RESEARCH ARTICLE

Accurate Identification of Common Pathogenic Nocardia Species: Evaluation of a Multilocus Sequence Analysis Platform and Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

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Abstract

Species identification of Nocardia is not straightforward due to rapidly evolving taxonomy, insufficient discriminatory power of conventional phenotypic methods and also of single gene locus analysis including 16S rRNA gene sequencing. Here we evaluated the ability of a 5-locus (16S rRNA, gyrB, secA1, hsp65 and rpoB) multilocus sequence analysis (MLSA) approach as well as that of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) in comparison with sequencing of the 5’-end 606 bp partial 16S rRNA gene to provide identification of 25 clinical isolates of Nocardia. The 5’-end 606 bp 16S rRNA gene sequencing successfully assigned 24 of 25 (96%) clinical isolates to species level, namely Nocardia cyriacigeorgica (n = 12, 48%), N. farcinica (n = 9, 36%), N. abscessus (n = 2, 8%) and N. otitidiscaviarum (n = 1, 4%). MLSA showed concordance with 16S rRNA gene sequencing results for the same 24 isolates. However, MLSA was able to identify the remaining isolate as N. wallacei and clustered N. cyriacigeorgica into three subgroups. None of the clinical isolates were correctly identified to the species level by MALDI-TOF MS analysis using the manufacturer-provided database. A small "in-house" spectral database was established incorporating spectra of five clinical isolates representing the five species identified in this study. After complementation with the “in-house” database, the remaining 20 isolates, 19 (95%) were correctly identified to species level (score ≥ 2.00) and one (an N. abscessus strain) to genus level (score ≥ 1.70 and < 2.00). In summary, MLSA showed superior discriminatory power compared with the 5’-end 606 bp partial 16S rRNA gene sequencing for species identification of Nocardia. MALDI-TOF MS can provide rapid and accurate identification but is reliant on a robust mass spectra database.
Introduction

Nocardia species are ubiquitous environmental bacteria that cause suppurative infections in humans, including in the lung, central nervous system and skin [1]. Nocardiosis typically occurs in immunosuppressed patients such as organ and stem cell transplantation, and malignancy, but also affects immunocompetent hosts [2–4]. Since there are species-specific differences with regard to geography and disease patterns, identification of Nocardia to species level is important to determine both epidemiology and clinical associations.

Conventional culture identification of Nocardia based on phenotypic methods and distinct (but limited) antimicrobial susceptibility profiles lacks sufficient discriminatory power, is slow and time-consuming and requires staff expertise [5, 6]. Thus identification of Nocardia isolates by molecular methods is increasingly used, of which PCR-based methods combined with DNA sequencing are the most popular. Of these, 16S rRNA gene sequencing is considered the “gold-standard” [6, 7]. However, 16S rRNA gene sequencing is unable to distinguish certain closely related species due to insufficient interspecies gene polymorphisms [6, 8], whilst also unable to resolve certain species, e.g. Nocardia nova due to the presence of multiple yet different copies of this gene [9]. To overcome this limitation, other gene polymorphisms have been evaluated, such as those within the β-subunit of the type II DNA topoisomerase gene (gyrB) [10, 11], the subunit A of the SecA preprotein translocase gene (secA1) [12, 13], the 65-kDa heat shock protein gene (hsp65) [14, 15], and the β subunit of DNA-dependent RNA polymerase gene (rpoB) [11]. A multilocus sequence analysis (MLSA) scheme analyzing sequence polymorphisms of multiple Nocardia genes was reported to be accurate not only for the identification of known Nocardia species but the unveiling of novel species [16].

Other than genomic approaches, proteomic methods, most notably matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) method, have also been evaluated to identify Nocardia species in clinical laboratories. However, the presence of aliphatic acids in the cell wall of Nocardia has posed an obstacle in achieving satisfactory protein profiles. Furthermore, although improvements are continuing, many databases provided by commercial MALDI-TOF MS systems contain only a limited number of archived Nocardia spectral profiles [17, 18]. Previous studies have reported identification to species level in only 14.9–80.4% of isolates [18–23]. Complementation with profiles provided by “in-house” databases, which in turn relies on knowledge of local epidemiology, hence may assist with identification to the species, or even, genus level.

In the present study, we firstly evaluated a published MLSA scheme [16] employing polymorphisms in the 16S rRNA, gyrB, secA1, hsp65 and rpoB loci for the identification of clinical Nocardia isolates in our laboratory; and secondly, determined the ability of MALDI-TOF MS for species assignment. Results were compared to those obtained by the 5’-end 606 bp 16S rRNA gene sequencing. An “in-house” database of Nocardia protein profiles was established and evaluated for its ability to complement a commercial database for identification of Nocardia species.

Materials and Methods

Ethics

The study was approved by the Human Research Ethics Committee of Peking Union Medical College Hospital (PUMCHBC-C-2-Q01-1). Written informed consent was obtained from patients for the use of the samples in research.

Nocardia strains and reference sequences

Twenty-five clinical strains were studied in the evaluation of the ability of an MLSA scheme [16] and MALDI-TOF MS to provide species identification. Isolates were cultured from patients admitted to
the Peking Union Medical College Hospital from January 2009 to January 2015 (Table 1). All isolates were identified by standard phenotypic methods [24]. Isolates were stored at -80°C and subcultured on Columbia blood agar for 72 h to 96 h at 37°C to ensure adequate growth before study.

In addition, the GenBank sequences of 16S rRNA, gyrB, secA1, hsp65 and rpoB loci corresponding to 20 Nocardia type strains were studied as the “validation cohort” to determine the ability of the MLSA typing scheme [7, 16] to identify Nocardia clinical isolates collected (see S1 Table).

**DNA extraction, PCR amplification and sequencing**

DNA extraction of all isolates was performed as previously described [8]. The Nocardia 16S rRNA gene was amplified using the primer pair 27F and 1522R [25]. The Nocardia gyrB, secA1, hsp65 and rpoB genes were amplified as previously described [16]. In all cases, amplified PCR products were sequenced in both directions using the amplification primers on the ABI 3730XL platform (Applied Biosystems, Foster City, CA).

**Species identification by the 5’-end 606 bp partial 16S rRNA gene sequencing and MLSA**

Molecular-based species identification was initially carried out by analysis of the 5’-end 606 bp partial 16S rRNA gene sequences as described using a percentage similarity (or identity) score of ≥ 99% as the criterion to strictly classify an isolate to species level [7]. MLSA was then carried

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**Table 1. Nocardia isolates examined (n = 25) in the present study.**

| Strains no. | Age (years) | Gender | Medical department | Specimen type      | Immuno-Compromised (Yes/No) |
|------------|-------------|--------|--------------------|--------------------|-----------------------------|
| PUNC001    | 53          | Male   | Outpatient         | PICC Drainage      | No                          |
| PUNC002    | 70          | Female | Gastroenterology   | Sputum             | No                          |
| PUNC003    | 53          | Male   | Immunology         | Lung tissue        | Yes                         |
| PUNC004    | 64          | Male   | Respiratory        | Subcutaneous nodule| Yes                         |
| PUNC005    | 72          | Male   | Nephrology         | Sputum             | Yes                         |
| PUNC006    | 31          | Female | Emergency          | Sputum             | Yes                         |
| PUNC007    | 43          | Female | Thoracic Surgery   | Sputum             | No                          |
| PUNC008    | 50          | Male   | Emergency          | Hydrothorax fluid  | Yes                         |
| PUNC009    | 67          | Male   | Emergency          | Sputum             | No                          |
| PUNC010    | 70          | Male   | Respiratory        | Sputum             | Yes                         |
| PUNC011    | 70          | Male   | Respiratory        | Sputum             | No                          |
| PUNC012    | 51          | Male   | Respiratory        | Sputum             | No                          |
| PUNC013    | 76          | Male   | Respiratory        | Sputum             | No                          |
| PUNC014    | 45          | Female | Respiratory        | Lung tissue        | No                          |
| PUNC015    | 61          | Female | Immunology         | Hydrothorax fluid  | Yes                         |
| PUNC016    | 81          | Male   | Outpatient         | Sputum             | No                          |
| PUNC017    | 71          | Male   | Respiratory        | Sputum             | Yes                         |
| PUNC018    | 56          | Female | Respiratory        | BALF               | Yes                         |
| PUNC019    | 53          | Male   | Orthopedics        | Pus                | No                          |
| PUNC020    | 46          | Female | Outpatient         | Pus                | No                          |
| PUNC021    | 57          | Male   | Immunology         | Sputum             | Yes                         |
| PUNC022    | 32          | Female | Respiratory        | BALF               | Yes                         |
| PUNC023    | 45          | Male   | Emergency          | Sputum             | No                          |
| PUNC024    | 37          | Male   | Emergency          | Pus                | No                          |
| PUNC025    | 65          | Male   | Emergency          | Sputum             | No                          |

Abbreviations: PICC, peripherally inserted central catheter; BALF, bronchoalveolar lavage fluid; SLE, systemic lupus erythematosus; COPD, chronic obstructive pulmonary disease.

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out examining nucleotide polymorphisms in the partial 16S rRNA gene at 5'-end (462 bp), as well as the secA1 (445 bp), gyrB (482 bp), rpoB (400 bp) and hsp65 (401 bp) genes [16]. The concatenated gyrB-16S-secA1-hsp65-rpoB sequences (2190 bp) were then used for phylogenetic analysis by the maximum-likelihood algorithm based on the Tamura-Nei model [26] with 1000 bootstrap replication to ensure robustness using MEGA software (version 6.0, MEGA Inc., Englewood, NJ). DnaSP software (version 5.1, University of Barcelona, Spain) was used to assess the haplotypes of each gene [27]. Determining of potential subtypes of *N. cyriacigeorgica* isolates was further carried out by analysis of the *Nocardia* hsp65 gene as previously described [28, 29].

Establishment and validation of an “in-house” *Nocardia* mass spectrum database for MALDI-TOF MS

All *Nocardia* isolates was pretreated using a mechanical disruption-ethanol extraction method as described by Dunne *et al.* [30]. MALDI-TOF MS analysis of 25 clinical *Nocardia strains* was first carried by the Bruker Autoflex Speed TOF/TOF MS system using Biotyper software version 3.1 (db 4613, Bruker Daltonics, Billerica, USA) according to the manufacturer’s instructions. A spectral score of $< 1.70$ was considered not to provide reliable identification. A score of $\geq 1.70$ but $< 2.00$ indicated identification at the genus level, and a score of $\geq 2.00$ was considered identification at the species level [18].

Because the identification results using the manufacturer-provided Biotyper database was unsatisfactory (see “Results” below), five of the 25 clinical isolates representing five different *Nocardia* species (as identified by DNA sequencing in the present study—strain ID no. PUNC001 (*Nocardia farcinica*), PUNC002 (*Nocardia abscessus*), PUNC006 (*Nocardia cyriacigeorgica*), PUNC020 (*Nocardia wallacei*), and PUNC024 (*Nocardia otitidiscaviarum*; Table 2) were used to generate an “in-house” mass spectrum fingerprint database following the methodology of Segawa *et al.* [18]. Each isolate was analyzed in replicates of eight (ie. inoculated in eight spots on the target plate), and each spot was interrogated three times. This procedure was repeated at least once on a different occasion under identical test conditions. The resulting data were averaged using the Biotyper software v3.1 (Bruker Daltonics), a single mass spectrum representing each isolate was generated, and the resultant profiles established as a small “in-house” database.

The 20 remaining clinical isolates were then analyzed against the Biotyper version 3.1 database (Bruker Daltonics) complemented with the “in-house” database. The technicians performing the analysis were blinded to the DNA sequencing results to avoid potential bias.

A main spectrum profile (MSP) dendrogram was further constructed using spectra of 25 *Nocardia* clinical strains in this study along with 37 reference spectra of 32 *Nocardia* species already contained in the Biotyper version 3.1 database for analysis of genetic relatedness.

Review for publications on validation of MALDI-TOF MS in identification of *Nocardia* species

Key words “*Nocardia*” and “matrix-assisted laser desorption ionization-time of flight mass spectrometry” were used to search related publications in English archived in NCBI PubMed database (http://www.ncbi.nlm.nih.gov/pubmed, till Dec 31st, 2015). All complete articles of the search (16 publications in all) were screened. After excluding case reports, six publications were reviewed in detail.

GenBank accession numbers

The DNA sequences of the full-length 16S rRNA, secA1, gyrB, rpoB and hsp65 genes of 25 *Nocardia* isolates studied have been submitted to GenBank database (accession numbers
KT985911 to KT985935, KU052160 to KU052184, KU052085 to KU052109, KU052135 to KU052159 and KU052110 to KU052134 for the above five genes, respectively, S2 Table).

Results
Identification by the 5’-end 606 bp partial 16S rRNA gene sequencing

Of 25 Nocardia clinical strains, 24 isolates representing four Nocardia species were identified unambiguously by the 5’-end 606 bp partial 16S rRNA gene sequencing. N. cyriacigeorgica (n = 12, 48%) and N. farcinica (n = 9, 36%) were the most common species, followed by N. abscessus (n = 2, 8%) and N. otitidiscaviarum (n = 1, 4%). However, the 5’-end 606 bp partial 16S rRNA gene sequence of strain PUNC020 showed 99% sequence similarity to both N. wallacei type strain DSM 45136T (606/606, 100%) and Nocardia transvalensis type strain NRRL B-16037T (600/606, 99.0%), though was identical to the former.

Table 2. Identification results of 25 Nocardia clinical isolates by DNA sequencing and MALDI-TOF MS after interrogation against the Biotyper database (version 3.1) and the “in-house” database.

| Strain ID no. | Identification by MLSA | Identification by conventional methods | Identification by MALDI-TOF MS* | First reported species | Score |
|---------------|------------------------|---------------------------------------|-------------------------------|------------------------|-------|
| PUNC003       | N. cyriacigeorgica     | N. cyriacigeorgica                    | N. cyriacigeorgica            | 2.160                  |       |
| PUNC004       | N. farcinica           | Nocardia sp.                          | Nocardia sp.                  | 2.332                  |       |
| PUNC005       | N. cyriacigeorgica     | Nocardia sp.                          | N. cyriacigeorgica            | 2.129                  |       |
| PUNC007       | N. cyriacigeorgica     | N. asteroides                         | N. cyriacigeorgica            | 2.393                  |       |
| PUNC008       | N. farcinica           | Nocardia sp.                          | N. farcinica                  | 2.411                  |       |
| PUNC009       | N. farcinica           | N. farcinica                          | N. farcinica                  | 2.284                  |       |
| PUNC010       | N. cyriacigeorgica     | N. cyriacigeorgica                    | N. cyriacigeorgica            | 2.011                  |       |
| PUNC011       | N. cyriacigeorgica     | N. cyriacigeorgica                    | N. cyriacigeorgica            | 2.232                  |       |
| PUNC012       | N. farcinica           | Nocardia sp.                          | N. farcinica                  | 2.196                  |       |
| PUNC013       | N. cyriacigeorgica     | N. cyriacigeorgica                    | N. cyriacigeorgica            | 2.126                  |       |
| PUNC014       | N. farcinica           | N. farcinica                          | N. farcinica                  | 2.124                  |       |
| PUNC015       | N. farcinica           | N. farcinica                          | N. farcinica                  | 2.055                  |       |
| PUNC016       | N. cyriacigeorgica     | N. cyriacigeorgica                    | N. cyriacigeorgica            | 2.422                  |       |
| PUNC017       | N. abscessus           | N. abscessus                          | N. abscessus                  | 1.894                  |       |
| PUNC018       | N. cyriacigeorgica     | Nocardia sp.                          | N. cyriacigeorgica            | 2.307                  |       |
| PUNC019       | N. cyriacigeorgica     | N. cyriacigeorgica                    | N. cyriacigeorgica            | 2.145                  |       |
| PUNC021       | N. cyriacigeorgica     | Nocardia sp.                          | N. cyriacigeorgica            | 2.079                  |       |
| PUNC022       | N. farcinica           | Nocardia sp.                          | N. farcinica                  | 2.498                  |       |
| PUNC023       | N. farcinica           | Nocardia sp.                          | N. farcinica                  | 2.431                  |       |
| PUNC025       | N. cyriacigeorgica     | Nocardia sp.                          | N. cyriacigeorgica            | 2.052                  |       |

Abbreviation: N/A, not applicable.
* Results by using the Bruker Biotyper version 3.1 (Bruker Daltonics) complementation with the “in-house” database (see Materials and Methods for detail).

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Phylogenetic analysis of concatenated gyrB-16S-secA1-hsp65-rpoB sequences obtained from the 25 clinical isolates and 20 Nocardia type strain sequences clearly revealed five major clusters with bootstrap threshold values of ≥ 90% (Fig 1). Each cluster corresponded to a unique Nocardia species, i.e., N. cyriacigeorgica, N. otitidiscaviarum, N. farcinica, and N. abscessus, except for clinical strain PUNC020 that was clustered together with type strains of N. wallacei and N. transvalensis. As strain PUNC020 was more phylogenetically closely related to N. wallacei strain DSM 45136^T compared with N. transvalensis strain NRRL B-16037^T (Fig 1), the isolate was assigned as N. wallacei.

Twelve N. cyriacigeorgica clinical isolates could further be classified into three subgroups: subgroup a (n = 6), b (n = 4) and c (n = 2), (Fig 1A). Comparing with results obtained by N. cyriacigeorgica genotyping using sequence analysis of the hsp65 gene [28, 29], isolates belonging to N. cyriacigeorgica MLSA subgroups a, b and c corresponded to N. cyriacigeorgica hsp65 genotype I, III and II, respectively (Fig 1B).

Overall, it was observed that the rpoB, gyrB and secA1 genes were the most polymorphic (14 to 16 haplotypes identified amongst 25 clinical strains, haplotype diversity ≥ 0.950), followed...
by hsp65 (10 haplotypes, haplotype diversity = 0.837), whilst the partial 16S rRNA gene was the least polymorphic (five haplotypes, haplotype diversity = 0.657) (Table 3 and S2 Table). Different degrees of intra-species micro-heterogeneity of different genes were also observed (Table 3). N. cyriacigeorgica isolates generally exhibited higher intra-species diversity than other species e.g. N. farcinica (Table 3).

Improvement of MALDI-TOF MS identification capacity by the “in-house” database

Of the five species identified by the 5’-end 606 bp partial 16S rRNA gene sequencing and MLSA from the present study which we selected to establish a mini “in-house” database, representation of four species (N. cyriacigeorgica, N. farcinica, N. abscessus and N. otitidiscaviarum) was already included in the Biotyper version 3.1 database (Bruker Daltonics); however, N. wallacei was not represented. When all 25 clinical isolates were interrogated using only the Biotyper database, only eight of 25 (32%) isolates was identified to genus level, none (0%) to species level, and 17 isolates were not identified (68%).

Using the “in-house” database to complement the Biotyper database, 19 of 20 (95%) Nocardia clinical isolates were correctly identified to species level with scores of ≥ 2.0 (Table 2). N. abscessus strain PUNC017, which yielded a MALDI-TOF MS score of 1.894, was identified to genus level (Table 2).

The MSP dendrogram indicated that the spectral profile of clinical isolates were distinct from the corresponding reference spectra for its species in the Biotyper database (Fig 2). The mass spectra differences between N. abscessus strain PUNC002 (for establishment of the “in-house” database) and N. abscessus strain PUNC017 (for test of the “in-house” database) also resulted in failure in identifying strain PUNC017 to specie level using the “in-house” database (Fig 2). Therefore, the mass spectra of strain PUNC017 was incorporated into the “in-house” database for future study. Similar divergence of mass spectra were also noted in N. otitidiscaviarum for clinical strain PUNC024 versus strain VA_01844_09 and type strain DSM 43242T that included in the commercial database (Fig 2), which led to failure of identifying strain PUNC024 to species level using the commercial database.

Literature review for Nocardia identification by MALDI-TOF MS

Of six studies reviewed, five had evaluated the Bruker Biotyper commercial database (version 3.1 or 3.0.2) and one, an Andromas (Paris, France) system (Table 4). The Biotyper database

Table 3. Genetic polymorphisms contained within the 16S rRNA, gyrB, secA1, hsp65 and rpoB genes for 25 clinical Nocardia isolates studied.

| Characters | gyrB | 16S rRNA gene | secA1 | hsp65 | rpoB |
|------------|------|---------------|-------|-------|------|
| All clinical isolates (n = 25) | | | | | |
| No. of haplotypes | 16 | 5 | 14 | 10 | 16 |
| Haplotype diversity | 0.957 | 0.657 | 0.950 | 0.837 | 0.963 |
| N. cyriacigeorgica (n = 12) | | | | | |
| No. of haplotypes | 8 | 1 | 6 | 5 | 8 |
| Haplotype diversity | 0.924 | N/A | 0.848 | 0.803 | 0.924 |
| N. farcinica (n = 9) | | | | | |
| No. of haplotypes | 4 | 1 | 5 | 1 | 5 |
| Haplotype diversity | 0.778 | N/A | 0.889 | N/A | 0.861 |

Abbreviation: N/A, not applicable.

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only correctly identified 14.9–52.8% of isolates to species level and an additional 6.9–52.7% of isolates to genus level. The performance of Andromas system (Paris, France) was better (80.4%)

Fig 2. The main spectrum profile (MSP) dendrogram constructed using spectra of 25 Nocardia clinical strains along with 37 reference spectra of 32 Nocardia species contained in the original Biotyper database (version 3.1; Bruker). Strain identification numbers of clinical isolates collected in the present study are shown underlined, and isolates used for establishment of the “in-house” database was labeled with asterisks.

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to species level and 17.6% to genus level). Three studies further developed local “in-house” databases, and the correct identification rate was significantly improved to 79.1–90.6% to species level and an additional 4.7–11.5% to genus level (Table 4).

A proposed algorithm for species identification of *Nocardia* in clinical laboratories

Although the MLSA scheme was definitively able to assign species, identify novel species, and to differentiate within species, it has the disadvantage of relatively high costs, and requiring staff time for analysis of results. We propose an algorithm for species identification of *Nocardia* in the clinical laboratory (Fig 3), recommending that identification by MALDI-TOF MS using a database complemented by “in-house” protein profiles be attempted in the initial instance. For those isolates that cannot be identified to species level by MALDI-TOF MS, the 5'–606 bp partial 16S rRNA sequencing may be attempted. For any isolates with < 99% gene sequence similarity to a known archived strain sequence or those with two or more “best hit” species identities, MLSA could be undertaken for a definitive identification.

**Discussion**

To date, more than 50 species of pathogenic *Nocardia* have been identified [31]. Since there are differences in prevalence of species between geographic regions, knowledge of species distribution is important. In the present study, *N. cyriacigeorgica* and *N. farcinica* were the most...
commonly encountered species, similar to that in Canada [16], but in contrast to a report from Taiwan where *Nocardia brasiliensis* was the most common [32]. In comparison, *Nocardia asteroides* was reported to be predominant in Switzerland [31], while *N. nova* was predominant in Australia [7].

Most studies examining identification of *Nocardia* have indicated that molecular techniques are required to achieve species assignment [6, 16, 31, 32]. Although polymorphisms in the 5'-end partial 16S rRNA gene is widely used to discriminate between *Nocardia* species, misidentification may occur due to high sequence similarity between certain species [6, 7, 16, 33], as exemplified by inability to distinguish *N. wallacei* from *N. transvalensis* in the present study, or due to multiple different copies of this gene, such as that in *N. nova* [9]. *N. wallacei* is very closely related to *N. transvalensis* and indeed was previously classified within the *N. transvalensis* complex. Using a more discriminatory method, in this case, a 5-locus MLSA, *N. wallacei* was assigned as an individual species, which supported findings by Conville *et al.* [12].

In this context then, MLSA has been carried out as an alternative technique for the identification of *Nocardia*. The most commonly used genes have been the 16S rRNA, *secA1*, *gyrB*, *rpoB* and *hsp65* genes. Different studies may employ different combination of the genes [11, 16, 34], yet it is notable that no single gene locus sequencing is sufficient to resolve a substantial number of *Nocardia* species [16]. In addition, the MLSA scheme can be used for examining intra-species genetic diversity [10, 11, 13]. Using this approach, Carrasco *et al.* has reported that species *N. nova* had highest intra-species heterogeneity, followed by *N. cyriacigeorgica* and *N. abscessus*, while *N. farcinica* was more conserved [11]. Although the diversity of *N. abscessus* and *N. nova* were not compared in this study due to only a limited number of isolates studied, we similarly found genetic heterogeneity within *N. cyriacigeorgica* where three subgroups were identified. Of note, these subgroups correspond to three *hsp65* genotypes proposed in previous studies [28, 29]. In the diagnostic algorithm proposed (Fig 3) for laboratory identification of *Nocardia*, MLSA has good clinical utility in definitive confirmative of species.

Application of sequencing-based methods in the routine work of clinical laboratories is restricted by high costs and the need for on-site sequencing facilities which potentially affect turn-around times. The recent introduction of MALDI-TOF MS platforms in clinical laboratories has revolutionized diagnostic microbiology [35]. However, its application in identification of *Nocardia* species required extra protein-extraction pretreatment of isolates [17, 30], and more importantly, may be limited by insufficient archiving of reference spectra within commercial MALDI-TOF MS databases [17–20]. In this study, the MSP analysis indicated
significant differences between spectra of the clinical isolates studied and reference spectra of corresponding species contained in the Biotyper version 3.1 database (Bruker Daltonics), which yielded suboptimal results in species identification when used as the only database. However, the establishment of even a small “in-house” database significantly improved the identification capacity of the MALDI-TOF MS system, as also found previously [18, 20, 22, 23]. To improve the identification capacity of MALDI-TOF MS, it is important for MS databases to contain more reference mass spectra from type strains of different bacteria species, and also spectra representing different strains of the same species [35], as strains of the same species may have divergent mass spectra e.g. the case of \textit{N. abscessus} clinical strain PUNC002 and PUNC017 found in the present study. With an ongoing expanded “in-house” database representing species that are the more frequently encountered, the utility and practicality of MALDI-TOF MS for \textit{Nocardia} identification will be improved. However, before expanding any “in-house” mass spectrum database, we recommend that \textit{Nocardia} isolates be analyzed by MLSA to ensure the high-quality of the “in-house” database (Fig 3). Moreover, comparing to MLSA, the MALDI-TOF MS system was unable to cluster \textit{N. cyriacigeorgica} into subgroups. In this regard, MLSA is superior to MALDI-TOF MS for subgrouping \textit{N. cyriacigeorgica}. Further studies addressing differences in MS profiles between \textit{N. cyriacigeorgica} strains are warranted. The major limitation of the current study is that relatively few isolates representing a small number of species was examined, and all isolates were from a single center and hence may not be generalizable across centers in China. Continuous expansion of the MALDI-TOF MS databases to include more species is necessary.

**Conclusions**

In conclusion, we have evaluated MLSA scheme and MALDI-TOF MS for identification of \textit{Nocardia} clinical isolates. MLSA showed superior discriminatory power compared with the 5’-end 606bp partial 16S rRNA gene sequencing for species identification of \textit{Nocardia}. MALDI-TOF MS has good utility in rapidly and accurately providing species identification but is contingent on building up a robust library of reference spectra.

**Supporting Information**

S1 Table. Species of \textit{Nocardia} and GenBank accession numbers of five gene sequences for six type strains of \textit{Nocardia} studied.

(DOCX)

S2 Table. Haplotype nomenclature based on sequencing of five \textit{Nocardia} genes for 25 clinical \textit{Nocardia} isolates.

(DOCX)

**Author Contributions**

Conceived and designed the experiments: MX LP YPZ YCX. Performed the experiments: MX LP XH JWC. Analyzed the data: MX LP XF. Contributed reagents/materials/analysis tools: LZ HXL. Wrote the paper: MX LP SC FK YCX.

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