by disseminated and extrapulmonary diseases, most commonly affecting the central nervous system, as well as primary skin and soft tissue infections [9]. This symptom overlap creates significant challenges for accurate clinical diagnosis [10]. Differentiating between various *Nocardia* species is challenging due to the limitations of conventional microbiological methods. Additionally, variability in organ involvement and clinical presentations that closely resemble other diseases such as mycobacteriosis, aspergillosis, and actinomycosis, further complicates diagnosis. Conventional identification methods, which rely on morphological characteristics and biochemical assays, are often labor-intensive and inconclusive. Identifying filamentous bacteria such as *Nocardia* spp. in laboratory settings is particularly challenging [11]. These difficulties stem from slow growth, hydrophobic cell walls, filamentous morphology, partial acid-fastness, and taxonomic complexity [12]. In fact, laboratory diagnosis of *Nocardia* spp. is limited by several critical challenges involving detection, identification and antimicrobial susceptibility testing (AST). First, *Nocardia* spp. are slow-growing microorganisms that often fail to grow in standard bacterial cultures, which are typically incubated for 48 h, resulting in delayed or missed diagnoses. Microscopic identification is complicated by the bacteria's Gram-positive, branching filamentous morphology and partial acid-fastness, which necessitate specialized staining techniques to differentiate them from fungi and mycobacteria. Frequent misidentifications occur due to overlapping features and the relative rarity of *Nocardia* spp. [13, 14]. In addition, the paraffin baiting culture method has proven effective for isolating *Nocardia* spp. from clinical specimens, even in the presence of contaminating microflora. When combined with other diagnostic approaches, this method significantly enhances diagnostic precision, an essential factor given the clinical overlap of nocardiosis with other pulmonary infections and the importance of selecting appropriate treatment strategies [15]. Although molecular techniques offer greater accuracy, they are time-consuming, expensive and often unavailable in routine clinical settings, limiting their widespread use. Furthermore, phenotypic and biochemical methods fail in providing reliable species-level identification due to overlapping traits within the species and inconsistent biochemical profiles [2, 9]. AST is particularly challenging. The slow growth and hydrophobicity of *Nocardia* spp. lead to bacterial clumping and uneven inoculum distribution, which complicates endpoint determination in the reference broth microdilution method. Antibiotics such as imipenem and ceftriaxone may produce false resistance results because of drug instability during prolonged incubations. This results in inconsistent and poorly reproducible susceptibility data, necessitating cautious interpretation and the use of reference strains to enhance the accuracy [16]. Therefore, therapeutic management is complicated by variable susceptibility patterns and absence of standardized treatment protocols. Sometimes, empirical antifungal therapy may be initiated when fungal infection is suspected, risking delay of appropriate antibacterial therapy. Overall, the combination of slow bacterial growth, complex taxonomy, limited identification methods and problematic susceptibility testing significantly challenge accurate laboratory diagnoses, as well as appropriate clinical management of *Nocardia* infections [17]. To overcome these diagnostic hurdles, numerous molecular techniques targeting the bacterial genome have been developed and implemented. Figure 1 provides a visual summary of these traditional and emerging diagnostic approaches, which are discussed in the following sections.

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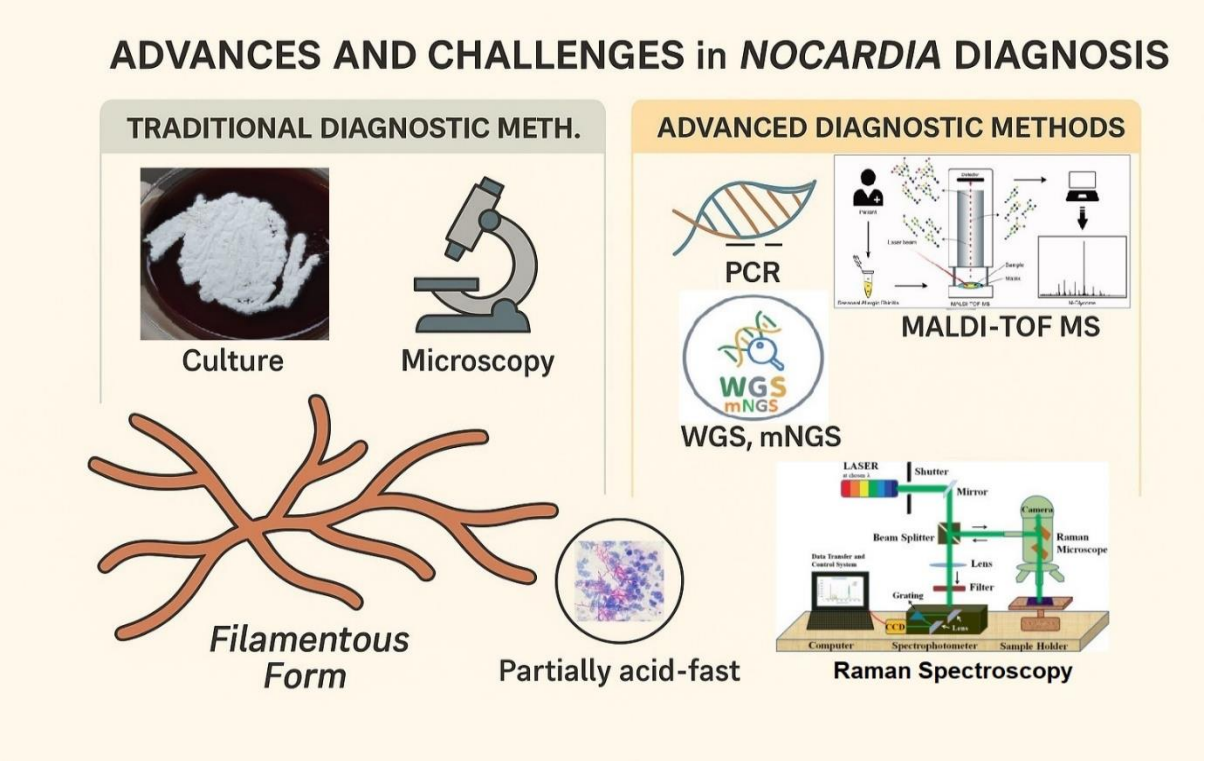


Fig. **1**. This graphic illustrates both traditional and modern laboratory approaches for diagnosing *Nocardia* infections. Traditional methods, such as culture and microscopy (left), are limited by the slow growth of the bacteria and the need for specialized expertise to interpret their distinctive morphology. The center panel highlights the filamentous structure of *Nocardia* spp. and their partial acid-fastness. On the right, advanced diagnostic techniques including PCR, MALDI-TOF MS, whole genome sequencing (WGS), and Raman spectroscopy are depicted. These modern methods offer improved speed, accuracy, and species-level identification; however, challenges such as cost, limited accessibility, and variability in performance hinder their widespread clinical adoption (The figure was created using Microsoft PowerPoint and Adobe Photoshop).

Real-time polymerase chain reaction (real-time PCR)

Real-time PCR is a rapid, reliable, molecular diagnostic tool for detecting *Nocardia* species, significantly decreasing the time needed for diagnosis, compared to traditional culture methods that may take weeks. This assay typically targets conserved regions of the *16S rRNA* gene, enabling specific detection of *Nocardia* species with high but variable sensitivity and specificity in clinical respiratory samples [18]. The time required to obtain results can be relatively short, facilitating earlier diagnosis and the timely initiation of appropriate antimicrobial therapies. However, a key disadvantage of real-time PCR in the diagnosis of *Nocardia* species is its potential lack of specificity, particularly in respiratory samples such as bronchoalveolar lavage (BAL) fluids from lung-transplant recipients. A key limitation is its inability to distinguish between active infection and airway colonization, as the assay detects *Nocardia* DNA regardless of bacterial viability. This can lead to clinically false-positive results and potential overdiagnosis, underscoring the necessity of strict clinical correlation to confirm invasive disease [19].

Multilocus sequence analysis (MLSA)

Advanced molecular methodologies such as MLSA have been shown to improve species discrimination. These techniques analyze multiple housekeeping genes, providing a more robust and reliable framework for species identification compared to single-gene targets or traditional phenotypic methods [4]. Molecular approaches, including MLSA targeting genes such as *16S rRNA*, *gyrB*, *secA1*, *hsp65*, and *rpoB*, enable accurate species-level identification of *Nocardia*. MLSA enhances the laboratory characterization of *Nocardia* infections by providing a highly reliable and discriminatory method for species-level identification from isolated colonies. By analyzing these housekeeping genes (*e.g., 16S rRNA*, *secA1*, *gyrB*), it effectively resolves closely related species where single-gene sequencing fails. This improved resolution supports better clinical management and strengthens epidemiological surveillance efforts [20].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

This method facilitates more precise identification of microorganisms at the species level by analyzing peptide and small protein profiles derived from the whole

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microbial cells, cell lysates and crude extracts [21]. MALDI-TOF MS facilitates rapid identification of *Nocardia* species, significantly reducing the necessary time from days to minutes, an essential advantage for timely clinical management. Once established, the technique is cost-effective and user-friendly, decreasing laboratory expenses. This provides reliable species-level discrimination by analyzing conserved protein spectra, allowing differentiation of closely related species that are difficult to identify using conventional methods [22]. The use of customized or expanded spectral databases further improves identification accuracy, particularly for rare and atypical species. However, the technique may demonstrate limited resolution in distinguishing closely related species with similar protein profiles, occasionally resulting in ambiguous identifications. Compared to Gram-negative bacteria, identification of Gram-positive microorganisms such as *Nocardia* spp. is less reliable and often necessitates additional sample preparation steps such as formic acid treatment [21]. Expanded and curated *Nocardia* reference spectra significantly improve accuracy. Although MALDI-TOF MS offers high accuracy in microbial identification, its effectiveness is limited by factors such as incomplete reference databases and intraspecies variability within *Nocardia* strains. Therefore, integrating advanced molecular methods with MALDI-TOF is essential to enhance diagnostic precision [23]. Additionally, the high cost of MALDI-TOF MS instrumentation restricts its accessibility to specialized reference laboratories [24].

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP is a molecular technique used for the rapid and accurate identification of *Nocardia* spp. by analyzing genetic polymorphisms in specific target genes, most commonly *hsp65* or *16S rRNA*. This method involves PCR amplification of a conserved gene fragment, followed by digestion with restriction enzymes that cut DNA molecules at specific sequences, producing unique fragment patterns that differentiate *Nocardia* spp. and their biotypes [25]. The restriction enzyme MspI is widely used to digest PCR-amplified fragments of the *hsp65* gene, due to its ability to produce species-specific cleavage patterns that facilitate differentiation among *Nocardia* species. BstEII is also commonly employed to improve discrimination between closely related species, although some polymorphisms have been observed in *N. asteroides* soil isolates. In addition, enzymes such as HaeIII and AluI have been used in studies targeting either the *16S rRNA* or *hsp65* gene, generating distinctive fragment profiles that aid in species identification [26]. The PCR-RFLP is a rapid, economical, and relatively simple molecular tool for the identification of *Nocardia* spp.; however, it can be limited by overlapping restriction patterns within species, necessitating verification methods for precise diagnosis [27]. Finally, a key limitation of PCR–RFLP versus WGS is that it detects polymorphisms only at selected restriction sites, missing variants elsewhere. As a result, many nucleotide variations outside these sites go undetected, limiting both its sensitivity and comprehensiveness. In contrast, WGS provides complete genome coverage, enabling the detection of all types of genetic variants, including single nucleotide polymorphisms (SNPs), insertions and deletions (indels), and structural variations [28].

Whole genome sequencing and next-generation sequencing (WGS/NGS)

WGS and NGS are powerful molecular tools that are increasingly used in the diagnosis and characterization of nocardial infections. The NGS technologies, including metagenomic NGS (mNGS), can detect *Nocardia* spp. directly from clinical specimens such as blood and tissue, often complementing culture-based methods. This is particularly valuable for slow-growing or difficult-to-cultivate strains [29], and the WGS enables detailed analysis of *Nocardia* genomes, revealing virulence factors, antimicrobial resistance genes and phylogenetic relationships among strains. This facilitates precise species identification and informs targeted treatment strategies by providing in-depth genomic characterization of isolated pathogens, while mNGS offers a culture independent, comprehensive approach to detect and identify all microbial and host genetic material in a sample, making it suited to different clinical and research needs [30]. Thus, these are distinct yet complementary sequencing approaches, each with unique applications in infectious disease diagnostics. Furthermore, WGS and NGS represent cutting-edge methodologies for *Nocardia* diagnosis, offering rapid, sensitive, and comprehensive genomic insights that are technically superior to conventional culture-based and single-locus molecular techniques. Their use enhances species-level identification, antimicrobial resistance profiling and detection of mixed infections, ultimately improving patient management. However, challenges associated with cost, data analysis and interpretation should be addressed to support broader clinical adoption [29, 31].

Flow cytometry (FC)

Flow cytometry is not a direct method for detecting *Nocardia* spp.; however, it is a valuable adjunctive tool for diagnosing nocardiosis by identifying underlying host immunodeficiencies that predispose individuals to this infection. It is widely used in diagnosis of primary immunodeficiencies by detecting specific protein deficiencies or functional defects in immune cells [32, 33]. While FC is not a direct diagnostic tool for detecting *Nocardia* spp., it can be used to analyze the immune status of patients with nocardiosis, particularly to identify underlying immunodeficiencies and immune dysfunctions that predispose individuals to infections. For example, FC can assess lymphocyte populations and immune cell functions in disseminated nocardiosis to rule

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out or detect immunodeficiencies and acquired immune defects such as anti-cytokine autoantibodies (*e.g.,* anti-GM-CSF antibodies), which increase susceptibility to *Nocardia* infections. Moreover, FC has been used in research settings to study cellular responses to *Nocardia* infections, including apoptosis analysis of infected cells by assessing markers such as annexin V and propidium iodide staining, which evaluate cell death pathways during infections [33, 34]. Flow cytometry supports evaluation of host immune competence in suspected nocardiosis but does not detect the pathogen. It is particularly valuable for identifying immune deficiencies such as chronic granulomatous disease, in which neutrophil oxidative burst activity is impaired. Through assays like the dihydrorhodamine 123 test, flow cytometry assesses NADPH oxidase function, which is essential for effective microbial killing, including against *Nocardia*. This functional evaluation aids in identifying patients at risk for severe nocardiosis by uncovering underlying immune defects. Moreover, flow cytometry can characterize immune cell phenotypes and activation states, offering insights into host-pathogen interactions. However, while it serves as a powerful adjunct, flow cytometry does not replace direct microbiological methods such as microscopy, culture, or molecular diagnostics for the definitive identification of *Nocardia* [33].

Fourier transform infrared spectroscopy (FTIR)

FTIR has been used for *Nocardia* spp., primarily for biochemical characterization rather than direct clinical diagnosis. The FTIR can identify functional groups and molecular components in *Nocardia* cells by detecting characteristic absorption bands corresponding to proteins, lipids, polysaccharides and other cellular constituents [35]. However, limited direct evidence exists supporting routine use of FTIR for clinical diagnosis or species-level identification of nocardial infections [36]. Most clinical microbiology uses of FTIR focus on rapid bacterial typing and outbreak investigations of other pathogens such as vancomycin-resistant *Enterococcus faecium*, where FTIR demonstrates high reproducibility and discriminatory power, compared to WGS. While FTIR offers advantages such as speed, affordability and ease of use, its uses in diagnosis of *Nocardia* spp. are still largely experimental and adjunctive, rather than a standard diagnostic tool [37]. Moreover, the application of FTIR spectroscopy in biofluid analysis (*e.g.,* blood, saliva, urine) suggests its potential for non-invasive screening of nocardiosis and monitoring of treatment response. When integrated with existing molecular diagnostic methods, FTIR could enhance diagnostic accuracy, particularly in complex or polymicrobial infections. However, widespread clinical adoption requires robust validation studies, standardized protocols for sample preparation, the development of user-friendly instrumentation, and adequate clinician training to bridge the gap between research and routine clinical practice [38].

Raman spectroscopy (RS)

Among emerging technologies, Raman spectroscopy or RS is an emerging technique with potential uses in identification and characterization of *Nocardia* species. Given that Raman spectroscopy has been successfully employed for the identification of *Mycobacterium tuberculosis*, and considering the structural similarities in the cell walls of *Mycobacterium* and *Nocardia* species, it shows potential for the detection and identification of *Nocardia* species, though direct evidence is limited [39]. This is an emerging, label-free technique that exploits unique biochemical signatures of bacteria for rapid identification and antibiotic susceptibility testing, in fact, Raman spectroscopy captures molecular vibrations to generate biochemical "fingerprints" of bacterial cells, enabling species-level identification without the need for labels or culture. These techniques analyze spectral differences from biochemical variations in bacterial cells such as proteins, lipids, nucleic acids and carbohydrates, allowing discrimination at the species or strain level [40]. The RS can detect subtle biochemical changes associated with microbial virulence factors, resistance mechanisms and biofilm formation, which may be valuable for the rapid clinical diagnosis of nocardiosis. The technique's ability to analyze small sample volumes and single cells enhances its suitability for clinical microbiology uses [41]. Although direct examples of Raman spectroscopy applied specifically to *Nocardia* are limited, the method's general principles and recent advances suggest promising potential for *Nocardia* diagnostics [42]. Although no specific clinical studies have directly applied Raman spectroscopy to *Nocardia*, the technique's ability to differentiate bacterial species based on their biochemical composition suggests it could distinguish *Nocardia* from other actinomycetes and pathogens. Additionally, it may enhance detection sensitivity for low numbers of *Nocardia* cells in clinical specimens, thereby aiding early diagnosis. Challenges include optimizing sample preparation to reduce background signals, ensuring reproducibility of Surface-Enhanced Raman Spectroscopy (SERS) spectra, and building robust spectral databases including *Nocardia* species for machine learning classification [43].

Electrochemical diagnosis

Among emerging diagnostic approaches, electrochemical diagnosis is a novel laboratory method showing promise for *Nocardia* detection. This approach can be used for the detection of *Nocardia* spp. through the use of sensitive electrochemical genosensors. These sensors use nucleic acid-functionalized nanoscale porous carbon materials to detect *Nocardia* DNA molecules in standard isolates, enabling rapid, specific identification of the pathogen. This technique offers a promising alternative to conventional molecular methods by providing high sensitivity and potential for point-of-care testing in clinical diagnostics [44, 45].

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Electrochemical diagnosis of *Nocardia* involves sensors that detect specific nucleic acids or antigens by measuring changes in electrical signals upon target binding. A key type of these sensors is genosensors, which employ nanoscale porous carbon materials functionalized with nucleic acid probes complementary to *Nocardia* DNA. Hybridization with target DNA induces measurable changes in electrical conductivity or current, enabling sensitive and specific detection [46]. Detection mechanisms typically measure changes in electron transfer, electrical potential (potentiometric), or impedance (impedimetric) resulting from target binding. Overall, electrochemical sensors offer rapid, sensitive, and potentially point-of-care detection of *Nocardia* by converting biological recognition events into quantifiable electrical signals. Despite their promise, electrochemical diagnostic techniques have several limitations, such as the need for standardized protocols and trained personnel to ensure accurate interpretation. Challenges such as interference from complex biological samples and need for signal enhancement to detect low bacterial loads may affect reliability. Additionally, these technologies are still emerging for *Nocardia* detection and may not be widely validated or available in routine clinical practice [47].

High-performance liquid chromatography (HPLC) of mycolic acids

HPLC has been used as a valuable tool in the laboratory identification and differentiation of *Nocardia* species by analyzing their mycolic acid profiles. Mycolic acids, which are long-chain fatty acids found in the cell walls of *Nocardia* and related genera, produce distinct chromatographic patterns when derivatized and separated by HPLC. These patterns enable differentiation between *Nocardia* species and from closely related genera such as *Rhodococcus* and *Corynebacterium*, and it is particularly valuable in research and reference laboratories for verifying *Nocardia* identification, especially when conventional phenotypic methods are inconclusive [48]. For example, HPLC analysis of *p*-bromophenacyl esters of mycolic acids extracted from whole organisms has demonstrated distinct chromatographic profiles for several *Nocardia* species, including *N. asteroides, N. otitidiscaviarum,* and *N. brasiliensis,* offering a complementary tool for species identification in reference laboratories. Although some degree of overlap exists with related species, the number and retention times of peaks generally enable clear discrimination at both the genus and species levels [49]. While HPLC provides valuable biochemical information for *Nocardia* identification, its disadvantages include technical complexity, longer turnaround time, limited specificity in clinical samples and restricted accessibility, which limit their widespread use in routine diagnosis [50].

Pulsed-field gel electrophoresis (PFGE)

For epidemiological studies, this method is valuable for tracking *Nocardia* species distribution and differentiation at the strain level. This method enables the characterization of genetic relationships between isolates by generating distinct chromosomal DNA fragment patterns, which classify isolates into genotypes below the species level. PFGE offers higher discriminatory power compared to phenotypic methods, making it invaluable in outbreak investigations and infection control [20]. For *Nocardia farcinica*, PFGE has been effectively employed to analyze isolates from clinical infections, such as postoperative wound infections, primarily for epidemiological investigations. For instance, in a hospital outbreak involving postoperative wound infections, PFGE was used to compare clinical isolates with environmental samples, successfully identifying endemic strains circulating within the hospital environment and pinpointing potential sources of infection. This approach facilitated the recognition of nosocomial transmission pathways, enabling targeted interventions to control the outbreak [51]. PFGE has been instrumental in differentiating *Nocardia* isolates from multiple patients within healthcare settings, confirming clonal relatedness and supporting epidemiological linkage. These findings highlight PFGE's critical role in tracking the spread of *Nocardia* strains in healthcare facilities, thereby aiding infection prevention efforts. However, despite its advantages, PFGE is technically demanding, time-consuming, and requires specialized equipment and expertise. The method's reproducibility for *Nocardia* isolates can be variable, sometimes producing ambiguous banding patterns that limit definitive strain typing. Additionally, the high GC content typical of *Nocardia* genomes complicates DNA digestion and fragment separation, presenting further challenges for PFGE analysis. To address these limitations, alternative molecular typing methods such as Amplified Fragment Length Polymorphism (AFLP) have been explored. AFLP may offer improved reproducibility and discriminatory power for *Nocardia* strain differentiation, although PFGE remains a valuable and widely recognized method ('gold standard' or 'cornerstone') in many epidemiological investigations [52]. In one comparative study, Random Amplified Polymorphic DNA (RAPD), PFGE, and AFLP techniques were employed to examine a suspected outbreak of *N. farcinica*, with AFLP demonstrating greater reliability and discriminatory power. PFGE is widely regarded as a 'gold standard' or 'cornerstone' method for molecular typing due to its high discriminatory power. Nonetheless, its performance appears to be variable, with empirical evidence from several studies suggesting that it may be less effective than AFLP for delineating particular clusters [53, 54].

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Table 1. Comparison of laboratory diagnostic methods for *Nocardia* spp.

Method	Principle	Advantages	Limitations
Culture & Microscopy	Growth on selective media; partial acid-fast staining	Basic, widely used	Slow growth; misidentification risk
Real‑time PCR	Amplifies *16S rRNA* or other conserved genes	Rapid, sensitive	May detect colonization; false positives
MLSA	Multilocus genetic sequencing (*e.g., 16S*, *gyrB*)	High resolution; reliable	Complex, costly
MALDI-TOF MS	Protein mass spectrometry	Fast, cost-effective after setup, database-dependent; requires optimized extraction	Limited for Gram-positive species; database-dependent
PCR-RFLP	Restriction enzyme digestion of PCR amplicons	Simple, cost-effective	Overlapping patterns; limited sensitivity
WGS/mNGS	Full genome or metagenomic sequencing	WGS: species ID + resistance profiling; mNGS: species ID + co-pathogens; limited resistance prediction	Expensive, data-heavy
Flow Cytometry	Host immune profiling	Adjunctive host immunodeficiency assessment (*e.g.*, CGD)	Indirect; not pathogen-specific
FTIR	Infrared absorption spectrum of cell components	Rapid, non-invasive	Experimental; low specificity for *Nocardia*
Raman Spectroscopy	Molecular fingerprint via laser scattering	Label‑free; potential species‑level ID (experimental)	Experimental; database and reproducibility issues
Electrochemical Diagnosis	DNA hybridization with signal detection	Rapid, sensitive; POC potential	Not widely validated
HPLC	Mycolic acid profile via chromatography	Species-level differentiation	Technically complex, limited specificity
PFGE	Chromosomal DNA fingerprinting	High discrimination; outbreak tracking	Time-consuming, equipment-intensive
AFLP / RAPD	DNA fragment polymorphism typing	High resolution (AFLP)	Reproducibility issues (RAPD)

Note: Sources: [2], [20–22], [28–32], [38], [42–45], [49], [52].

DISCUSSION

Table 1 summarizes each method's principle, advantages, and limitations. Molecular and proteomic technologies have significantly advanced the laboratory diagnosis of *Nocardia* species. In resource-limited settings, traditional methods such as culture, microscopy, and biochemical assays remain essential. However, their limitations, especially in sensitivity, specificity, and turnaround time have driven the adoption of more sophisticated techniques. Real-time PCR offers rapid and sensitive detection of infection, though it can produce false positives in cases of colonization, highlighting the need for careful clinical correlation [18]. MLSA provides highly accurate species-level identification with strong discriminatory power, but it requires specialized bioinformatics skills and additional resources [55]. MALDI-TOF MS has revolutionized microbial identification in many laboratories due to its speed and affordability. Still, its effectiveness for identifying *Nocardia* species is limited by incomplete databases and challenges associated with Gram-positive organisms, often necessitating molecular confirmation [22]. PCR–RFLP is a simple and cost-effective method for distinguishing *Nocardia* species, though it lacks the resolution of WGS. Both WGS and mNGS offer deep genetic insights, but their high cost, complexity, and computational demands have restricted widespread use [56-58]. Flow cytometry, while not a direct diagnostic method for *Nocardia*, offers valuable insights into host immune status and immune dysfunctions predisposing to infection, complementing microbiological diagnostics [33]. Emerging technologies such as Raman spectroscopy, electrochemical sensors, and FTIR spectroscopy show potential for rapid, non-invasive detection, although they remain largely experimental [59]. While not diagnostic in itself, flow cytometry plays a useful role in assessing host immunodeficiency, a key risk factor for nocardiosis [33]. HPLC of mycolic acids enables species differentiation via mycolic acid profiling, mainly in reference laboratories, limited by the technical demands and accessibility constraints. PFGE and AFLP serve as powerful tools for strain-level epidemiological investigations, though PFGE's technical complexity and *Nocardia* genomic characteristics pose challenges [51, 52].

PERSPECTIVES AND CONCLUSIONS

In conclusion, laboratory diagnosis of *Nocardia* spp. remains challenging within clinical microbiology due to multiple factors, including nonspecific clinical manifestations and diagnostic limitations. First, clinical manifestations of nocardiosis are often nonspecific and heterogeneous, frequently overlapping with those of other infectious and non-infectious diseases, such as tuberculosis, fungal infections, *Rhodococcus equi* infections, and malignancies. This clinical ambiguity complicates the initial suspicion and timely consideration of *Nocardia* spp. as causative agents. Second, conventional culture-based diagnostic methods are limited by the slow growth rate of *Nocardia* spp., resulting in prolonged incubation times that often exceed standard laboratory protocols. Addressing diagnostic and therapeutic challenges will require: (1) in-depth study of resistance mechanisms to refine AST, and (2) integrated multi-omics (genomics, transcriptomics, proteomics,

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metabolomics) to strengthen diagnostics and therapeutics. Future research could leverage transcriptomics to identify viability-specific RNA signatures, creating a molecular test capable of differentiating live, infecting *Nocardia* from dead organisms or colonizers—a critical gap that current DNA-based assays such as PCR cannot address. Similarly, metabolomics could uncover unique volatile organic compounds produced by *Nocardia in vivo*, paving the way for non-invasive breath-based diagnostics. Moreover, these advanced techniques can facilitate not only precise species identification but also real-time assessments of virulence factors and resistance determinants, ultimately enhancing clinical decision-making and patient care. Systematic assessments of resistance patterns in diverse clinical isolates and geographic regions could further inform evidence-based treatment guidelines and reduce risks of therapeutic failures.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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No generative artificial intelligence tools were used in the preparation of this manuscript. Standard software tools such as word processors, grammar checkers, and reference managers were used only for editing and citation management.

DATA AVAILABILITY

All data are presented within the manuscript.

AUTHORS' CONTRIBUTIONS

SR: Investigation; Writing – Original Draft. MS: Writing – Review & Editing. KJ: Writing – Review & Editing. AF: Supervision; Project Administration; Formal Analysis; Validation. RMNF: Writing – Review & Editing. All authors reviewed and approved the final version of the manuscript.

ETHICS STATEMENT

Not applicable, as this is a review article based on published literature.

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