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Article

Identification of *Aspergillus* Species by Matrix-Assisted Laser Desorption Ionisation–Time-of-Flight Mass Spectrometry

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Abstract: Matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI–TOF MS) has already been used routinely in clinical laboratories for rapid bacterial and yeast identification. However, its routine use for *Aspergillus* identification is still controversial because of a lack of unification of the culture method and a suboptimal database. In this study, we first examined the difference in using broth and agar plate cultures for growing the aspergilli before protein extraction and found that agar culture was much superior to broth culture in generating interpretable protein mass spectra. Then, based on the solid culture method, we examined the accuracy, with *benA* and/or *CaM* sequencing as the identification gold standard, in using MALDI–TOF MS for *Aspergillus* identification. Overall, the original Bruker Library could only identify 38.2% and 22.8% of the strains characterised at the genus/section and species level, respectively. When the Bruker Library was expanded with reference mass spectra generated from reference *Aspergillus* species in-house, the identification rates were improved to 61.0% and 48.0%, respectively. The MSI database, freely available online,

outperformed the former two libraries for *Aspergillus* identification at all levels (genus: 95.1%, section: 92.7%, species: 74.8%). Agar plate culture and the MSI database should be used for MALDI–TOF MS identification of aspergilli in clinical laboratories.

Keywords: *Aspergillus*; rare species; cryptic species; MALDI–TOF MS; identification

1. Introduction

Aspergillus infections have become a major focus of clinical microbiology and infectious disease, as the number of patients infected with *Aspergillus* species has risen dramatically in recent years [1,2]. In immunocompetent hosts, *Aspergillus* species rarely cause serious illnesses, except for aspergilloma in patients with pre-existing chronic lung diseases and a few related chronic pulmonary diseases, such as chronic cavitary pulmonary aspergillosis, chronic fibrosing aspergillosis, and subacute invasive aspergillosis (previously chronic necrotising aspergillosis) [3]. Recently, aspergillosis complicated influenza has emerged and is associated with significant mortality [4]. On the other hand, invasive aspergillosis is one of the most important causes of morbidity and mortality in immunocompromised patients, such as patients with haematological malignancies undergoing chemotherapy, as well as marrow and solid organ transplant recipients [3]. Among the known *Aspergillus* species, *A. fumigatus*, *A. flavus*, *A. niger,* and *A. terreus* are the most common ones causing human infections [5].

Traditionally, identification of *Aspergillus* strains was achieved through microscopic examination of fungal colonies and recognition of the characteristic features. In recent years, the increasing use of molecular methods, such as sequencing of the β-tubulin gene (*BenA*), calmodulin gene (*CaM*), and other housekeeping genes, for fungal identification has revealed that a significant proportion of *Aspergillus* infections are actually caused by *Aspergillus* species that were not previously frequently recognised to cause aspergillosis. For example, we observed that around 25% of *A. flavus* reported by our clinical microbiology laboratory were indeed *A. nomiae* or *A. tamarii* [6], a diversity of *Aspergillus* species other than *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus* were recovered from patients with various types of *Aspergillus* infections [7,8], and a novel *Aspergillus* species, *A. hongkongensis*, was discovered [7]. However, identification by DNA sequencing is still labour-intensive, time-consuming, expensive, and require laboratory staff with expertise on these molecular technologies. Although artificial intelligence, such as chatbots and image recognition algorithms, is being increasingly used in providing knowledge on *Aspergillus* infections or identifying *Aspergillus* species, their applications are still under an explorative nature and await further development and maturation [9,10].

Matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry (MALDI–TOF MS) has recently emerged as a revolutionary technique for rapid bacterial and yeast identification at a low cost and has been adopted in a lot of clinical microbiology laboratories for such purpose. However, the routine use of this technology for *Aspergillus* identification in clinical microbiology laboratories is still controversial because of a lack of unification of the culture method and a suboptimal database. In this study, we first examined the difference in using broth and agar plate cultures for growing the *Aspergillus* before protein extraction. Then, based on the agar plate culture method, we tested the accuracy, with *benA* and/or *CaM* sequencing as the identification gold standard, in using MALDI–TOF MS for identification of both the *Aspergillus* species classically associated with clinical infections (*A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*) as well as other rare/cryptic *Aspergillus* species using the original manufacturer's library, the manufacturer's library expanded with our own in-house mass spectral database as well as an online free-access web application for fungal identification by MALDI–TOF MS [11,12].

2. Materials and Methods

2.1. Fungal Strains

A total of 123 *Aspergillus* strains were retrieved from the collection of our laboratory (Table S1). Amongst these strains, 16 were *A. flavus*, 19 were *A. fumigatus*, six were *A. niger*, five were *A. terreus,* and 77 were rare *Aspergillus* species. Except for 31 rare *Aspergillus* strains, which were identified by molecular method in our previous study [8], the identities of the other 92 *Aspergillus* strains from our collection were confirmed by DNA sequencing as the gold standard in this study as described below. These *Aspergillus* strains were isolated from clinical specimens or the environment and were sent to our laboratory from the clinical microbiology laboratories of four hospitals in Hong Kong, one hospital in Shenzhen, China, as well as one hospital in Shanghai, China.

In addition, 18 reference strains were obtained from culture collections for the in-house generation of reference protein mass spectra. The reference strains *A. amoenus* NRRL 4838^{T}, *A. austroafricanus* NRRL 233^{T},

A. brunneoviolaceus NRRL 4912^T, *A. clavatus* NRRL 1^T, *A. lentulus* NRRL 35552^T, *A. nidulans* NRRL 187^T, *A. restrictus* NRRL 154^T, *A. sydowii* NRRL 250^T, *A. tabacinus* NRRL 4791^T, and *A. tubingensis* NRRL 4875^T were obtained from the Agricultural Research Service (ARS) Culture Collection (NRRL), Department of Agriculture, USA; whereas the reference strains *A. griseoauranticus* CBS 138191^T, *A. neoniger* CBS 115656^T, *A. nomiae* CBS 260.88^T, *A. pseudocaelatus* CBS 117616^T, *A. salwanensis* CBS 138172^T, *A. sublatus* (synonym: *A. latus* [13]) CBS 492.65, *A. tamarii* CBS 104.13^T, and *A. welwitschiae* CBS 139.54^T were obtained from the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands. All the *Aspergillus* strains were cultured on Sabouraud dextrose agar (SDA; Difco, BD Diagnostics Systems, Franklin Lakes, NJ, USA) supplemented with chloramphenicol (50 μg/mL; Calbiochem, San Diego, CA, USA) or Sabouraud dextrose broth (SDB; Difco) supplemented with chloramphenicol (50 μg/mL). This study was approved by the Institutional Review Board (IRB) of The University of Hong Kong/Hospital Authority Hong Kong West Cluster, Hong Kong East Cluster Research Ethics Committee, the IRB of The University of Hong Kong-Gleaneagles Hospital Hong Kong, the Medical Ethics Committee of The University of Hong Kong-Shenzhen Hospital as well as the Shanghai Jiaotong University School of Medicine Ruijin Hospital Ethics Committee.

2.2. DNA Sequencing

DNA extraction and polymerase chain reaction (PCR)–DNA sequencing for the *Aspergillus* strains were performed according to our previous publication [14]. In brief, fungal materials from cultures on SDA were harvested in distilled water and then subjected to bead-beating for 10 min with Tissuelyser II (Qiagen, Hilden, Germany). Total DNA was subsequently extracted using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instruction. PCR was performed using the iProof™ High-Fidelity PCR Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The primer pair Bt2a/Bt2b [15] was used to amplify the partial *BenA* whereas the primer pair CF1M/CF4 [16] was used to amplify the partial *CaM* for the *Aspergillus* strains. The PCR products were sequenced using ABI 3730xl DNA Analyzer (Applied Biosystems, Waltham, MA, USA) with the PCR primers. Sequencing results were visualised with Chromas 2.6.5 (Technelysium, Brisbane, Australia) and manually edited. The DNA sequences were then compared with other sequences in the DDBJ/ENA/GenBank databases for identification using BLAST [17]. They were also deposited to the DDBJ/ENA/GenBank databases with nucleotide accession numbers listed in Table S1.

2.3. Fungal Identification by MALDI–TOF MS

MALDI–TOF MS was performed using the Microflex LT platform (Bruker Daltonics, Bremen, Germany). For sample preparation using liquid media cultures, the *Aspergillus* strains were first cultured in SDB supplemented with 50 μg/mL of chloramphenicol with or without agitation at 250 rpm at 25 °C for 2–4 d. Since *Aspergillus* strains do not grow uniformly in broth, the broth cultures were mixed vigorously using a pipette to break up clumps. For each of the strains, 1 mL of the culture was then removed into a 1.5 mL microcentrifuge tube. After centrifugation at 14,000× *g* for 2 min, the supernatant was removed, and 800 μL of 70% ethanol were then added to resuspend the fungal materials. For sample preparation using solid agar plate cultures, the *Aspergillus* strains were first cultured on SDA supplemented with 50 μg/mL of chloramphenicol at 25 °C for 4–7 d days and subcultured again to prevent potential contamination. For each strain, a cotton swab was wetted with 0.1% Tween 20 and used to gently collect approximately 1 cm^2 of fungal materials into a 1.5 mL microcentrifuge tube containing 800 μL of 70% ethanol. For both methods, the fungal material-ethanol suspension was then centrifuged at 14,000× *g* for 2 min, and the supernatant was removed by decantation. The remaining ethanol was allowed to evaporate within a biosafety cabinet for 15 min. The pellet was resuspended using 40 μL of 70% formic acid and allowed to rest for 10 min. An equal volume of 100% acetonitrile was added, and the mixture rested for an additional 5 min. The mixture was then centrifuged again at 14,000× *g* for 2 min. One microlitre of the supernatant was transferred in duplicate to individual spots on an MSP 96 steel plate and allowed to dry. Once dried, each sample was then overlaid with a 100% α-cyano-4-hydroxycinnamic acid matrix and allowed to dry. The plate was then transferred to the microflex LT system. Spectra acquisition was performed using default parameters (linear positive-ion acquisition detection of 2000–20,000 Da mass range) using Biotyper Compass 4.1.8 (Bruker Daltonics) and FlexControl 3.4 (Bruker Daltonics). The Filamentous Fungi Library 3.0 was selected within the Biotyper Compass Software. Log score outputs below 1.7 were considered invalid, while scores ≥1.7 were recorded. In addition, following the completion of spectra acquisition, each of the runs was exported as a .zip file. The compressed files were then uploaded to the mass spectrometry identification (MSI) platform (https://msi.happy-dev.fr/) and analysed against the online database [11,12]. MSI score percentages above 20 were recorded, and were considered equivalent to Bruker log scores of ≥2.3 [18].

2.4. Construction and Evaluation of In-House Mass Spectral Database for Cryptic Aspergillus Species

Ex-type/reference strains of the 18 rare *Aspergillus* species included in this study were selected to improve the Bruker library. Sample preparation to create the in-house database was identical to the solid agar plate culture method previously mentioned for fungal identification. One microlitre of the formic acid-extracted supernatant was spotted on four consecutive positions on an MSP 96 plate and overlaid with the matrix. FlexControl 3.4 was used to acquire six profiles for each spot, for a total of 24 profiles for each strain. At least 20/24 non-empty profiles were used to generate mass spectral profiles (MSPs) following post-processing and normalisation using Biotyper Compass Explorer 4.1.8 (Bruker Daltonics) for each strain. The Bruker Filamentous Fungi Library 3.0 supplemented with the in-house constructed database was then used to retrospectively analyse every recorded identification run using Biotyper Compass Explorer 4.1.8.

2.5. Statistical Analyses

The MALDI–TOF MS identification rates between different categorical groups, such as common species versus rare/cryptic species or different libraries/databases, were compared by Fisher's exact test using QuickCalcs (GraphPad, Boston, MA, USA; available at: https://www.graphpad.com/quickcalcs/contingency1/ accessed on 11 June 2024).

3. Results

3.1. Confirmation of Fungal Identities

Sequencing of the partial *BenA* and/or *CaM* confirmed the species identities of the *A. flavus*, *A. fumigatus*, *A. niger* and *A. terreus* strains. DNA sequencing also revealed that the 77 rare *Aspergillus* strains belonged to 18 different *Aspergillus* species, namely *A. sydowii* (n = 18), *A. salwaensis* (n = 11), *A. tubingensis* (n = 8), *A. welwitschiae* (n = 7), *A. amoenus* (n = 4), *A. tabacinus* (n = 5), *A. brunneovialaceus* (n = 4), *A. nidulans* (n = 4), *A. tamarii* (n = 4), *A. pseudocaelatus* (n = 3), *A. griseoauranticus* (n = 2), *A. austroafricanus* (n = 1), *A. clavatus* (n = 1), *A. costaricensis*/*A. neoniger* (n = 1), *A. lentulus* (n = 1), *A. nomiae*/*A. pseudonomiae* (n = 1), *A. restrictus* (n = 1), and *A. sublatus* (n = 1). Althogether, the 123 *Aspergillus* strains from our collection (both common and rare/cryptic species) belonged to a total of eight *Aspergillus* sections, namely *Nidulantes* (n = 35), *Nigri* (n = 26), *Flavi* (n = 24), *Fumigati* (n = 20), *Circumdati* (n = 11), *Terrei* (n = 5), *Clavati* (n = 1), and *Restricti* (n = 1) (Table S1).

3.2. MALDI–TOF MS Analysis Using Broth Cultures Versus Agar Plate Cultures

Initially, protein extraction for MALDI–TOF MS analysis was performed on broth cultures. This sample preparation approach was first tested for six reference strains of six different cryptic species (*A. griseoaurantiacus* CBS 138191^T, *A. neoniger* CBS 115656^T, *A. pseudocaelatus* CBS 117616^T, *A. salwaensis* CBS 138172^T, *A. sublatus* CBS 492.65, and *A. welwitschiae* CBS 139.54^T). In broth culture with agitation, these strains grew solely as gelatinous spherical macrostructures (Figure 1a,b). Without agitation, the same strains retained the gelatinous structure but did not form spheres, they formed a biofilm-like substance at the liquid-air interface (Figure 1c). Distinct protein peaks were detected only once (*A. neoniger* CBS 115656^T) from the formic acid extract of these six strains tested during MALDI–TOF MS (Figure 2a), rendering identification of these *Aspergillus* strains by MALDI–TOF MS using this approach unfeasible. Furthermore, runs without sufficient distinct peaks were automatically deleted by FlexControl and instead generated empty spectra files, which could not be processed further (Figure 2b).

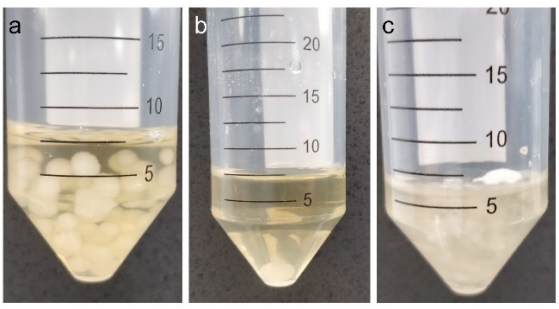

Figure 1. Examples of growth patterns of rare *Aspergillus* species in liquid cultures. (**a**) *Aspergillus sublatus* CBS 139.54 and (**b**) *A. neoniger* CBS 115656^T cultured with shaking at 250 rpm. The fungi formed gelatinous spherical macrostructures. (**c**) *Aspergillus sublatus* CBS 139.54 cultured without agitation. Biofilm-like substance at the liquid-air interface was formed. All cultures were grown in Sabauroud's dextrose broth supplemented with chloramphenicol (50 μg/mL) at 25 °C for 2–4 d.

To overcome this problem, the same six *Aspergillus* strains were cultured on agar plates, and fungal materials were harvested from solid cultures for sample preparation instead. The subsequent protein extraction method remained the same. Using this sample preparation approach, protein peaks could be detected from the cell lysate extract of all *Aspergillus* strains included in this study (Figure 2b). Notably, the peaks obtained from strains grown on solid medium differed from peaks obtained from liquid culture (Figure 2c).

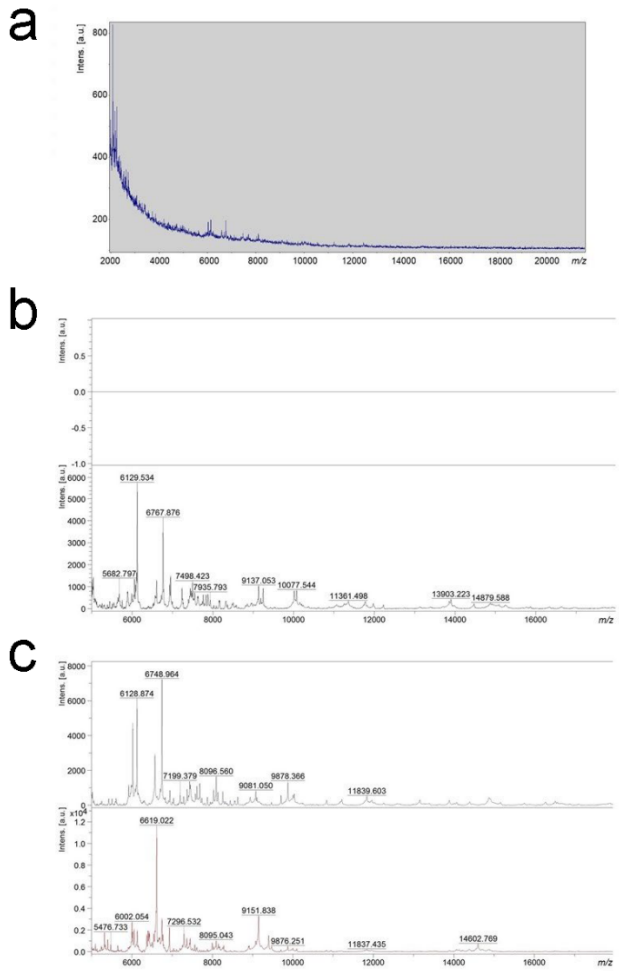

Figure 2. Cultivation method effects on MALDI–TOF MS for *Aspergillus* species. (**a**) Screen capture of MALDI–TOF MS mass spectra acquisition of *Aspergillus sublatus* CBS 139.54 grown in liquid medium for 4 d without agitation. (**b**) Post-acquisition comparison of mass spectra for *Aspergillus sublatus* CBS 139.54 grown on liquid medium (top) versus solid medium (bottom). No interpretable mass spectrum was obtained for liquid culture. (**c**) Post-acquisition comparison of mass spectra for *Aspergillus neoniger* CBS 115656^{T} grown on solid medium (top) versus liquid medium (bottom). Different protein peaks were detected.

3.3. MALDI–TOF MS Identification Using Bruker Library

When the mass spectra from the *Aspergillus* strains were matched against the Bruker Filamentous Fungi Library 3.0 and using a score of 1.7 as the cutoff value, amongst all the 123 strains from our collection, both genus- and section-level identifications could be achieved for 47 strains (38.2%) whereas species-level identification could only be achieved for 28 strains (22.8%). Species-level identification was successful for all the common species (*A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus*) strains which scored ≥1.7, whereas out of the 22 rare/cryptic species strains which scored ≥1.7 species-level identification could only be achieved for three of them (two for *A. nidulans* and one for *A. tamarii*). The identification rates at the genus/section and species levels for the common species (both 54.3%) were significantly higher than those for the rare/cryptic species (28.6% and 3.9%, respectively) ($p = 0.0069$ and $p < 0.0001$, respectively). Amongst the four common species, the rate of species-level identification was the highest for *A. fumigatus* (84.2%), followed by *A. niger* (83.3%), *A. terreus* (20%), and *A. flavus* (18.8%). For the rare/cryptic species, reliable identification at genus level or below could not be achieved for strains of the species *A. brunneovialaceus*, *A. clavatus*, *A. costaricensis*/*A. neoniger*, *A. griseoauranticus*, *A. lentulus*, *A. nomiae*/*A. pseudonomiae*, *A. pseudocaelatus*, *A. restrictus*, *A. tabacinus*, and *A. tubingensis* (Tables 1 and S2).

Table 1. MALDI–TOF MS identification results for the *Aspergillus* strains characterised in this study using different mass spectral libraries/databases.

Section	Species	Bruker Filamentous Fungi Library 3.0			Bruker Library Expanded with In-House Constructed Database			MSI Database		
		Species-Level ID (*n*, %)	Section-Level ID (*n*, %)	Genus-Level ID (*n*, %)	Species-Level ID (*n*, %)	Section-Level ID (*n*, %)	Genus-Level ID (*n*, %)	Species-Level ID (*n*, %)	Section-Level ID (*n*, %)	Genus-Level ID (*n*, %)
Circumdati (*n* = 11)	*A. salwaensis* (*n* = 11)	(0, 0)	(7, 63.6)	(7, 63.6)	(6, 54.5)	(9, 81.8)	(9, 81.8)	(0, 0)	(10, 90.9)	(10, 90.9)
Clavati (*n* = 1)	*A. clavatus* (*n* = 1) *,†	(0, 0)	(0, 0)	(0, 0)	(1, 100)	(1, 100)	(1, 100)	(1, 100)	(1, 100)	(1, 100)
Flavi (*n* = 24)	*A. flavus* (*n* = 16) *,†	(3, 18.8)	(3, 18.8)	(3, 18.8)	(4, 25)	(4, 25)	(4, 25)	(16, 100)	(16, 100)	(16, 100)
	A. nomiae/*A. pseudonomiae* (*n* = 1) *,†	(0, 0)	(0, 0)	(0, 0)	(1, 100)	(1, 100)	(1, 100)	(1, 100)	(1, 100)	(1, 100)
	A. pseudocaelatus (*n* = 3)	(0, 0)	(0, 0)	(0, 0)	(1, 33.3)	(1, 33.3)	(1, 33.3)	(0, 0)	(1, 33.3)	(2, 66.6)
	A. tamarii (*n* = 4) *,†	(1, 25)	(1, 25)	(1, 25)	(3, 75)	(3, 75)	(3, 75)	(3, 75)	(3, 75)	(3, 75)
Fumigati (*n* = 20)	*A. fumigatus* (*n* = 19) *,†	(16, 84.2)	(16, 84.2)	(16, 84.2)	(14, 73.7)	(14, 73.7)	(14, 73.7)	(19, 100)	(19, 100)	(19, 100)
	A. lentulus (*n* = 1) *,†	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(1, 100)	(1, 100)	(1, 100)
Nidulantes (*n* = 35)	*A. amoenus* (*n* = 4) †	(0, 0)	(3, 75)	(3, 75)	(0, 0)	(3, 75)	(3, 75)	(2, 40)	(3, 60)	(4, 100)
	A. austroafricanus (*n* = 1) †	(0, 0)	(1, 100)	(1, 100)	(0, 0)	(1, 100)	(1, 100)	(0, 0)	(1, 100)	(1, 100)
	A. griseoauranticus (*n* = 2) †	(0, 0)	(0, 0)	(0, 0)	(2, 100)	(2, 100)	(2, 100)	(2, 100)	(2, 100)	(2, 100)
	A. nidulans (*n* = 4) *,†	(2, 50)	(2, 50)	(2, 50)	(1, 25)	(3, 75)	(3, 75)	(2, 50)	(4, 100)	(4, 100)
	A. sublatus (*n* = 1) †	(0, 0)	(1, 100)	(1, 100)	(1, 100)	(1, 100)	(1, 100)	(1, 100)	(1, 100)	(1, 100)
	A. sydowii (*n* = 18) *,†	(0, 0)	(4, 22.2)	(4, 22.2)	(11, 61.1)	(12, 66.7)	(12, 66.7)	(14, 77.8)	(17, 94.4)	(17, 94.4)
	A. tabacinus (*n* = 5) †	(0, 0)	(0, 0)	(0, 0)	(2, 40)	(5, 100)	(5, 100)	(3, 60)	(5, 100)	(5, 100)
Nigri (*n* = 26)	*A. brunneoviolaceus* (*n* = 4) †	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(3, 75)	(3, 75)	(4, 100)
	A. niger (*n* = 6) *,†	(5, 83.3)	(5, 83.3)	(5, 83.3)	(4, 66.7)	(4, 66.7)	(4, 66.7)	(5, 83.3)	(6, 100)	(6, 100)
	A. costaricensis/*A. neoniger* (*n* = 1) †	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(0, 0)	(0, 0)
	A. tubingensis (*n* = 8) †	(0, 0)	(0, 0)	(0, 0)	(6, 75)	(6, 75)	(6, 75)	(8, 100)	(8, 100)	(8, 100)
	A. welwitschiae (*n* = 7) †	(0, 0)	(3, 42.9)	(3, 42.9)	(0, 0)	(3, 42.9)	(3, 42.9)	(5, 71.4)	(6, 85.7)	(6, 85.7)
Restricti (*n* = 1)	*A. restrictus* (*n* = 1) †	(0, 0)	(0, 0)	(0, 0)	(1, 100)	(1, 100)	(1, 100)	(1, 100)	(1, 100)	(1, 100)
Terrei (*n* = 5)	*A. terreus* (*n* = 5) *,†	(1, 20)	(1, 20)	(1, 20)	(1, 20)	(1, 20)	(1, 20)	(5, 100)	(5, 100)	(5, 100)

* These species are included in the Bruker Filamentous Fungi Library 3.0 [19]. † These species were included in the MSI database [11,12].

3.4. MALDI–TOF MS Identification Using the Bruker Library Expanded with In-House Constructed Database

Again, using a score of 1.7 as the cutoff value, amongst all the 123 strains from our collection both genus- and section-level identifications could be achieved for 75 strains (61.0%) whereas species-level identification could be achieved for 59 strains (48.0%) using the Bruker Library expanded with our in-house constructed database. Species-level identification was successful for all the common species strains which scored ≥1.7, except that *A. flavus* PW4043 was misidentified as *A. pseudocaelatus*; although the overall success rates turned lower for *A. flavus* and *A. terreus*. On the other hand, out of the 52 rare/cryptic species strains that scored ≥1.7, species-level identification could only be achieved for 36 strains. The identification rates at the genus/section and species levels for the common species (both 50.0%) were similar to those for the rare/cryptic species (67.5% and 46.8%, respectively) (p = 0.0592 and p = 0.8522, respectively). Amongst the rare/cryptic species, all strains from *A. clavatus*, *A. griseoauranticus*, *A. nomius*/*A. pseudonomiae*, *A. restrictus*, and *A. sublatus* could be successfully identified down to the species level, followed by *A. tamarii* (75%) and *A. tubingensis* (75%), *A. sydowii* (61.1%), *A. salwaensis* (54.5%), *A tabacinus* (40%), *A. pseudocaelatus* (33.3%), and *A. nidulans* (25%). Reliable identification at the genus level or below remained unsuccessful for strains of the species *A. brunneovialaceus*, *A. costaricensis*/*A. neoniger*, and *A. lentulus* (Tables 1 and S2).

3.5. MALDI–TOF MS Identification Using the Online MSI Database

Using the online MSI database with a score of 20 as the cutoff value, 117 (95.1%), 114 (92.7%), and 92 (74.8%) strains out of the 123 strains from our collection could be identified down to the genus, section, and species level, respectively. While the identification rates at the genus level of the common species and the rare/cryptic species were similar (100% and 92.2%, respectively) (p = 0.0828); the identification rates at the section and species levels of the common species (100% and 97.8%, respectively) were significantly higher than those for the rare/cryptic species (88.3% and 61.0%, respectively) (p = 0.0258 and p < 0.0001, respectively). Amongst the four common species, identification was successful for all strains of *A. flavus*, *A. fumigatus*, and *A. terreus* down to the species level. Although all *A. niger* strains were correctly identified to the section level (*Nigri*), one out of the six strains were misidentified as *A. welwitschiae*. As for the rare/cryptic species, species-level identification was the most successful for *A. clavatus*, *A. griseoauranticus*, *A. lentulus*, *A. nomiae*/*A. pseudonomiae*, *A. restrictus*, *A. sublatus*, and *A. tubingensis* (all 100%), followed by *A. sydowii* (77.8%), *A. brunneiviolaceus* and *A. tamarii* (both 75%), *A. welwitschiae* (71.4%), *A. tabacinus* (60%), *A. nidulans* (50%), and *A. amoenus* (40%). Species-level identification was not successfully for *A. austroafricanus*, *A. costaricensis*/*A. neoniger*, *A. pseudocaelatus*, and *A. salwaensis* (Tables 1 and S2).

3.6. Comparison of the Performance of the Bruker Library, Bruker Library Expanded with In-House Database, and Online MSI Database

Comparing the original Bruker Library with the Bruker Library expanded with our in-house constructed database, genus and section level identifications for all the 123 *Aspergillus* strains from our collection were both more successful (original: both 38.2% vs. expanded: both 61.0%; p = 0.0005) after library expansion with mass spectra from the reference *Aspergillus* strains. Similarly, the expanded library also performed better for species-level identification (original: 22.8% vs. expanded: 48.0%; p < 0.0001). While expansion of the library using mass spectra from reference strains belonging to rare/cryptic species did not affect identification of the common species strains (original: 54.3% for genus/section/species level vs. expanded: 50.0% for genus/section/species levels; p = 0.8348), inclusion of these extra reference spectra significantly improved identifications of these rare/cryptic species at all three levels after library expansion (original: 28.6% for genus/section level and 3.9% for species level vs. expanded: 67.5% for genus/section level and 46.8% for species level; both p < 0.0001).

The MSI database is freely accessible online and includes 166 *Aspergillus* species [12]. Therefore, its utility in identifying our cohort of *Aspergillus* strains was also evaluated. Compared with both the original Bruker Library and the in-house expanded library, the MSI database outperformed for identification at all levels (genus level: 95.1% vs. 38.2% for Bruker original [p < 0.0001] and 61.0% for Bruker expanded [p < 0.0001]; section level: 92.7% vs. 38.2% for Bruker original [p < 0.0001] and 61.0% for Bruker expanded [p < 0.0001]; species level: 74.8% vs. 22.8% for Bruker original [p < 0.0001] and 48.0% for Bruker expanded [p < 0.0001]). This was also the case when strains of common species were identified using the MSI database (genus and section levels: both 100% vs. both 54.3% for Bruker original [p < 0.0001] and both 50.0% for Bruker expanded [p < 0.0001]; species level: 97.8% vs. 54.3% for Bruker original [p < 0.0001] and 50.0% for Bruker expanded [p < 0.0001]). As for the rare/cryptic species, the MSI database performed better for identification at the genus and section levels as well when compared with both the Bruker original and in-house expanded libraries (genus level: 92.2% vs. 28.6% for

Bruker original [$p < 0.0001$] and 67.5% for Bruker expanded [$p = 0.0002$]; section level: 88.3% vs. 28.5% for Bruker original [$p < 0.0001$] and 67.5% for Bruker expanded [$p = 0.0032$]). However, for species-level identification, although the MSI database still outperformed the Bruker original library (61.0% vs. 3.9%; $p < 0.0001$), its performance was similar to the in-house expanded library (61.0% vs. 46.8%; $p = 0.1057$).

4. Discussion

The use of the solid agar plate culture method for sample preparation and the corresponding protein mass spectra database helped enhance the utility of MALDI–TOF MS for identification of *Aspergillus* species. In our initial attempt of using broth cultures for sample preparation in this study, interpretable protein mass spectral profiles could only be obtained for one out of the six reference strains analysed. This is similar to our previous study in that quite a number of *Aspergillus* strains, especially those of the rare/cryptic species, could not be analysed and identified by MALDI–TOF MS when cultured in broth due to the absence of any interpretable protein profile [7]. Such failure may be attributed to the formation of gelatinous structures acting as a physical barrier to cell lysis via formic acid digestion (Figure 1). Although a previous study showed that fungal aggregates from broth cultures might be dispersed by repeated washing of fungal pellets to facilitate subsequent cell lysis, the technical and biological reproducibilities of this procedure for mass spectral peak generation were poor [20]. In contrast, in our subsequent sample preparation attempts using solid agar plate cultures, proteins could be detected for all the *Aspergillus* strains analysed with good signals (Figure 2b,c), and the resultant protein mass spectral profiles could then be compared with those in the database for species identification. Interestingly, for strains that gave interpretable protein mass spectral profiles from both broth and solid agar plate cultures, variable mass peak lists were found (Figure 2c). This suggested that proteins extracted for MALDI–TOF MS analysis may be composed of non-ribosomal proteins and could be affected by the culture conditions [21]. This probably helped explain why the Bruker Filamentous Fungi Library 3.0 evaluated in this study still failed to identify 63.2% of the *Aspergillus* strains when only species included in the Library were considered (Table 1), since reference mass spectra in the Library were created from broth cultures [19]. On the other hand, when protein mass spectra from strains of these same ten species included in the Bruker Library were analysed by the online MSI database, the identification success rate increased significantly (86.8%, $p < 0.0001$). Such a performance improvement was most likely a result of the fact that the online MSI database was constructed using reference protein mass spectra prepared from solid agar plate cultures as well [11,12].

Inclusion of more reference mass spectra from a more diverse spectrum of *Aspergillus* species is also necessary to improve the accuracy of MALDI–TOF MS for the identification of this group of fungi. Although the Bruker Filamentous Fungi Library 3.0 contains reference mass spectra for 29 *Aspergillus* species (4 common and 25 rare/cryptic species), only ten of these species (*A. clavatus*, *A. flavus*, *A. fumigatus*, *A. lentulus*, *A. nidulans*, *A. niger*, *A. nomiae*/*A. pseudonomiae*, *A. sydowii*, *A. tamarii*, and *A. terreus*) were characterised in this study, and the other 12 species analysed (*A. amoenus*, *A. austroafricanus*, *A. brunneoviolaeus*, *A. costaricensis/A. neoniger*, *A. griseoauranticus*, *A. pseudocaelatus*, *A. restrictus*, *A. salwanensis*, *A. sublatus*, *A. tabaciunus*, *A. tubingensis*, and *A. welwitschiae*) are not included in this Bruker Library. This explained why identification was not possible for these species. When reference mass spectra were generated in-house to expand the Bruker Library using the ex-type/reference strains of these species, identification for seven of these species became possible (*A. griseoauranticus*, *A. pseudocaelatus*, *A. restrictus*, *A. salwanensis*, *A. sublatus*, *A. tabaciunus*, *A. tubingensis*), although the success rate was not always 100%. Although the remaining five species could not be identified by the Bruker Library expanded with an in-house constructed database, three of them (*A. amoenus*, *A. brunneoviolaeus*, and *A. welwitschiae*) became identifiable when the online MSI database was used instead. This was probably because reference protein mass spectra from a larger number of strains were included for each species in the MSI database, while only one reference protein mass spectrum from one strain was used for each species in our in-house database. The inclusion of more reference protein mass spectra from more different strains for a particular species could accommodate more spectral variations within a species and thus could facilitate identification. Amongst the three mass spectral libraries/databases evaluated in this study, the online MSI database gave the best overall performance. This was because it contains the reference protein mass spectra for 18 out of the 22 species characterised in this study. As for the remaining four species (*A. austroafricanus*, *A. costaricensis*/*A. neoniger*, *A. pseudocaelatus*, and *A. salwaensis*), theoretically they would also be identifiable if their reference protein mass spectra are also included in the online MSI database. This could be evidenced by the fact that after the reference protein mass spectra from two of these species were used to construct our in-house database for Bruker Library expansion, species identification for both *A. pseudocaelatus* and *A. salwaensis* became possible. Although the online MSI database contains reference protein mass spectra from a remarkable number of *Aspergillus* species ($n = 166$)

as well as other filamentous fungi, further database expansion is essential to cover a wider range of fungal species, especially the less commonly encountered ones.

With the markedly enhanced accuracy using fungal proteins extracted from solid agar plate cultures and a more robust database, the routine application of MALDI–TOF MS for laboratory diagnosis of filamentous fungal infection would improve the management of these patients, particularly in hospitals without laboratory expertise in medical mycology. The traditional fungal identification method in clinical microbiology laboratories relies heavily on manual morphological recognition, which requires technical staff proficient in mycological identification. However, such expertise is lacking all over the world, and a long training time and extensive experience are required. With the advancement of molecular technologies, DNA sequencing is gaining popularity for fungal identification in clinical microbiology laboratories. Although the internal transcribed spacer region (ITS) and translation elongation factor 1α gene (*TEF1α*) are regarded as the primary and secondary DNA barcodes for fungi and sequence databases, such as the International Society for Human and Animal Mycology (ISHAM) Barcoding Databases, are available freely for pathogenic fungi, these two DNA markers may not be able to provide sufficient resolution for species identification for some groups of fungi, in particular *Aspergillus* species. As such, sequencing of additional DNA markers, such as *benA* and/or *CaM* for *Aspergillus* species, is needed for accurate species identification. The use of species-specific DNA markers would inconvenience the routine molecular work in clinical laboratories, increase the identification cost, and lengthen the turn-around-time. In this study, we demonstrated that with an improved protein extraction method and a comprehensive database MALDI–TOF MS could serve as a cheaper and faster replacement to morphological recognition or DNA sequencing to achieve rapid identification of *Aspergillus* species. Therefore, patient management could be greatly facilitated as accurate diagnosis of the aetiological fungal agents could help inform physicians of the proper treatment strategies and choice of antifungal regimens, especially since certain cryptic *Aspergillus* species may exhibit different antifungal resistance profiles. Furthermore, with the precise identification of *Aspergillus* species, epidemiological studies can also be performed to understand disease patterns and transmission.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/260401141004307 7/eMicrobe-25110126-SI.pdf. Table S1. *Aspergillus* strains characterised in this study. Table S2. Detailed MALDI–TOF MS identification results for the *Aspergillus* strains characterised in this study using different mass spectral libraries/databases.

Author Contributions

Conceptualisation, C.-C.T. and P.C.Y.W.; Data curation, C.-C.T., W.C. and J.Y.M.T.; Formal analysis, C.-C.T., W.C. and J.Y.M.T.; Funding acquisition, C.-C.T., S.K.P.L. and P.C.Y.W.; Investigation, W.C., J.Y.M.T., O.H.Y.C., Y.-W.L., T.W.S.H., T.Z., C.W.H.L., C.Y., Y.-N.T., A.H.Y.N., J.H.K.C., H.Y., F.X., S.K.F.L., C.X., L.H., D.M.W.T., C.K.C.L., A.K.L.W. and T.-L.Q.; Methodology, C.-C.T., W.C. and J.Y.M.T.; Project administration, S.K.P.L. and P.C.Y.W.; Resources, S.K.P.L. and P.C.Y.W.; Supervision, C.-C.T., S.K.P.L. and P.C.Y.W.; Validation, C.-C.T., W.C. and J.Y.M.T.; Visualization, C.-C.T., W.C., J.Y.M.T., S.K.P.L. and P.C.Y.W.; Writing—original draft, C.-C.T., W.C., J.Y.M.T., S.K.P.L. and P.C.Y.W.; Writing—review & editing, C.-C.T., W.C., J.Y.M.T., O.H.Y.C., Y.-W.L., T.W.S.H., T.Z., C.W.H.L., C.L., C.Y., Y.-N.T., A.H.Y.N., J.H.K.C., H.Y., F.X., S.K.F.L., C.X., L.H., D.M.W.T., C.K.C.L., A.K.L.W., T.-L.Q., S.K.P.L. and P.C.Y.W. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

This study was approved by the Institutional Review Board (IRB) of The University of Hong Kong/Hospital Authority Hong Kong West Cluster, Hong Kong East Cluster Research Ethics Committee, the IRB of The University of Hong Kong-Gleaneagles Hospital Hong Kong, the Medical Ethics Committee of The University of

Hong Kong-Shenzhen Hospital as well as the Shanghai Jiaotong University School of Medicine Ruijin Hospital Ethics Committee.

Informed Consent Statement

Patient consent was waived since only leftover microorganisms isolated from clinical specimens were included in this study. No patient intervention was involved, and no patient information was collected.

Data Availability Statement

Nucleotide sequences generated in this study were deposited to the DDBJ/ENA/GenBank databases with nucleotide accession numbers listed in Table S1.

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Conflicts of Interest

Patrick C.Y. Woo has provided scientific advisory/laboratory services for Gilead Sciences, Incorporated; International Health Management Associates, Incorporated; Merck & Corporation, Incorporated, Micología Molecular S.L. and Pfizer, Incorporated. Christopher K. C. Lai has provided scientific advisory services from GenMark Diagnostics. The other authors report no conflicts of interest. The funding sources had no role in study design, data collection, analysis, interpretation, or writing of the report.

Given the role as Editor-in-Chief, Patrick C.Y. Woo had no involvement in the peer review of this paper and had no access to information regarding its peer-review process. Full responsibility for the editorial process of this paper was delegated to another editor of the journal. The authors alone are responsible for the content and the writing of the manuscript.

Use of AI and AI-Assisted Technologies

No AI tools were utilised for this paper.

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