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Comparison of MALDI-TOF MS and GenoType CM/AS/NTM-DR for Routine Identification of Non-Tuberculous Mycobacteria Using 16S rRNA Gene Sequencing as the Comparator Method

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Abstract

Accurate identification of non-tuberculous mycobacteria (NTM) is essential because closely related species may differ in clinical significance and treatment. To compare matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and the GenoType® Mycobacterium CM/AS/NTM-DR line probe assay for the routine identification of NTM, using 16S rRNA gene sequencing as a comparator method. Consecutive, non-duplicate clinical NTM isolates recovered between January and April 2025 at a tertiary hospital in Granada, Spain, were identified by both methods during routine diagnostics and retrospectively subjected to full-length 16S rRNA sequencing. Agreement was assessed at species and complex level using Cohen's kappa. Of 57 sequenced isolates, 50 yielded adequate sequences. MALDI-TOF MS achieved species-level identification in all 50 isolates and GenoType® in 46 (92.0%); the remaining four (*M. kumamotonense*, *M. mageritense* [$n = 2$] and *M. neoaurum*) were identified using MALDI-TOF MS in agreement with 16S gene sequencing. Among the 46 isolates identified by both methods, species-level agreement was 89.1% ($\kappa = 0.86$) and complex-level agreement 97.8% ($\kappa = 0.96$). Four of five discordances occurred within the same complex; 16S sequencing supported the GenoType® identification in one (*M. avium*) and could not resolve the remaining three. GenoType® NTM-DR assigned all five *M. abscessus* isolates to subspecies. Both methods showed high mutual agreement and complementary strengths: MALDI-TOF MS provided broader species coverage, whereas GenoType® offered subspecies resolution, supporting complementary strategies for closely related species and discordant results. These findings should be confirmed in larger, multicenter studies.

Keywords: non-tuberculous mycobacteria; MALDI-TOF MS; GenoType CM/AS/NTM-DR; 16S rRNA gene sequencing; species identification; *Mycobacterium avium* complex

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Academic Editors: Kwang-sun Kim and Zehra Edis

Received: 27 October 2026
Revised: 19 November 2026
Accepted: 26 November 2026
Published: 29 November 2026

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1. Introduction

Non-tuberculous mycobacteria (NTM) are a group of approximately 200 species that are ubiquitous in the environment and can behave as opportunistic pathogens for both animals and humans [1]. From their environmental niches, NTM can cause a wide range of infections, including pulmonary disease, lymphadenitis, and disseminated infections. These are more frequently associated with slow-growing NTM in patients with underlying lung conditions or immunosuppression, or with skin and soft tissue infections linked to rapidly growing NTM [2]. The incidence of diseases caused by NTM is increasing worldwide, highlighting their growing importance as a public health issue [3,4]. In Spain, these infections are not notifiable diseases, so their true incidence is not precisely known. However, regional studies indicate an increase in NTM infections, as observed in other parts of the world [5–7].

Given the variability in clinical significance, resistance patterns, and treatment regimens among different species and subspecies, accurate identification of NTM is essential for proper clinical management. For this reason, organizations such as the American Thoracic Society and the Infectious Diseases Society of America recommend that clinically relevant NTM should be identified down to the species level [3,8].

Recently, a practical nomenclature has been proposed that restricts the term "complex" to genetically closely related species that cannot be readily distinguished by commonly used identification methods, and discourages the use of the term "group" [9]. Imprecise identification leads to inconsistencies in microbiological reports and confusion in clinical management. A critical example is *Mycobacterium abscessus*, where precise identification of its three subspecies is essential due to the presence or absence of a functional and inducible *erm*(41) gene, which directly influences variability in macrolide susceptibility and the success of therapeutic regimens [10]. Consequently, mycobacteriology laboratories should aim to achieve the highest possible resolution in identifying their isolates at the species and subspecies level, reserving the use of the term "complex" only for cases where diagnostic methods do not reach their maximum taxonomic resolution [9].

Traditionally, the identification of NTM has been based on phenotypic characteristics such as growth rate, colony pigmentation, optimal growth temperature, and biochemical tests. These tests were poorly reproducible and required subcultures that needed several weeks of incubation before species-level identification could be achieved [11]. High-performance liquid chromatography (HPLC) made it possible to identify NTM in groups or complexes without the need for subcultures, but it was not specific enough for the identification of most species [12]. In most mycobacteriology laboratories, molecular and proteomic techniques have now become the standard for NTM identification. Proteomic methods have faced challenges in their implementation due to the nature of the mycobacterial cell wall and the difficulty in culturing some NTM species. Nevertheless, in recent years there have been advances in the standardization of protein extraction protocols and improvements in databases, which are consolidating matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as a rapid and relatively cost-effective tool for NTM identification [13]. Currently, two commercial MALDI-TOF MS systems are available: the MALDI Biotyper (Bruker Daltonics, Billerica, MA, USA) and the Vitek MS (bioMérieux, Durham, NC, USA).

On the other hand, commercial molecular systems, particularly reverse-hybridization strip assays (line probe assays) such as GenoType® Mycobacterium CM/AS/NTM-DR (Hain Lifescience) and INNO-LiPA Mycobacteria v2 assay (Fujirebio Europe, Ghent, Belgium), allow the identification of clinically relevant NTM and the molecular detection of resistance to macrolides and aminoglycosides in *Mycobacterium chelonae*, members of the *Mycobacterium avium* complex, and species within *Mycobacterium abscessus*. Recently, a

new molecular assay, FluoroType Mycobacteria VER 1.0 (Hain Lifescience GmbH, Nehren, Germany), based on LiquidArray technology and combining multiplex asymmetric PCR with melting curve analysis, has been developed. It allows differentiation of 32 clinically relevant NTM species from positive cultures [14]. Finally, sequencing of conserved genes (such as *rpoB*, *hsp65*, or 16S rRNA) is a valuable tool for the identification of non-tuberculous mycobacteria; however, its discriminatory power may be insufficient for the accurate differentiation of certain closely related species within specific complexes. In addition, due to its cost, technical complexity, and longer turnaround time, sequencing is not well suited for routine workflow in most mycobacteriology laboratories.

The aim of this study is to evaluate MALDI-TOF MS and the commercial GenoType® Mycobacterium CM/AS/NTM-DR method for the identification of NTM, two methodologies routinely used in our laboratory, by comparing their results with Sanger sequencing of 16S rRNA as the comparator method, acknowledging its limited discriminatory power in specific NTM complexes. By evaluating these methods, we aim to compare their performance in a real-world clinical collection and to analyze the situations in which the methods provide concordant, discrepant, or complementary results.

2. Materials and Methods

2.1. Study Design and Isolate Selection

Between January and April 2025, all consecutive, non-duplicate clinical NTM isolates recovered during the study period from respiratory and extrapulmonary samples of patients attended within the healthcare area of the Hospital Universitario Virgen de las Nieves (Granada, Spain) were included.

The study was designed and conducted in accordance with the principles outlined in the Declaration of Helsinki. The experimental protocols were approved by the local Ethics Committee of the Hospital Universitario Virgen de las Nieves in Granada (Spain), with reference number 2024/003-1. Patient consent was waived, as the methodology involved the collection of microbiological and epidemiological data without influencing or altering clinical decision-making.

Sample processing was carried out according to routine diagnostic practice, including microscopic examination by auramine staining and/or Ziehl–Neelsen staining, as well as culture in liquid medium (BD Bactec™ MGIT™, Becton Dickinson, Sparks, MD, USA) and solid medium (Löwenstein–Jensen Pyruvate Medium, RPD Microbiology, Barcelona, Spain). The isolates were characterized using the commercial molecular method GenoType® CM/AS/NTM-DR (Hain Lifescience, Nehren, Germany) and MALDI-TOF MS from isolated colonies in solid medium and subsequently stored at −20 °C in CRYOBANK™ tubes (Bacterial Culture Freezing System, Copan Diagnostics Inc., Murrieta, CA, USA). For retrospective comparison, eligible isolates were recovered from storage and subcultured on Columbia blood agar (Columbia blood agar base, bioMérieux, France) and NTM Elite® agar (bioMérieux, Marcy-l'Étoile, France), incubated in parallel at 37 °C and 30 °C. Incubation was maintained for 5 to 7 days in the case of rapidly growing mycobacteria and from 7 to 14 days for slow-growing ones until optimal growth was achieved to perform full-length 16S rRNA gene sequencing. Inclusion criteria were independent of the species-level result originally obtained by GenoType® and MALDI-TOF MS. Isolates identified as members of the *Mycobacterium tuberculosis* complex and contaminated or non-viable stored cultures were excluded. In the absence of a reference method, 16S rRNA gene sequencing was used as a comparator, not as a gold standard.

2.2. MALDI-TOF MS Analysis

The commercial Biotyper® MBT Mycobacteria method (Bruker Daltonics GmbH, Bremen, Germany) was used according to the manufacturer's instructions for protein extraction. All steps requiring manipulation of mycobacteria prior to inactivation were carried out inside a biosafety level 2 cabinet. Briefly, using a sterile 1 µL loop, mycobacterial colonies grown on solid medium were transferred into an Eppendorf tube containing beads and 300 µL of washing solution. Then, 900 µL of inactivation solution was added and the mixture was incubated for 30 min at room temperature. After vortexing, the tubes were centrifuged at 14,000 rpm for 2 min and the supernatant was discarded. The Eppendorf tubes were centrifuged again at 14,000 rpm for 2 min and all residual liquid was completely removed. The pellet was resuspended in 100 µL of acetonitrile, and 100 µL of pure formic acid was added. The tubes were vortexed for 5 min. They were then centrifuged again at 13,000 rpm for 2 min, and the supernatant was used for MALDI-TOF MS analysis.

One microliter of each supernatant was used and spotted in duplicate onto a MALDI plate position and left to dry at room temperature. One microliter of matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was then pipetted onto each spot and allowed to dry again before inserting the plate into the MALDI-TOF MS.

Spectra were acquired at a laser frequency of 60 Hz within a mass-to-charge (m/z) range of 2000–20,000 Da. The protein profile was obtained using FlexControl 3.4 software (Bruker Daltonics GmbH, Bremen, Germany). The spectra were analyzed by comparison with those in the Bruker MALDI Biotyper Mycobacteria Library version 7.0 (Bruker Daltonics, Bremen, Germany) using the MALDI Biotyper software (v3.1; Bruker Daltonics).

This process assigned a logarithmic score ranging from 0 to 3 and determined the best match based on the m/z ratio and the relative size of each ionized protein. The manufacturer considers scores ≥1.80 as high-confidence and scores of 1.60–1.79 as low-confidence identifications. In this study, species-level identification was accepted when the score exceeded 1.85, the threshold above which agreement with sequencing was optimal in a previous evaluation [13]. Both duplicate spots were analyzed; the highest score was used for interpretation.

Isolates that did not reach the required score in the initial analysis of the duplicate spots underwent repeat protein extraction, followed by a second MALDI-TOF MS analysis.

2.3. GenoType® Mycobacterium CM/AS/NTM-DR Assays

The GenoType® assay includes a complete procedure covering DNA extraction, followed by multiplex PCR and subsequent reverse hybridization using line probe assay technology. The GenoType® assay and the identification of the isolates were performed according to the manufacturer's instructions. The resulting banding patterns were interpreted visually. Initial identification was attempted using the GenoType Mycobacterium CM assay. For isolates yielding inconclusive results with the CM assay, the GenoType Mycobacterium AS assay was subsequently performed. Differentiation of species within the M. avium complex and of subspecies of M. abscessus was achieved using the GenoType NTM-DR assay.

2.4. Nucleic Acid Extraction for 16S rRNA Gene Sequencing

Nucleic acid extraction was performed using the automated MagXtract 3200 system (Chroma ATE Inc., Taoyuan, Taiwan), following the manufacturer's protocol. In brief, biomass from isolated colonies grown on solid medium was collected using an inoculation loop and transferred into tubes containing 700 µL of distilled water and a small amount of glass beads. The tubes were heated at 95 °C for 15 min.

Subsequently, three vortex pulses of 40 s each were applied. The tubes were then loaded into the extraction system, which uses the TANBead Nucleic Acid Extraction kit (Taiwan Advanced Nanotech Inc., Taoyuan, Taiwan), where nucleic acids were purified through lysis, binding to magnetic microbeads, several washing steps, and a final elution in 50 µL of buffer.

2.5. 16S rRNA Gene Amplification and Sequencing

Amplification of the 16S rRNA gene was performed by PCR using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), yielding an amplicon of approximately 1500 bp. The reaction was carried out in nuclease-free Eppendorf tubes with the following composition: 0.5 µL forward primer (0.5 µM), 0.5 µL reverse primer (0.5 µM), 0.2 µL TEMPase Hot Start Polymerase (5 U/µL, VWR Life Science, Radnor, PA, USA), 2.5 µL 10× Combination Buffer (15 mM $MgCl_2$, VWR Life Science), 0.5 µL dNTPs (10 mM, VWR Life Science), and 20.3 µL nuclease-free H_2O to reach a final volume of 25 µL. One microliter of the corresponding eluate was added to each reaction tube.

The amplification reaction was performed in a Veriti™ 96-well Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA) under the following conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. Finally, an elongation step at 72 °C for 7 min was carried out. To verify the size of the PCR products, 5 µL was analyzed by electrophoresis on a 1% agarose gel.

A total of 11.5 µL of purified PCR product was used for sequencing with the GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start kit, following the manufacturer's instructions (Beckman Coulter, Inc., Brea, CA, US, using the following program: 30 cycles of denaturation at 96 °C for 20 s, primer annealing at 50 °C for 20 s, and extension at 60 °C for 4 minutes. The sequencing primers were the same as those used for amplification. The products of the sequencing reactions were read on an automated sequencer (CEQ™ 8000 Genetic Analysis System, Beckman Coulter) according to the standard protocol provided by the manufacturer. Raw sequence chromatograms were visually inspected, and base calling quality was assessed using Phred quality scores. Only sequences with a minimum acceptable Q score ≥ 20 across the analyzed region were included. Ambiguous base calls and low-quality regions at both ends were trimmed prior to analysis.

Trimmed sequences (approximately 1400 bp; minimum accepted length 1300 bp) were compared with the National Center for Biotechnology Information (NCBI) database (accessed November 2025) using the Basic Local Alignment Search Tool (BLASTn, BLAST+ 2.17.0), excluding uncultured sample sequences. Only alignments covering ≥98% of the query sequence were considered. Hits were ranked by percentage identity, and sequences deposited without a species designation (e.g., "*Mycobacterium* sp.") were disregarded. Identification was based on the number of mismatches with the closest reference sequence, following the criteria described by Cisse et al. [15]: a species name was assigned when the sequence showed 100% identity (no mismatches) with a single species; the result was reported at the complex level when 1–3 mismatches with the closest species were observed or when 100% identity was shared by two or more species; and the result was reported as *Mycobacterium* sp. when more than 3 mismatches were found.

2.6. Statistical Analysis

The obtained data were analyzed using MedCalc software (version 23.3.4, MedCalc Software Ltd., Ostend, Belgium). Agreement between the species identification methods MALDI-TOF MS and GenoType® CM/AS/NTM-DR was assessed using a tiered approach, including complete agreement at species level, agreement at complex level and disagree-

ment. In addition, agreement was assessed by calculating Cohen's kappa coefficient, which was interpreted according to the criteria proposed by Landis and Koch [16]. Isolates with non-identification results by MALDI-TOF MS or genus-level identification only by GenoType® CM/AS/NTM-DR were excluded from the agreement and Cohen's kappa analyses. Complex-level categories were defined a priori according to the nomenclature proposed by Wengenack et al. [9]: *M. avium* complex (MAC), *M. abscessus*, *M. chelonae* complex, *M. fortuitum* complex and *M. terrae* complex; species not included in any of these complexes were analysed as separate categories. GenoType® subspecies results were considered concordant with the corresponding MALDI-TOF MS species. Exact (Clopper–Pearson) 95% confidence intervals (CIs) were calculated for proportions.

3. Results

3.1. Study Population and Isolates

During the study period, a total of 772 clinical specimens from patients with suspected mycobacterial infection were processed in the laboratory. Of these, 125 (16.2%) yielded a positive mycobacterial culture. After excluding cultures positive for the *Mycobacterium tuberculosis* complex ($n = 49$), duplicate isolates and isolates meeting the predefined exclusion criteria ($n = 19$), 57 clinical NTM isolates underwent 16S rRNA gene sequencing. Successful amplification and acceptable sequence coverage were achieved for 50/57 isolates (87.7%). These 50 isolates from 50 patients constituted the final collection included in the comparative analysis (Figure 1).

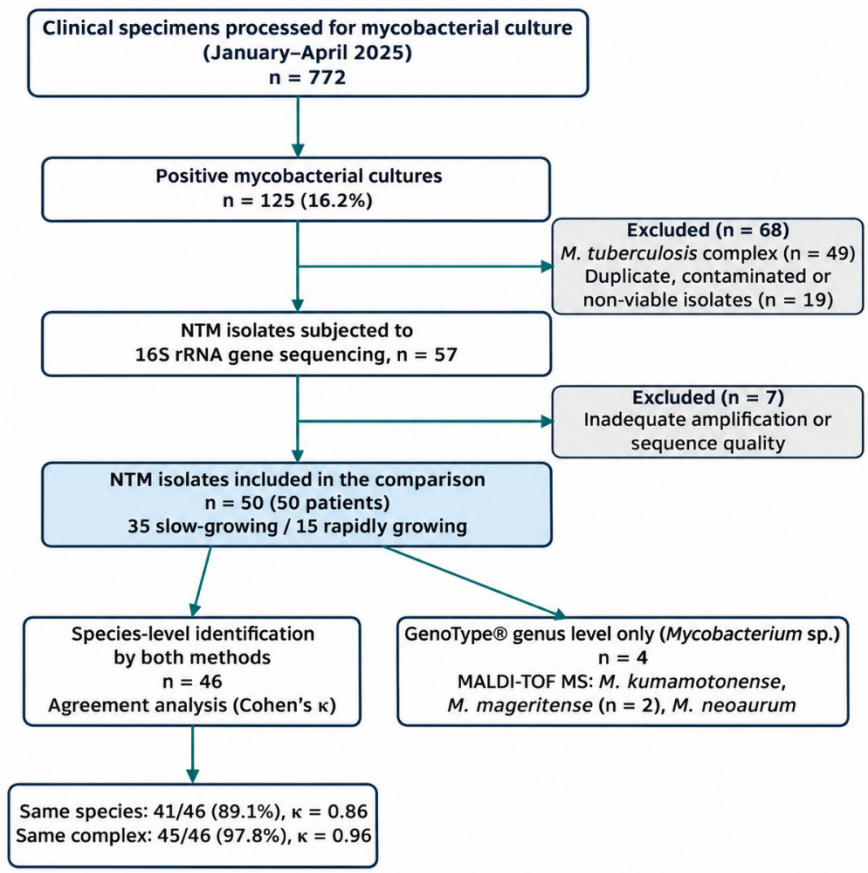


Figure 1. Flow chart of specimen processing and isolate selection. NTM, non-tuberculous mycobacteria.

These included 26 men (52%) and 24 women (48%), with an age range from 1 to 98 years and a mean age of 52 years. The isolates came from 45 respiratory samples

(90%) and 5 non-respiratory samples, including 2 blood cultures, one lymph node biopsy, one urine sample, and one ascitic fluid sample. Thirty (60.0%) isolates belonged to the *Mycobacterium avium* complex (MAC), 5 (10.0%) were *M. abscessus*, 2 (4.0%) belonged to the *M. chelonae* complex, 5 (10.0%) belonged to the *M. fortuitum* complex and 1 *M. kumamotonense* belonged to the *M. terrae* complex.

The remaining 7 isolates (14.0%) did not belong to any of the complexes considered: *M. mageritense* ($n = 2$), *M. scrofulaceum* ($n = 2$), *M. lentiflavum* ($n = 1$), *M. malmoense* ($n = 1$) and *M. neoaurum* ($n = 1$). At complex level, each of these species was analysed as a separate category.

3.2. Identification Performance and Agreement Between Methods

MALDI-TOF MS achieved a conclusive species-level identification for all 50 isolates (100%), whereas GenoType® CM/AS/NTM-DR achieved a conclusive species-level identification for 46 (92.0%). The four isolates that GenoType reported as *Mycobacterium* sp. were identified by MALDI-TOF MS as *M. kumamotonense*, *M. mageritense* ($n = 2$) and *M. neoaurum*, in agreement with the 16S result. These isolates were excluded from concordance analyses. MALDI-TOF MS identifications were compatible with the 16S result in 48 of 50 isolates (96.0%; 95% CI, 86.3–99.5), the exceptions being isolates 3 and 47. GenoType® identifications were compatible in 46 of 50 (92.0%; 95% CI, 80.8–97.8); the remaining four were reported at genus level only, and none was incompatible.

Species-level agreement was assessed in the 46 isolates identified to species level by both methods. MALDI-TOF MS and GenoType reported the same species in 41 of 46 isolates (89.1%; 95% CI, 76.4–96.4). Cohen's kappa was 0.86 (95% CI, 0.75–0.97), corresponding to almost perfect agreement. Complex-level agreement was assessed in the same 46 isolates, with species not belonging to any complex treated as separate categories. MALDI-TOF MS and GenoType assigned the same complex in 45 of 46 isolates (97.8%; 95% CI, 88.5–99.9), with a Cohen's kappa of 0.96 (95% CI, 0.89–1.00). Detailed identification results obtained by 16S rRNA sequencing, MALDI-TOF MS, and GenoType® CM/AS/NTM-DR for slow-growing NTM are shown in Table 1, and those for rapidly growing NTM in Table 2.

Table 1. Results of identification of slow-growing non-tuberculous mycobacteria with MALDI-TOF MS, GenoType® CM/AS/NTM-DR assays and the 16S rRNA gene sequencing comparator.

Isolate No.	16S rRNA Sequencing	MALDI-TOF MS	GenoType® CM/AS/NTM-DR
1	*Mycobacterium avium*	*M. avium*	*M. avium*
2	*Mycobacterium avium*	*M. avium*	*M. avium*
3	*Mycobacterium avium*	*M. intracellulare*	*M. avium*
4	*Mycobacterium avium*	*M. avium*	*M. avium*
5	*Mycobacterium avium*	*M. colombiense*	*M. intracellulare*
6	*Mycobacterium avium*	*M. avium*	*M. avium*
7	*Mycobacterium avium*	*M. avium*	*M. avium*
8	*Mycobacterium avium*	*M. marseillense*	*M. intracellulare*
9	*Mycobacterium avium*	*M. avium*	*M. avium*
10	*Mycobacterium avium*	*M. avium*	*M. avium*

Table 1. *Cont.*

Isolate No.	16S rRNA Sequencing	MALDI-TOF MS	GenoType® CM/AS/NTM-DR
11	*Mycobacterium avium*	*M. avium*	*M. avium*
12	*Mycobacterium avium*	*M. avium*	*M. avium*
13	*Mycobacterium avium*	*M. avium*	*M. avium*
14	*Mycobacterium avium*	*M. avium*	*M. avium*
15	*Mycobacterium avium*	*M. avium*	*M. avium*
16	*Mycobacterium avium*	*M. avium*	*M. avium*
17	*Mycobacterium avium*	*M. avium*	*M. avium*
18	*Mycobacterium avium*	*M. avium*	*M. avium*
19	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
20	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
21	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
22	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
23	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
24	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
25	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
26	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
27	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
28	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
29	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
30	*Mycobacterium intracellulare*	*M. intracellulare*	*M. intracellulare*
31	*M. kumamotonense*	*M. kumamotonense*	*Mycobacterium* sp.
32	*M. lentiflavum*	*M. lentiflavum*	*M. lentiflavum*
33	*M. malmoense*	*M. malmoense*	*M. malmoense*
34	*M. scrofulaceum*	*M. scrofulaceum*	*M. scrofulaceum*
35	*M. scrofulaceum*	*M. scrofulaceum*	*M. scrofulaceum*

Table 2. Results of identification of rapidly growing non-tuberculous mycobacteria with MALDI-TOF MS, GenoType® CM/AS/NTM-DR assays and the 16S rRNA gene sequencing comparator.

Isolate No.	16S rRNA Sequencing	MALDI-TOF MS	GenoType® CM/AS/NTM-DR
36	*M. abscessus*/*M. chelonae*	*M. chelonae*	*M. chelonae*
37	*M. abscessus*/*M. chelonae*	*M. abscessus*	*M. abscessus* subsp. *bolletii*
38	*M. abscessus*/*M. chelonae*	*M. chelonae*	*M. chelonae*
39	*M. abscessus*/*M. chelonae*	*M. abscessus*	*M. abscessus* subsp. *bolletii*
40	*M. abscessus*/*M. chelonae*	*M. abscessus*	*M. abscessus* subsp. *massiliense*
41	*M. abscessus*/*M. chelonae*	*M. abscessus*	*M. abscessus* subsp. *abscessus*
42	*M. abscessus*/*M. chelonae*	*M. abscessus*	*M. abscessus* subsp. *abscessus*

Table 2. *Cont.*

Isolate No.	16S rRNA Sequencing	MALDI-TOF MS	GenoType® CM/AS/NTM-DR
43	*M. fortuitum complex*	*M. fortuitum*	*M. fortuitum*
44	*M. fortuitum complex*	*M. fortuitum*	*M. fortuitum*
45	*M. fortuitum complex*	*M. senegalense*	*M. fortuitum*
46	*M. fortuitum complex*	*M. fortuitum*	*M. fortuitum*
47	*M. fortuitum complex*	*M. mageritense*	*M. fortuitum*
48	*M. mageritense*	*M. mageritense*	*Mycobacterium* sp.
49	*M. mageritense*	*M. mageritense*	*Mycobacterium* sp.
50	*M. neoaurum*	*M. neoaurum*	*Mycobacterium* sp.

3.3. Discordant and Complementary Results

Assignment was concordant for all isolates of MAC ($n = 30$), *M. abscessus* ($n = 5$) and the *M. chelonae* complex ($n = 2$), for 4 of 5 isolates of the *M. fortuitum* complex, and for the four isolates outside any complex included in this analysis. Four of the five species-level discordances therefore fell within the same complex. In isolate 3, MALDI-TOF MS identified *M. intracellulare* (score 1.98) and GenoType identified *M. avium*. In isolates 5 and 8, MALDI-TOF MS identified *M. colombiense* and *M. marseillense*, respectively, whereas GenoType reported *M. intracellulare* in both. In isolate 45, MALDI-TOF MS identified *M. senegalense* and GenoType *M. fortuitum*. In isolate 3, 16S sequencing identified *M. avium*, in agreement with GenoType® but not with MALDI-TOF MS. In isolates 5, 8 and 45, the 16S result reached only complex level and was compatible with both identifications, so these discordances could not be resolved. In isolate 47, MALDI-TOF MS identified *M. mageritense* (score 1.99), which was analysed as a category outside the complexes, whereas GenoType identified *M. fortuitum*. The 16S result (*M. fortuitum* complex) was compatible with GenoType and incompatible with MALDI-TOF MS, and the isolate was assigned to the *M. fortuitum* complex. Notably, the two MALDI-TOF MS identifications not supported by 16S sequencing (isolates 3 and 47) had scores just below 2.00.

Complementary results were also observed. GenoType further assigned all five *M. abscessus* isolates to subspecies (*M. abscessus* subsp. *abscessus*, $n = 2$; subsp. *bolletii*, $n = 2$; subsp. *massiliense*, $n = 1$), a level of resolution not provided by MALDI-TOF MS.

4. Discussion

This study compared GenoType® CM/AS/NTM-DR and MALDI-TOF MS for the routine identification of NTM in 50 clinical isolates, including 35 slow-growing and 15 rapidly growing mycobacteria. Full-length 16S rRNA gene sequencing was used as a comparator.

In our study, MALDI-TOF MS and GenoType® CM/AS/NTM-DR showed a high level of agreement. Of the 46 isolates identified to species level by both methods, 41 (89.1%) were concordant. Four of the five discordances occurred within the same complex, and the remaining one between complexes.

Although in our series MALDI-TOF MS and GenoType® CM/AS/NTM-DR showed excellent concordance, relevant differences were observed in their taxonomic resolution capacity. For example, MALDI-TOF MS was able to achieve species-level identification in one isolate of *Mycobacterium kumamotonense*, whereas GenoType® CM/AS/NTM-DR only reported *Mycobacterium* sp. Similarly, MALDI-TOF MS enabled species-level identification in two isolates of *M. mageritense* and one of *M. neoaurum*, while GenoType® CM/AS/NTM-DR only reached genus-level identification. In these isolates the MALDI-TOF MS species

matched the species-level 16S result. These findings suggest broader species coverage of MALDI-TOF MS for species not represented in the molecular panels and are in agreement with Rodríguez-Temporal et al., who showed that MALDI-TOF MS can identify a broader range of NTM species than reverse hybridization assays [17].

Despite this broader species coverage, MALDI-TOF MS also showed limitations: in isolate 3 (*M. intracellulare* by MALDI-TOF MS (score 1.98); *M. avium* by GenoType® and 16S) and isolate 47 (*M. mageritense* by MALDI-TOF MS (1.99); *M. fortuitum* by GenoType® and *M. fortuitum* complex by 16S), the MALDI-TOF MS result was not supported by the other two methods. These findings highlight the importance of the score thresholds used to validate identification by MALDI-TOF MS. In this regard, Costa-Alcalde et al. proposed a threshold of >1.85, above which agreement with sequencing was optimal [13]. In a larger collection, Rodríguez-Temporal et al. showed that most isolates were correctly identified with scores ≥1.60, that all isolates with scores ≥2.00 were correctly identified, and that only a few species required this higher threshold [18]. Similarly, Rodriguez-Pazmiño et al. suggested a more conservative criterion (≥2.00) to consider species-level identification reliable [19]. These differences reflect the lack of current consensus on the interpretation of scores for validating NTM identification by MALDI-TOF MS. These findings highlight that MALDI-TOF MS performance is not only method-dependent but also strongly influenced by database completeness and score interpretation criteria.

Among slow-growing NTM, the discrepancies were concentrated in isolates belonging to the MAC (isolates 3, 5 and 8). In isolates 5 and 8, MALDI-TOF MS identified *M. colombiense* and *M. marseillense*, respectively, whereas GenoType® CM/AS/NTM-DR reported *M. intracellulare* in both; in isolate 3, MALDI-TOF MS reported *M. intracellulare* and GenoType® CM/AS/NTM-DR reported *M. avium*. In isolate 3, 16S sequencing identified *M. avium*, supporting the GenoType® result; in isolates 5 and 8 it reached only the MAC level and was compatible with both identifications.

This finding is consistent with recent studies indicating that both proteomic techniques and commercial molecular assays have difficulties resolving less frequent, phylogenetically closely related species [14,19,20]. In the case of GenoType® CM/AS/NTM-DR, these discrepancies could be explained by the high phylogenetic proximity between the species included in the panel and other less frequent species not covered by the assay [20]. In line with this, cross-reactivity of the *M. intracellulare* probe with most MAC species has been reported as the cause of the misidentification of *M. marseillense* as *M. intracellulare* by GenoType® CM/AS [21]. In MALDI-TOF MS, these limitations may be related to the low representation or insufficient discrimination of corresponding spectra in the Mycobacteria Library v7.0. However, in the absence of a reference method, these discordances in isolates 16 and 28 could not be resolved in our study.

Other discrepancies observed among rapidly growing mycobacteria, such as the identification of *M. senegalense* by MALDI-TOF MS and of *M. fortuitum* by GenoType® CM/AS/NTM-DR (isolate 45), are more likely related to the limited discriminatory capacity of current assays for phylogenetically closely related species. Here 16S sequencing could not arbitrate either, since partial 16S sequences have been reported to be identical across several species of the *M. fortuitum* complex, including *M. fortuitum* and *M. senegalense* [22]. In isolate 47, by contrast, GenoType® CM/AS/NTM-DR and 16S sequencing (*M. fortuitum* complex) agreed against the MALDI-TOF MS identification of *M. mageritense*, which suggests a MALDI-TOF MS misidentification, although this could not be confirmed with a reference method. *M. mageritense* is not included in the *M. fortuitum* complex in the nomenclature adopted [9]; had it been included, complex-level agreement between MALDI-TOF MS and GenoType® CM/AS/NTM-DR would have been 100% (46/46). By contrast, four isolates of

M. lentiflavum, *M. malmoense* and *M. scrofulaceum* ($n = 2$) were identified concordantly at species level by the three methods.

From a clinical perspective, the impact of these discrepancies is likely not uniform. In some cases, misclassification among closely related MAC species may have limited therapeutic impact, as many of these species share similar diagnostic and treatment approaches. However, inaccurate identification can be clinically relevant in certain scenarios. This is illustrated by isolate 3, in which MALDI-TOF MS reported *M. intracellulare* for an isolate identified as *M. avium* by GenoType® and 16S. It has been described that patients with infection caused by *Mycobacterium intracellulare* may present more severe clinical manifestations, with a higher frequency of fibro-cavitary forms, positive smear microscopy, and lower microbiological response rates to treatment compared to those infected with *Mycobacterium avium* [23]. Likewise, if GenoType® misidentified *M. colombiense* as *M. intracellulare*, as MALDI-TOF MS suggested in isolate 5, this could have relevant clinical implications. *M. colombiense* has been described as an emerging pathogen capable of causing severe disseminated disease in both immunocompromised and immunocompetent patients [24]. In rapidly growing mycobacteria, in our series, the seven isolates of *M. abscessus* and the *M. chelonae* complex were identified at species level and concordantly by MALDI-TOF MS and GenoType® NTM-DR (*M. abscessus*, $n = 5$; *M. chelonae*, $n = 2$). In addition, GenoType® NTM-DR enabled the identification of the three *M. abscessus* subspecies (subsp. *abscessus*, $n = 2$; *bolletii*, $n = 2$; *massiliense*, $n = 1$) highlighting the added discriminatory value of this assay for this species. Moreover, subspecies identification of *M. abscessus* may have important implications for predicting macrolide resistance patterns and clinical prognosis.

4.1. Limitations of the Study

This study was conducted in a single center and included only 50 clinical isolates, which limits the precision of the agreement estimates and the robustness of the conclusions. Several clinically relevant species were represented by very few isolates (five *M. abscessus*), and species frequently encountered in other settings (e.g., *M. kansasii* and *M. xenopi*) were not represented. Therefore, our findings cannot be generalized to the broader NTM population or to individual species and should be confirmed in larger, multicenter collections. This is particularly relevant for the subgroup of rapidly growing NTM, which included only 15 isolates, in which discrepancies may have a disproportionate impact on the overall performance estimates and could lead to biased interpretations. Furthermore, 16S rRNA sequencing reached species level in 36 of 50 isolates (72.0%) and complex level in the remaining 14, so it could not arbitrate three of the five discordances (isolates 5, 8 and 45). It has been shown that phylogenetic analysis based on a single marker has intrinsic limitations [13,19]. The implementation of a multilocus sequencing approach, such as the combination of 16S + rpoB genes, would have been a more accurate strategy for identifying these isolates [19]. In addition, MALDI-TOF MS and GenoType® results were obtained during routine diagnostics, whereas 16S rRNA gene sequencing was performed retrospectively on subcultures of stored isolates. Finally, although this study focused on isolates from solid media, future research should evaluate the performance of these identification methods directly from liquid culture, which would further optimize turnaround times in the microbiology laboratory.

Beyond agreement, factors such as turnaround time, cost, and technical complexity, which were not assessed in this study, should also be considered when selecting identification methods in routine clinical laboratories. In this context, MALDI-TOF MS offers rapid results and broad species coverage, while GenoType® CM/AS/NTM-DR provides standardized workflows and the potential for simultaneous detection of resistance markers, which was not evaluated here.

4.2. Perspectives

Future studies should include larger, multicenter collections with adequate representation of less frequent species and a robust reference method, such as multilocus sequencing (e.g., 16S rRNA + rpoB or hsp65) or whole-genome sequencing, to determine the accuracy of each method and to resolve discordances within closely related taxa. The performance of both methods directly from positive liquid cultures should also be assessed, as it could further shorten turnaround times.

5. Conclusions

MALDI-TOF MS and GenoType® CM/AS/NTM-DR showed a high level of agreement with each other (89.1% at species level and 97.8% at complex level), and their identifications were compatible with the 16S comparator in 96.0% (48/50) and 100% (46/46) of isolates, respectively. MALDI-TOF MS identified four isolates of species not covered by the GenoType® panels, whereas GenoType® NTM-DR resolved *M. abscessus* to subspecies level. Because no reference method was available, the accuracy of either method could not be determined, and species-level discordances within closely related taxa remained unresolved. The discriminatory capacity of GenoType® is linked to the species represented in its panels, whereas that of MALDI-TOF MS depends on library content and score interpretation criteria. Clinical microbiologists should be aware of the limitations of the identification method used and resort to additional techniques when the clinical context requires a higher level of identification, particularly for closely related species or isolates with discordant results.

Author Contributions: Conceptualization, J.M.S.R. and J.R.-G.; methodology, J.M.S.R., and Y.Y.F.V.; validation, J.M.S.R., R.M.R. and M.T.L.; formal analysis, J.M.S.R.; investigation, J.M.S.R., Y.Y.F.V., R.M.R., M.T.L. and J.F.G.-B.; resources, J.A.G.-S., A.S.M. and J.M.N.M.; data curation, J.M.S.R.; writing—original draft preparation, J.M.S.R.; writing—review and editing, J.F.G.-B., A.S.M., J.M.N.M. and J.R.-G.; visualization, J.M.S.R.; supervision, J.R.-G. and J.M.N.M.; project administration, J.R.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was designed and conducted in accordance with the principles outlined in the Declaration of Helsinki. The experimental protocols were approved by the local Ethics Committee of the Hospital Universitario Virgen de las Nieves in Granada (Spain) on 27 November 2024 (reference number 2024/003-1).

Informed Consent Statement: Patient consent was waived, as the methodology involves the collection of microbiological and epidemiological data without influencing or altering clinical decision-making.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Acknowledgments: During the preparation of this manuscript, the authors used Claude (Opus 5.5, Anthropic) for language editing and generation of the code used to produce Figure 1. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BLAST	Basic Local Alignment Search Tool
CI	confidence interval
HPLC	high-performance liquid chromatography
MAC	*Mycobacterium avium* complex

MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MGIT	Mycobacteria Growth Indicator Tube
NCBI	National Center for Biotechnology Information
NTM	non-tuberculous mycobacteria
PCR	polymerase chain reaction
rRNA	ribosomal RNA
κ	Cohen's kappa coefficient

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