Application of MALDI-TOF mass spectrometry for identification of *Nocardia* species

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Abstract

Background Nocardiosis, despite its rarity and underreporting, is significant due to its severe impact, characterized by high morbidity and mortality rates. The development of a precise, reliable, rapid, and straightforward technique for identifying the pathogenic agent in clinical specimens is crucial to reduce fatality rates and facilitate timely antimicrobial treatment. In this study, we aimed to identify *Nocardia* spp. in clinical isolates, using MALDI-TOF MS as the primary method, with molecular methods as the gold standard. Clinical *Nocardia* isolates were identified using *16S rRNA/hsp65/gyrB/secA1/rpoB* gene sequencing. Identification performance of the Bruker MALDI Biotyper 3.1 (V09.0.0.0_8468) and MBT Compass 4.1 (V11.0.0.0_10833) for *Nocardia* identification was evaluated.

Results Seventy-six Nocardia isolates were classified into 12 species through gene sequencing. The MALDI Biotyper 3.1 (V09.0.0.0_8468) achieved 100% genus-level accuracy and 84.2% species accuracy (64/76). The MBT Compass 4.1 with the BDAL Database (V11.0.0.0_10833) improved species identification to 98.7% (75/76). The updated database enhanced species level identification with scores > 1.7, increasing from 77.6% (59/76) to 94.7% (72/76), a significant improvement ($P = 0.001$). The new and simplified extraction increased the proportion of strains identified to the species level with scores > 1.7 from 62.0% (18/29) to 86.2% (25/29) ($P = 0.016$). An in-house library construction ensured accurate species identification for all isolates.

Conclusions The Bruker mass spectrometer can accurately identify *Nocardia* species, albeit with some variations observed between different database versions. The MALDI Biotyper 3.1 (V09.0.0.0_8468) has limitations in identifying *Nocardia brasiliensis*, with some strains only identifiable to the genus level. MBT Compass 4.1 (V11.0.0.0_10833) effectively addresses this shortfall, improving species identification accuracy to 98.7%, and offering quick and reliable identification of *Nocardia*. Both database versions incorrectly identified the clinically less common *Nocardia sputorum* as *Nocardia araoensis*. For laboratories that have not upgraded their databases and are unable to achieve satisfactory identification results for Nocardia, employing the new and simplified extraction method can provide a degree of improvement in identification outcomes.

Keywords MALDI-TOF MS, Nocardia, Rapid identification

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Background

Nocardia species are aerobic opportunistic pathogens characterized by beaded and branched hyphae that are widely distributed in soil, dust, and decaying plants in nature as well as in freshwater, seawater, and animal excretions [1, 2]. Currently, 168 *Nocardia* spp. have been validly described from the environment samples (http://www.bacterio.CICT.FR/N/Nocardia.HTML). In recent years, new *Nocardia* species have been continually isolated from human specimens, and an increasing number of *Nocardia* species isolated from the environment, such as *Nocardia huaxiensis* and *Nocardia takedensis*, have been recently reported to infect humans. [3, 4] More than 40 valid *Nocardia* species are considered clinically relevant (https://www.cdc.gov/nocardiosis/health-care-workers/index.html). As an aerobic opportunistic pathogen, a significant proportion of the Nocardia species can cause infections in humans [5, 6]. According to data from the Centers for Disease Control and Prevention (CDC), there are approximately 500 to 1,000 new cases of Nocardia infection in the United States each year, with about 60% of these cases being associated with pre-existing immune compromise. Although the incidence rate data are limited, the number of cases may be rising due to an increase in individuals with severe immune compromise. (https://www.cdc.gov/nocardiosis/hcp/clinical-overview/index.html). It is important to note that due to the lack of systematic surveillance, the true prevalence of Nocardia infection is unknown. Despite being commonly classified as opportunistic, Nocardia species can also infect immunocompromised patients [7–10]. While pulmonary and disseminated nocardial diseases typically occur in immunocompromised patients, primary cutaneous nocardiosis is generally an infection found in immunocompetent individuals. Although any species of Nocardia species can cause primary cutaneous infections, N. brasiliensis is the predominant species, isolated from the majority (approximately 80%) of cases involving primary cutaneous or subcutaneous nocardiosis [1]. According to research reports, 33-42% of Nocardia infection cases do not exhibit significant immune deficiencies [7, 8].

As *Nocardia* has a slow growth rate and infections may show few symptoms, it can be challenging to detect them in clinical samples [11]. Therefore, laboratory diagnosis of *Nocardia* infection is of great significance in clinical practice. Identifying *Nocardia* isolates at the species level is crucial for providing the right level of care to patients because different *Nocardia* species display varying geographic prevalence, pathogenic characteristics, and patterns of susceptibility to antimicrobial agents [12].

Among the traditional microbiological methods for diagnosing *Nocardia* infections, the sensitivity of smear microscopy is low. It also has the drawback of not being able to identify species. Biochemical methods are inadequate for accurately differentiating among clinically significant species due to the vast number of identified *Nocardia* species.

Gene sequencing is the most precise molecular method for identifying *Nocardia* at the species level. The 16 S rRNA gene sequencing method is widely recognized as the most reliable technique, but it has limitations in distinguishing closely related species because it lacks adequate gene polymorphisms [1, 13]. Several additional housekeeping genes, including DNA gyrase subunit B (*gyrB*), ATPase secretory protein (*secA1*), heat shock protein gene (*hsp65*), and B-subunit of RNA polymerase (*rpoB*), were selected as supplementary genetic markers. When used in combination with 16 S rRNA, these genes establish a precise and highly specific method for the molecular identification of *Nocardia* [1, 14–21]. *SecA1* is a housekeeping gene responsible for the production of the indispensable protein SecA1. Analysis of a specific 468-bp segment of this gene has proven to be effective in distinguishing between different *Nocardia* species [18]. The *hsp65* gene, with a 441-bp sequence, is useful for identifying *Nocardia* species and offers more variability than the *16 S rRNA* gene, allowing the discrimination of closely related species [19, 22]. The *gyrB* gene, which encodes the subunit B of DNA gyrase, has been utilized in the identification and phylogenetic analyses of *Nocardia* isolates [21, 23]. The B subunit of the RNA polymerase is encoded by *rpoB*, which has proven to be a valuable tool for identifying *Nocardia* isolates [21]. Although molecular methods have proven their ability to identify new species, distinguish within species, and accurately assign species, they have certain drawbacks such as relatively high costs and the need for staff to spend time analyzing the results.

Over the past couple of decades, the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) has completely transformed the way bacteria are identified in microbiology laboratories, enabling quick, effortless, low-cost, and reliable identification from culture growth. Early studies have shown that commercial databases are insufficient for accurate identification because they are inconsistent with sequencing methods (0–47.3%) [15, 24–26]. Recent studies have shown that VITEK ®-MS (MALDI-TOF), based on the VITEK ®-MS IVD database, performs well in the identification of *Nocardia* [27–30]. However, there have been limited studies evaluating the capability of Bruker mass spectrometry system to identify *Nocardia*, and prior research has indicated that Bruker mass spectrometry system may not be optimal for *Nocardia* identification, with only a range of 14.9–95.9% of the isolates being identifiable at the species level [25, 31–34]. Existing studies have utilized the manufacturer-recommended formic acid extraction method as a sample pretreatment,

Liu *et al. BMC Microbiology*   (2024) 24:358

Page 3 of 11

which is not only complex and time-consuming to execute, but also challenging to integrate into the daily workflow of clinical laboratories.

In this study, we examined the effectiveness of MALDI-TOF MS (Bruker Daltonics Inc., Billerica, MA, USA) for the identification of *Nocardia* species. Simultaneously, we aimed to explore a streamlined, rapid, and accurate workflow for identifying *Nocardia* strains that can be easily integrated into routine microbiology laboratory practices.

Methods

Nocardia isolates

This study analyzed 76 non-duplicate strains of *Nocardia* isolated from clinical specimens at Sichuan University West China Hospital from January 2016 to November 2022. All strains were stored at −80℃ and revived by transferring onto blood agar plates and cultured at 35℃. The length of time for different strains to culture varied slightly; however, typical colony characteristics were observed in most strains within 48–72 h.

DNA extraction, PCR, *16S rRNA/hsp65/ gyrB/secA1/rpoB* sequencing-based *Nocardia* Identification

Purified bacterial colonies were processed for DNA extraction using a TIAamp Bacteria DNA Kit (TIAN-GEN Biotech, Beijing, China), a centrifugal column-based kit, according to the manufacturer's instructions. Sangon Biotech Co., Ltd. (Shanghai, China) synthesized the partial *16S rRNA, gyrB, secA1, hsp65, and rpoB.*

Primers for *16S rRNA, gyrB, secA1, hsp65, and rpoB* Table 1 lists the primers and PCR cycling conditions. After PCR amplification, the products were sent to Sangon Biotech Co. Ltd. (Shanghai, China) for bidirectional sequencing. The bidirectional sequence results were assembled using the DNASTAR/SeqMan software (7.1.0.44), and the sequences were cross-referenced with the type strains of *Nocardia* species present in the GenBank database using the BLAST program at NCBI for analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

According to the CLSI MM18, to classify two strains of aerobic actinomycetes as the same species, they must have a sequence similarity of at least 99.6%, with a minimum separation of 0.4% between different species. Furthermore, this document suggests that a sequence similarity of 99.0 to 99.5% is appropriate for identifying the genus. If a similarity of 99.0% in the 16 S rRNA gene sequence was detected between *Nocardia* species and multiple strains, species identification was performed by sequencing several other housekeeping genes: *gyrB, secA1, hsp65,* and *rpoB.*

MALDI-TOF MS identification

The MALDI biotyper microflex LT (Bruker Daltonics, Inc., Billerica, MA, USA) was used to analyze all *Nocardia* spp. isolates. To ensure calibration and quality control, a Bacterial Test Standard from the manufacturer was added each day. MALDI Biotyper 3.1 software (Bruker Daltonics) was used with the BDAL database (V09.0.0.0_8468) to identify *Nocardia* through extended direct transfer and a new, simplified extraction sample preparation. The MBT Compass 4.1 software (Bruker Daltonics) was used with the BDAL database (V11.0.0.0_10833) to identify *Nocardia* through extended direct transfer sample preparation. Two spot on the MALDI target plate was used for each strain, with the maximum score and the corresponding identification as determined by MALDI-TOF MS being documented. The experiment utilized two preprocessing methods, extended direct transfer and the new and simplified extraction sample preparation, to identify 29 Nocardia strains using the MALDI Biotyper 3.1 software with the BDAL database (V09.0.0.0_8468). Additionally, for the identification of 76 Nocardia strains, the extended direct transfer method was applied in conjunction with both the MALDI Biotyper 3.1 software using the BDAL database (V09.0.0.0_8468) and the MBT Compass 4.1 software along with the updated BDAL database (V11.0.0.0_10833).

The extended direct transfer method was carried out as follows. A small quantity of bacteria was deposited onto a MALDI target plate. 70% formic acid was poured over the samples, which were allowed to dry before the

Table 1 PCR primers and DNA amplification conditions

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)	PCR cycling conditions	Reference or source
16S rRNA	GCTTAACA CATGCAAG TCG	GAATTCC AGTCTCC CCTG	95 ℃, 5 min; (95 ℃, 10 s; 55 ℃, 10 s; 72 ℃, 30 s) 40 cycles; 72 ℃, 1 min.	[16]
rpoB	CGACCAC TTCGGCAA CCG	TCGATCG GGCACAT CCGG	95 ℃, 5 min; (95 ℃, 10 s; 55 ℃, 10 s; 72 ℃, 50 s) 40 cycles; 72 ℃, 1 min.	[21]
gyrB	CTTCGCCA ACACCATC AACAC	TGATGATC GACTGGA CCTCG	95 ℃, 5 min; (95 ℃, 10 s; 60 ℃, 10 s; 72 ℃, 50 s) 40 cycles; 72 ℃, 1 min.	[20]
secA1	GCGACGC CGAGTGGA TGG	TTGGCCT TGATGGC GTTGTTC	95 ℃, 5 min; (95 ℃, 10 s; 65 ℃, 10 s; 72 ℃, 30 s) 40 cycles; 72 ℃, 1 min.	[20]
hsp65	ACCAACGA TGGTGTGT CCAT	CTTGTCG AACCGCA TACCCT	95 ℃, 5 min; (95 ℃, 10 s; 60 ℃, 10 s; 72 ℃, 30 s) 40 cycles; 72 ℃, 1 min.	[20]

addition of 1 μl of a matrix solution known as α-cyano-4-hydroxycinnamic acid (α-HCCA).

A new and simplified extractionwas carried out as follows, 30 μL of 70% formic acid solution was added to a sterilized EP tube (Eppendorf tube), and the bacterial sample was scraped into the tube for thorough mixing. The mixture was left to stand for 10 min, and 1 μL of the mixture was applied onto MALDI target plate and once the drying process was completed, 1 μL of HCCA matrix was added, as instructed by the manufacturer. All the samples were identified using MALDI-TOF MS, and the highest score was recorded.

An analysis was conducted to compare the outcomes of the extended direct transfer method and the new and simplified extraction method. Strains identified at the species level with a confidence score exceeding 1.7 were classified as 'a'. In contrast, those that failed to be identified at the species level and exhibited a confidence score below 1.7 were designated as 'b'. The counts of strains for each method were recorded, and McNemar's test was employed to ascertain whether there was a statistically significant disparity in the identification capacities of the two methods for *Nocardia* spp. A *P*-value<0.05 was considered statistically significant. All analyses were performed using the Statistical Package for the Social Sciences version 24.0 software (IBM SPSS Inc., Chicago, IL, USA).

In-house library creation

According to the steps described in the "IVD MALDI Biotyper 2.3 User Manual for the fully automated rapid biomarker mass spectrometry detection system", *Nocardia* species that could not be identified at the species level by the V09.0.0.0_8468 database version were added as MSP of the strains that produced identification results that were obtained using gene sequencing.

Before acquiring the spectral data, a freshly prepared bacterial test standard (Bruker Daltonics) was used to calibrate the instrument. Individual colonies were placed at eight different locations within the target plate, and readings were taken three times per position. Any isolate included as a reference MSP in the in-house library was required to have at least 20 indistinguishable spectra. The fingerprint spectra were manually collected, accumulated until the signal intensity reached over 10,000, and saved in a folder. The MALDI Biotyper software was used for library creation.

Results

Identification by genes sequencing

Sequencing of the 16*S rRNA/hsp65/gyrB/secA1/rpoB* genes allowed the identification of a combined total of 76 *Nocardia* isolates. Twelve different species of *Nocardia spp.* were identified. The detailed alignment results

of all sequencing data are provided in Supplementary Table 1 (Sequencing of the *16 S rRNA*, *gyrB*, *hsp65*, *rpoB*, and *secA1* genes was conducted and compared against the GeneBank database using the BLAST software). The results of bidirectional sequencing were assembled and then uploaded to the BLAST software for sequence alignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The result with the highest percent identity (Per.Ident) score was recorded. In cases where different identification outcomes were obtained and the difference in Per. Ident scores between them was less than 0.4%, a definitive identification could not be established, and both or multiple results were included in the final reference range. Results with a Per. Ident score below 95% were not considered for reference. For instance, the strain HX001 exhibited sequence alignment results for the *16 S rRNA* gene with both *Nocardia farcinica* (Per. Ident: 100%) and *Nocardia kroppenstedtii* (Per. Ident: 100%). The *gyrB* gene sequencing alignment results included *Nocardia farcinica* (Per. Ident: 100%), *Nocardia jejuensis* (Per. Ident: 100%), *Nocardia globerula* (Per. Ident: 100%), and *Nocardia exalbida* (Per. Ident: 99.66%), none of which provided a definitive identification. The final identification was achieved by integrating the *hsp65* gene result, identifying *Nocardia farcinica* (Per. Ident: 100%), the *rpoB* gene result as *Nocardia farcinica* (Per. Ident: 99.15%), and the *secA* gene result (Per. Ident: 99.38%), leading to the conclusive identification of the strain as *Nocardia farcinica*. The final identification was determined by integrating the test results of the *16 S rRNA* gene with the other four genes. All isolated strains were unequivocally identified to the species level through sequencing. The data from this study was deposited in NCBI Sequence Read Archive under accession SRA data: PRJNA1091814. The numbers and composition ratios of the species are shown in Fig. 1.

Comparison of extended direct transfer and a new and simplified sample preparation using maldi biotyper 3.1 with BDAL database (V09.0.0.0_8468) for Nocardia identification

Using Extended direct transfer and a new and simplified sample preparation method, 29 Nocardia spp. isolates were identified using MALDI Biotyper 3.1 software was applied with the BDAL database (V09.0.0.0_8468). The specific identification results and scores are listed in Supplementary Table 2. We compiled the quantitative data on species and genus identifications by the two methods across different bacterial strains, including the distribution of confidence scores for species-level identifications, as shown in Table 2. Strains identified at the species level with a confidence score exceeding 1.7 were classified as 'a'. In contrast, those that failed to be identified at the
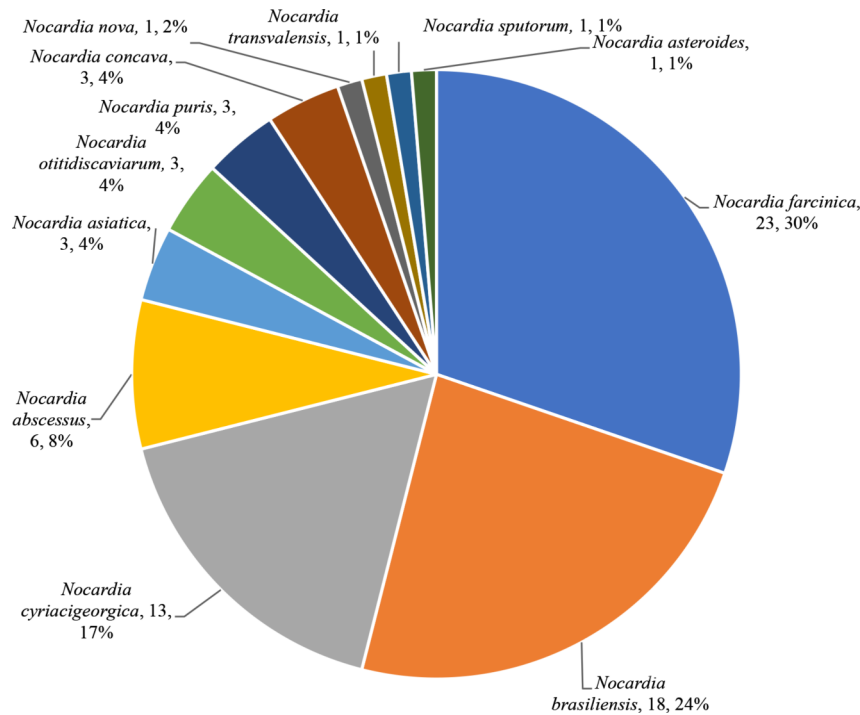
Fig. 1 Species identification of 76 *Nocardia* isolates, through sequencing of *16S rRNA/hsp65/gyrB/secA1/rpoB* gene

Table 2 Comparison of extended direct transfer and a new simplified extraction method for Sample Preparation: identification of *Nocardia Spp.* Using the BDAL database V09.0.0.0_8468

Identification by genes sequencing	Number of isolates	the Extended direct transfer					A new and simplified extraction				
		Score > 2.0	Score 1.99–1.7	Score < 1.7	Species level	Genus level	Score > 2.0	Score 1.99–1.7	Score < 1.7	Species level	Genus level
Nocardia brasiliensis	13	/	6	1	7	6[a]	1	8	1	10	3[b]
Nocardia farcinica	4	3	1	/	4	/	4	/	/	4	/
Nocardia cyriacigeorgica	4	1	1	2	4	/	1	3	/	4	/
Nocardia abscessus	3	3	/	/	3	/	2	1	/	3	/
Nocardia otitidiscaviarum	2	2	/	/	2	/	2	/	/	2	/
Nocardia puris	1	1	/	/	1	/	1	/	/	1	/
Nocardia concava	1	/	/	1	1	/	/	1	/	1	/
Nocardia transvalensis	1	/	/	1	1	/	1	/	/	1	/
Total	29	10	8	5	23	6	12	13	1	26	3

Identification is done through: (i) extended direct transfer, and (ii) a new and simplified extraction sample preparation method

[a] Based on the sequencing results, six isolates of *Nocardia brasiliensis* were classified as *Nocardia* sp. using the MALDI Biotyper 3.1 BDAL database V09.0.0.0_8468

[b] Based on the sequencing results, three isolates of *Nocardia brasiliensis* were classified as *Nocardia* spp. utilizing the MALDI Biotyper 3.1 BDAL database V09.0.0.0_8468

Table 3 Frequency distribution of identifications by extended direct transfer and the new simplified extraction method

the Extended direct transfer	A new and simplified extraction	Frequency
a	a	18
a	b	0
b	b	4
b	a	7

a: Identified to the species level with a confidence score > 1.7

b: Not identified to the species level or had a confidence score < 1.7

species level and/or exhibited a confidence score below 1.7 were designated as 'b'. The statistical results are presented in Table 3.

The identification results for 29 *Nocardia spp.* isolates using the two methods showed that with the MALDI Biotyper 3.1 and BDAL Database (V09.0.0.0_8468), we achieved 100% genus identification accuracy. However species identification varied with the sample preparation method: it was 79.3% (23/29) using the extended direct transfer method, with six strains identified as *Nocardia spp.* and confirmed as *Nocardia brasiliensis* by

sequencing (Table 2). Conversely, the new and simplified extraction method improved the species identification accuracy to 89.7% (26/29), with three strains identified as *Nocardia spp.* and confirmed as *Nocardia brasiliensis* by sequencing (Table 2). Compared to the extended direct transfer method, the new and simplified extraction method increased the proportion of strains identified at the species level with a confidence score > 1.7 from 62.0% (18/29) to 86.2% (25/29), an improvement of 24.2% (7/29). McNemar's test revealed a statistically significant difference in the accuracy of *Nocardia spp.* preprocessing between the two experimental methods, with $P=0.016$.

The strains HX007, HX018, and HX029 were identified as *Nocardia* spp. by MALDI-TOF MS. Sequencing identified these species as *Nocardia brasiliensis*. These strains were added as MSPs to an in-house library for future reference and retesting. The results confirmed their identification and the scores showed a significant improvement (Table 4).

Comparison of *Nocardia spp.* identification capabilities between maldi biotyper 3.1 with BDAL database (V09.0.0.0_8468) and MBT compass 4.1 with BDAL database (V11.0.0.0_10833) using extended direct transfer sample preparation

Using the Extended Direct Transfer Sample Preparation Methods, 76 *Nocardia* spp. isolates were identified using MALDI Biotyper 3.1 with BDAL Database (V09.0.0.0_8468) and MBT Compass 4.1 software with the BDAL database (V11.0.0.0_10833). The specific identification results and scores refer to (Table 5).

Strains identified at the species level with a confidence score exceeding 1.7 were classified as 'a'. In contrast, those that failed to be identified at the species level and/or exhibited a confidence score below 1.7 were designated as 'b'. The statistical results are presented in Table 6.

Compared to the sequencing outcomes, MALDI Biotyper 3.1 with the BDAL Database (V09.0.0.0_8468) achieved a genus identification accuracy of 100% and a species identification accuracy of 84.2% (64/76) for Nocardia spp. Among the results at the species level, 47.37% (36/76) had scores above 2.0, 30.26% (23/76) fell within the range of 1.7 to 1.99, and 6.58% (5/76) had scores below 1.7. 14.5% (11/76) of strains were identified as Nocardia spp., and according to the sequencing

results, all eleven were identified as Nocardia brasiliensis (Table 5). However, one strain of Nocardia sputorum was erroneously identified as Nocardia araoensis.

Compared to the sequencing results, MBT Compass 4.1 with the BDAL Database (V11.0.0.0_10833) achieved a genus identification accuracy of 100% and 98.7% accuracy (75/76) in Nocardia species identification. At the species level, 64.47% (49/76) scored more than 2.0, 30.26% (23/76) scored between 1.7 and 1.99, and 3.95% (3/76) scored less than 1.7. One strain of Nocardia sputorum was misidentified as *Nocardia araoensis*.

Eleven isolates were identified as *Nocardia* sp. using the MALDI Biotyper 3.1 BDAL database (V09.0.0.0_8468) and were correctly identified as *Nocardia brasiliensis* using MBT Compass 4.1 software with the BDAL database (V11.0.0.0_10833). Comparing the identification capabilities of the two database versions for Nocardia, the MBT Compass 4.1 with the updated BDAL Database (V11.0.0.0_10833) showed an improvement over the MALDI Biotyper 3.1 BDAL database (V09.0.0.0_8468) in the proportion of *Nocardia* species accurately identified to the species level with a score > 1.7, increasing from 77.6% (59/76) to 94.7% (72/76). McNemar's Test indicated a significant enhancement in identification capability after the database upgrade, with a $P=0.001$, indicating a statistically significant difference between the two methods. Both databases incorrectly identified one strain of Nocardia sputorum as Nocardia araoensis. For the remaining strains, no species misidentifications occurred, even when the confidence scores were below 1.7.

Discussion

The MALDI-TOF MS identification technique has significantly transformed clinical bacteriology by offering a swift and dependable method for bacterial identification, which is crucial for clinicians in making informed treatment decisions. This technology holds particular relevance for the identification of Nocardia species, as it allows for the inference of antibiotic susceptibility profiles directly from the identification process [27].

The 16S rRNA sequence analysis is commonly used for bacterial identification. However, studies have shown that this sequence cannot effectively distinguish closely related *Nocardia* species [1, 13]. Consistent with previous reports [15], this study also revealed that 16S rRNA sequencing was unable to accurately distinguish between certain *Nocardia* species. For instance, the 16 S rRNA identification results for 16 strains of *Nocardia farcinica*, including HX001, HX004, HX006, etc., were identified as both *Nocardia kroppenstedtii* and *Nocardia farcinica*, with Per.Ident scores all exceeding 99.0% and the difference in scores being less than 0.4%. Similarly, the 16S rRNA identification results for three strains of *Nocardia*

Table 4 Identification results and scoring comparison of three *Nocardia spp.* After added as MSPs to the in-house library

Strains no.	BDAL 8468 database	Score	BDAL 8468 database + In-house library	Score
HX007	*Nocardia* sp.	1.793	*Nocardia brasiliensis*	2.379
HX018	*Nocardia* sp.	1.608	*Nocardia brasiliensis*	2.395
HX029	*Nocardia* sp.	1.839	*Nocardia brasiliensis*	2.283

Liu *et al. BMC Microbiology*   (2024) 24:358

Page 7 of 11

Table 5 Identification of Nocardia isolates using DNA sequencing and Bruker MALDI-TOF MS through extended direct transfer sample preparation

Strains no.	Identification by genes sequencing	Identification by MALDI-TOF MS(BDAL database V09.0.0.0_8468)	Score	Identification by MALDI-TOF MS(BDAL database V11.0.0.0_10833)	Score
HX001	*Nocardia farcinica*	*Nocardia farcinica*	2.2	*Nocardia farcinica*	2.37
HX002	*Nocardia concava*	*Nocardia concava*	1.633	*Nocardia concava*	1.72
HX003	*Nocardia brasiliensis*	*Nocardia sp*	1.878	*Nocardia brasiliensis*	2.24
HX004	*Nocardia farcinica*	*Nocardia farcinica*	2.144	*Nocardia farcinica*	1.87
HX005	*Nocardia brasiliensis*	*Nocardia brasiliensis*	1.805	*Nocardia brasiliensis*	2.12
HX006	*Nocardia farcinica*	*Nocardia farcinica*	1.946	*Nocardia farcinica*	1.62
HX007	*Nocardia brasiliensis*	*Nocardia sp*	1.793	*Nocardia brasiliensis*	1.86
HX008	*Nocardia brasiliensis*	*Nocardia brasiliensis*	1.768	*Nocardia brasiliensis*	2.31
HX009	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	2.209	*Nocardia cyriacigeorgica*	1.95
HX010	*Nocardia abscessus*	*Nocardia abscessus*	2.265	*Nocardia abscessus*	2.05
HX011	*Nocardia otitidiscaviarum*	*Nocardia otitidiscaviarum*	2.179	*Nocardia otitidiscaviarum*	2.2
HX012	*Nocardia brasiliensis*	*Nocardia brasiliensis*	1.963	*Nocardia brasiliensis*	2.08
HX013	*Nocardia puris*	*Nocardia puris*	2.056	*Nocardia puris*	2.28
HX014	*Nocardia brasiliensis*	*Nocardia brasiliensis*	1.83	*Nocardia brasiliensis*	2.07
HX015	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	1.571	*Nocardia cyriacigeorgica*	1.86
HX016	*Nocardia abscessus*	*Nocardia abscessus*	2.305	*Nocardia abscessus*	1.8
HX017	*Nocardia transvalensis*	*Nocardia transvalensis*	1.604	*Nocardia transvalensis*	1.89
HX018	*Nocardia brasiliensis*	*Nocardia sp*	1.608	*Nocardia brasiliensis*	1.88
HX019	*Nocardia farcinica*	*Nocardia farcinica*	2.174	*Nocardia farcinica*	2.01
HX020	*Nocardia brasiliensis*	*Nocardia brasiliensis*	1.658	*Nocardia brasiliensis*	2.17
HX021	*Nocardia abscessus*	*Nocardia abscessus*	2.324	*Nocardia abscessus*	2.15
HX022	*Nocardia brasiliensis*	*Nocardia sp*	2.028	*Nocardia brasiliensis*	2.22
HX023	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	1.611	*Nocardia cyriacigeorgica*	1.69
HX024	*Nocardia brasiliensis*	*Nocardia sp*	1.41	*Nocardia brasiliensis*	1.62
HX025	*Nocardia brasiliensis*	*Nocardia brasiliensis*	1.998	*Nocardia brasiliensis*	2.04
HX026	*Nocardia brasiliensis*	*Nocardia brasiliensis*	1.767	*Nocardia brasiliensis*	2.31
HX027	*Nocardia otitidiscaviarum*	*Nocardia otitidiscaviarum*	2.365	*Nocardia otitidiscaviarum*	2.36
HX028	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	1.967	*Nocardia cyriacigeorgica*	1.82
HX029	*Nocardia brasiliensis*	*Nocardia sp*	1.839	*Nocardia brasiliensis*	2.02
HX030	*Nocardia brasiliensis*	*Nocardia sp*	1.85	*Nocardia brasiliensis*	2.23
HX031	*Nocardia farcinica*	*Nocardia farcinica*	2.16	*Nocardia farcinica*	2.16
HX032	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	2.18	*Nocardia cyriacigeorgica*	2.18
HX033	*Nocardia asiatica*	*Nocardia asiatica*	1.86	*Nocardia asiatica*	2.02
HX034	*Nocardia brasiliensis*	*Nocardia sp*	1.95	*Nocardia brasiliensis*	2.41
HX035	*Nocardia puris*	*Nocardia puris*	2.16	*Nocardia puris*	2.16
HX036	*Nocardia abscessus*	*Nocardia abscessus*	2.28	*Nocardia abscessus*	2.28
HX037	*Nocardia sputorum*	*Nocardia araoensis*	1.96	*Nocardia araoensis*	1.96
HX038	*Nocardia farcinica*	*Nocardia sp*	1.76	*Nocardia farcinica*	1.85
HX039	*Nocardia farcinica*	*Nocardia farcinica*	2.08	*Nocardia farcinica*	2.08
HX040	*Nocardia concava*	*Nocardia concava*	1.83	*Nocardia concava*	1.83
HX041	*Nocardia concava*	*Nocardia concava*	1.93	*Nocardia concava*	1.93
HX042	*Nocardia brasiliensis*	*Nocardia sp*	1.72	*Nocardia brasiliensis*	2.22
HX043	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	2.25	*Nocardia cyriacigeorgica*	2.25
HX044	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	2.29	*Nocardia cyriacigeorgica*	2.29
HX045	*Nocardia farcinica*	*Nocardia farcinica*	1.96	*Nocardia farcinica*	1.96
HX046	*Nocardia farcinica*	*Nocardia farcinica*	1.92	*Nocardia farcinica*	1.92
HX047	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	1.96	*Nocardia cyriacigeorgica*	1.96
HX048	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	2.25	*Nocardia cyriacigeorgica*	2.25
HX049	*Nocardia nova*	*Nocardia nova*	2.03	*Nocardia nova*	2.03
HX050	*Nocardia abscessus*	*Nocardia abscessus*	2.36	*Nocardia abscessus*	2.36
HX051	*Nocardia otitidiscaviarum*	*Nocardia otitidiscaviarum*	2.38	*Nocardia otitidiscaviarum*	2.38

Liu *et al. BMC Microbiology*   (2024) 24:358

Page 8 of 11

Table 5 (continued)

Strains no.	Identification by genes sequencing	Identification by MALDI-TOF MS(BDAL database V09.0.0.0_8468)	Score	Identification by MALDI-TOF MS(BDAL database V11.0.0.0_10833)	Score
HX052	*Nocardia farcinica*	*Nocardia farcinica*	2.23	*Nocardia farcinica*	2.23
HX053	*Nocardia farcinica*	*Nocardia farcinica*	2.22	*Nocardia farcinica*	2.22
HX054	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	2.09	*Nocardia cyriacigeorgica*	2.09
HX055	*Nocardia farcinica*	*Nocardia farcinica*	1.96	*Nocardia farcinica*	1.96
HX056	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	2.12	*Nocardia cyriacigeorgica*	2.12
HX057	*Nocardia asteroides*	*Nocardia asteroides*	2.2	*Nocardia asteroides*	2.2
HX058	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	2.25	*Nocardia cyriacigeorgica*	2.25
HX059	*Nocardia asiatica*	*Nocardia asiatica*	1.94	*Nocardia asiatica*	1.94
HX060	*Nocardia farcinica*	*Nocardia farcinica*	2.02	*Nocardia farcinica*	2.02
HX061	*Nocardia farcinica*	*Nocardia farcinica*	1.93	*Nocardia farcinica*	1.93
HX062	*Nocardia farcinica*	*Nocardia farcinica*	1.87	*Nocardia farcinica*	1.87
HX063	*Nocardia farcinica*	*Nocardia farcinica*	2.09	*Nocardia farcinica*	2.09
HX064	*Nocardia farcinica*	*Nocardia farcinica*	2.4	*Nocardia farcinica*	2.4
HX065	*Nocardia farcinica*	*Nocardia farcinica*	1.94	*Nocardia farcinica*	1.94
HX066	*Nocardia farcinica*	*Nocardia farcinica*	2.21	*Nocardia farcinica*	2.21
HX067	*Nocardia farcinica*	*Nocardia farcinica*	2.05	*Nocardia farcinica*	2.05
HX068	*Nocardia farcinica*	*Nocardia farcinica*	2.2	*Nocardia farcinica*	2.2
HX069	*Nocardia farcinica*	*Nocardia farcinica*	2.13	*Nocardia farcinica*	2.13
HX070	*Nocardia farcinica*	*Nocardia farcinica*	2.23	*Nocardia farcinica*	2.23
HX071	*Nocardia brasiliensis*	*Nocardia sp*	1.92	*Nocardia brasiliensis*	2.34
HX072	*Nocardia brasiliensis*	*Nocardia brasiliensis*	1.87	*Nocardia brasiliensis*	2.25
HX073	*Nocardia asiatica*	*Nocardia asiatica*	1.89	*Nocardia asiatica*	1.89
HX074	*Nocardia puris*	*Nocardia puris*	1.89	*Nocardia puris*	1.89
HX075	*Nocardia abscessus*	*Nocardia abscessus*	2.14	*Nocardia abscessus*	2.14
HX076	*Nocardia cyriacigeorgica*	*Nocardia cyriacigeorgica*	1.94	*Nocardia cyriacigeorgica*	1.94

Table 6 Frequency distribution of identifications by MALDI biotyper 3.1 with BDAL Database (V09.0.0.0_8468) and MBT compass 4.1 with BDAL database (V11.0.0.0_10833)

The Extended direct transfer	A new and simplified extraction	Frequency
a	a	58
a	b	1
b	b	3
b	a	14

a: Identified at the species level with a confidence score > 1.7

b: Not identified at the species level or had a confidence score < 1.7

concava, numbered HX002, HX040, HX041, were identified as both *Nocardia sp.* and *Nocardia concava*, with Per.Ident scores all exceeding 99.0% and the difference in scores being less than 0.4%. Additionally, the 16S rRNA identification results for 11 strains of *Nocardia brasiliensis*, including HX003, HX005, HX007, etc., were identified as *Nocardia brasiliensis*, *Nocardia sp.*, and *Nocardia vulneris*, with Per.Ident scores all exceeding 99.0% and the difference in scores being less than 0.4%. However, it is worth mentioning that sequencing a single gene locus is not sufficient to accurately identify a significant proportion of *Nocardia* species [20]. *Nocardia* species can

be accurately identified by combining the *16S rRNA* gene with the *hsp65, gyrB, secA1, and rpoB* genes.

In the present study, *Nocardia farcinica* (30%), *Nocardia brasiliensis* (24%), and *Nocardia cyriacigeorgica* (17%) were the three most common clinical *Nocardia* species. *N. cyriacigeorgica* and *N. farcinica* have been the most commonly reported clinical strains in previous studies [26, 27, 35], which is consistent with the results of the present study. However, in studies conducted by Xiao et al. [26] and Wang et al. [35], the proportion of *Nocardia brasiliensis* was low. In another study conducted by Lao et al. in Taiwan [36], *Nocardia brasiliensis* showed the highest clinical isolation rate. In this study, *Nocardia brasiliensis* ranked second in terms of the isolation rate, raising the question whether *Nocardia* species causing human infection vary from one geographical region to another.

The high costs and requirements of sequencing facilities hinder the implementation of sequencing-based methods in routine clinical laboratory operations, potentially impacting the efficiency and timeliness of result generation. The emergence of mass spectrometry technology has enabled the rapid and accurate clinical identification of *Nocardia* bacteria.

Liu *et al. BMC Microbiology*   (2024) 24:358

Page 9 of 11

Previous studies have shown that accurate identification of *Nocardia* species requires the use of in-house libraries developed [24, 26, 33, 37], or additional tools and methods such as VITEK®- PICKMETM pen and a formic acid-based protein extraction [27] (with identification accuracies of 93.7–95.9%). With the expansion of commercial databases, the identification capabilities of *Nocardia* mass spectrometry have gradually improved. According to the research by M. Marín et al., the accuracy of identifying *Nocardia* species using MALDI-TOF MS with the updated BDAL database (Bruker Daltonics) containing 6,903 MSPs reached 94.5% [33]. Although this version of the database precedes the (V09.0.0.0_8468) version, our study revealed that the accuracy of identifying *Nocardia* species with the 8468 MSPs database was 84.2% at the species level (64 out of 76), indicating a noticeable discrepancy. The primary analysis for this discrepancy suggests that our study included a larger number of *N. brasiliensis* isolates, totaling 18, whereas M. Marín's study had a smaller representation of this species with only 2 isolates. The insufficient diversity of strains in the earlier version (V09.0.0.0_8468) may have failed to uncover its limitations in identifying *N. brasiliensis*, highlighting a potential flaw in the database's ability to accurately identify this species.

In the study by Gema Carrasco and colleagues, *Nocardia* species were categorized into three groups based on their prevalence in Spain [25]. The consistency of MALDI-TOF MS identification results compared to 16 S rRNA analysis for high and intermediate prevalence *Nocardia* species was 76% and 45%, respectively, indicating that the proteomic technique has room for improvement in accurately identifying *Nocardia* species. However, in our study, which included strains from the high and intermediate prevalence groups, excluding *N. nova* and *N. carnea*, we achieved a species identification accuracy of 98.7% (75/76) using the MBT Compass 4.1 (V11.0.0.0_10833). This suggests that the upgraded MALDI-TOF MS database has significantly enhanced its capability for identifying common *Nocardia* species.

Similar to the study by Melanie L. Yarbrough [34], our research has shown that the use of the Bruker mass spectrometry system with the direct on-target extraction method yields excellent identification results for *Nocardia* species. Yarbrough's study compared three extraction methods: mycobacterial extraction, ethanol formic acid extraction, and direct on-target extraction, with the latter proving to be the superior approach. In line with their findings, our study found that for databases version V11.0.0.0_10833 and above, the direct on-target extraction, also known as Extended Direct Transfer, provides reliable identification results. In practical applications, due to the dry nature of *Nocardia*, which can be challenging to collect from agar plates, we can perform two spotting attempts on the target plate and record the highest score as the final result, obviating the need for more complex extraction methods. However, when using lower version databases or when Extended Direct Transfer does not yield high identification scores (>1.7), the new and simplified extraction method can be employed. This method is less cumbersome than ethanol formic acid extraction and can enhance the reliability of the identification certain degree.

In this study, based on the species included in the BDAL database (V11.0.0.0_10833), the performance of Bruker MS using MBT Compass 4.1 software in identifying *Nocardia* through extended direct transfer sample preparation was highly effective. This method allowed for quick and reliable *Nocardia* identification in 98.7% (75/76) of the isolates using the same laboratory process as for conventional bacteria. The HX037 strain is *Nocardia sputorum*., which was newly discovered as a *Nocardia species* in 2023 by Hamada M et al., [38] was misidentified as *Nocardia araoensis*. The analysis revealed that the reason for this misidentification is the absence of the strain's spectral profile in the current V11.0.0.0_10833 of the BDAL database. Among the identified results, 64.47% (49/76) had a score of 2.0 or higher, 30.26% (23/76) had a score ranging from 1.7 to 1.99, and 3.95% (3/76) had a score below 1.7. According to the manufacturer's recommendations, a spectral score of ≥2.00 was considered species-level identification, a score of 1.700-1.999 indicated identification at the genus level, and a score of <1.70 was deemed unreliable for identification purposes. In this study, 34.2% of the *Nocardia* strains had identification scores below 2.0. However, with the exception of a single case where the clinically less common *Nocardia sputorum* was misidentified, when the highest score among all identification results was in agreement with the molecular identification results, MBT Compass 4.1 with the BDAL Database (V11.0.0.0_10833) fulfilled the clinical requirement for the rapid and accurate identification of Nocardia species. Similar to the findings of Melanie L. et al. [34]. , for *Nocardia spp.*, which is a challenging pathogen due to its difficult cell wall breakdown, adjusting the confidence threshold to 1.8 or 1.7 can yield satisfactory identification results with Bruker MS.

Database upgrades come at a certain cost, and unfortunately, not all laboratories have had the opportunity to undergo these important updates. Our research has shown that the extended direct transfer sample preparation method using Bruker MALDI-TOF MS (BDAL database v09.0.0.0_8468 database) has an identification accuracy of 100% for *Nocardia* at the genus level and 84.2% (64/76) at the species level. The main limitation of this database is the lack of diversity in MSP for *Nocardia brasiliensis* strains, resulting in some strains being identified only at the genus level. However, this

issue was effectively resolved in the most recent iteration of the database (BDAL database V11.0.0.0_10833) with supplementary improvements. For laboratories still using the v09.0.0.0_8468 database and unable to identify *Nocardia* to the genus level or with no identification, a new and simplified extraction sample preparation method is recommended. Although this pre-processing method may be cumbersome, it is simpler than formic acid-based protein extraction and easier to implement in clinical practice. This new and simplified extraction sample preparation method has the potential to improve identification success rates. However, if a new and simplified extraction sample preparation method fails to yield reliable identification, conditional laboratories should use molecular methods as an initial step to accurately identify strains. If the laboratory encounters difficulties in sequencing using four or five genes, it should at least perform sequencing based on *16 S rRNA*, along with *gyrB* and *secA* [15, 20] or sequence the full length of the *16 S rRNA* gene(1500 bp), and then add any one or more of the *gyrB*, *secA1*, *hsp65*, and *rpoB* for identification [15, 39]. Additionally, the laboratory should incorporate MSP into its in-house library using a standardized procedure with the aim of enhancing the accuracy of *Nocardia* identification.

This study had certain limitations. Some rare *Nocardia* species such as *N. wallacei*, *N. pseudobrasiliensis*, *N. veterana*, *N. carnea*, *N. elegans*, and *N. thailandica*, which were rarely found in other studies [27, 33], were not detected at our research center. Therefore, it was not possible to evaluate the identification effectiveness of the current database for these rare *Nocardia* species.

Conclusion

In conclusion, we evaluated the ability of the Bruker MALDI-TOF MS to identify *Nocardia* spp. The Bruker MALDI-TOF MS provides fast and reliable results for identifying *Nocardia* in the BDAL database V11.0.0.0_10833. The identification accuracy of the most common *Nocardia* species in this region was 98.7%. However, for the BDAL database V09.0.0.0_8468, there was a deficiency in the identification efficiency for *Nocardia brasiliensis*, and it was necessary to add the MSP into the in-house library to accurately identify *Nocardia* species.

Abbreviations

MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MALDI	Matrix-Assisted Laser Desorption/Ionization
TOF	Time-of-Flight
MS	Mass Spectrometry
16S rRNA	16 S Ribosomal RNA
hsp65	Heat Shock Protein 65
gyrB	DNA Gyrase Subunit B
secA1	ATPase Secretory Protein
rpoB	RNA Polymerase B Subunit
BDAL	Bruker Daltonics Automated Library
MBT Compass	MALDI Biotyper Compass
CDC	Centers for Disease Control and Prevention
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
CLSI	Clinical and Laboratory Standards Institute
SRA	Sequence Read Archive
MSP	Matrix Spectra Profile
EP Tube	Eppendorf Tube
HCCA	α-Cyano-4-Hydroxycinnamic Acid
SPSS	Statistical Package for the Social Sciences

Supplementary Information

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Supplementary Material 1

Author contributions
Y.L. led the conception and design of the study, performed experiments, created figures, analyzed data, and prepared the manuscript. S.Y.W., J.D., and N.W. also contributed to the experimental work. Z.K.W participated in the experimental sequencing of this study. N.W., Y.T., and Y.L.X. played key roles in data analysis. W.L. Z. and Q.F.L. conceived the study and performed experiments. Additionally, M. K. substantively revised the manuscript and approved the final version for submission.

Data availability
The datasets supporting the conclusions of this article are included within the article (and its additional files).

Declarations

Ethical approval
This study has been approved by the Biomedical Research Ethics committee of West China Hospital of Sichuan University (approval number: 2021 (252)).

Consent for publication
Consent for publication is not applicable to this study as it does involve any individual person's data.

Competing interests
The authors declare no competing interests.

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