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Identification Potency of Clinical Isolates in Aspergillus Species Using MALDI-TOF MS

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Abstract

It is important to make accurate identification of genus *Aspergillus* including cryptic species, because they could exhibit drug resistance to multiple antifungal agents. Recent advances in molecular diagnosis extended to clinical mycology, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been applied as an attractive methodology. Herein we evaluated the ability of MALDI-TOF MS for the identification of genus *Aspergillus*. A total of 42 strains of *Aspergillus* were genetically identified by the sequence analysis of PCR amplicons and simultaneously followed by MALDI-TOF MS. The concordance rate for *Aspergillus* using MALDI-TOF MS was 90.5 % in comparison with sequencing of PCR amplicons. Thirty eight registered strains in the MALDI Biotyper standard library showed the perfect concordance rate (100%). Notablely, azoles-resistant *Aspergillus lentulus* and *Aspergillus felis,* which were not registered in the MALDI Biotyper standard library, failed to be identified as the correct species. Moreover, these species with low score values in the MALDI-TOF MS analysis potentially resisted to a variety of antifungal agents. These results suggest that additional drug susceptibility testing should be further considered in poor yields with MALDI-TOF MS analysis.

Keywords: *matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Aspergillus felis; Aspergillus lentulus; Aspergillus tubingensis; cryptic species*

Introduction

The genus *Aspergillus* is one of the ubiquitous fungi that exhibit wide clinical spectrums of diseases in humans. It is a causative pathogen from an allergic response to subacute invasive life-threatening diseases, depending on the host immune conditions. Chronic aspergillosis including aspergilloma is usually found in patients with previously formed lung cavities and/or with mild immunocompromised status. Especially the lives of immunocompromised patients (receiving organ transplantation and anti-cancer chemotherapy) could be threatened by invasive aspergillosis (IA). Additionally, as IA often results in a fatal outcome, the early diagnosis and immediate treatment for IA with optimal chemotherapy lead to improvement of patient's prognosis [1,2].

Antifungal agents often exhibit variable activities against fungal organisms. For example, a few strains of genus *Aspergillus* could acquire drug resistance to multiple antifungal drug classes in accordance with failure of the antifugal treatment [3]. In addition, it has also been reported that azole-resistant cases of *Aspergillus fumigatus* have been increasing in number with several mutations in azole target genes [4]. Furthermore, cryptic species of *A. fumigatus* tend to resist azole antifungals, leading to the reduced treatment efficacy [5]. Therefore, in an effort to survey aspergillosis appropriately and to treat the patients with optimal antifungals, it is quite important to make accurate identification of genus *Aspergillus* including cryptic species.

Recently, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, BD, Bremen, Germany) has also been applied to the identification of fungal pathogens in clinical settings [6]. This methodology is based on applying a laser to the mixed crystals of the clinical specimens and matrix, before implementing acceleration with an electric field [7]. The flight time is then measured to determine the molecular weight of specimens and the pathogens could be identified with the reference of the previously registered molecular patterns in the library. While the use of MALDI-TOF MS has a great potential as one of the reliable methods to detect pathogens, the information on its detection sensitivity and specificity remains to be limited. In this study, we demonstrate the possibility and the current limitation for MALDI-TOF MS-mediated identification of genus *Aspergillus*.

Materials and methods

Our current study was conducted with clinical strains of genus *Aspergillus* that were previously isolated and identified in our hospital

between April 2014 and August 2016. The isolated strains, cultured at 37°C on Sabouraud dextrose broth for 2 days, were suspended in Sabouraud dextrose broth with 20% glycerol and then were stored at -80°C until the use.

For genetic identification of genus *Aspergillus*, stored samples were re-harvested on Potato Dextrose Agar (PDA) for 3 days, and each obtained colony was washed with 3 mL of phosphate buffered saline (PBS). DNA was then extracted from the pellet using the DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was used for the conventional sequence analysis of the following PCR products for the identification of genus *Aspergillus*. Segments of the internal transcribed spacer (ITS) and D1/D2 region were amplified using the primers ITS1 and NL4 [8], a segment of the beta-tubulin gene was amplified using the primers bT2a and bT2b [9] and a segment of the calmodulin gene was additionally amplified using the primers cmd5 and cmd6 [10]. The sequencing analysis of these PCR products with blastn (v2.5.0) algorism against database nucleotide collection (nr/nt) confirmed the species of *Aspergillus*. Additionally, to distinguish *Aspergillus oryzae* and *Aspergillus flavus*, a segment of the transcriptional regulator gene of the aflatoxin biosynthesis genes was additionally amplified and performed sequencing analysis using the primers *aflR* F2 and *aflR* R2 [11].

Analysis with the MALDI-TOF MS system was applied to above obtained each colony according to the manufacturer's instructions with a MALDI Biotyper 3.1 RTC software and MALDI Biotyper 4.0 standard library (Bruker Daltonics, Bremen, Germany). Any score value (SV) of less than 1.7 was deemed insufficient for identification.

Drug susceptibilities were confirmed in accordance with the modified M38-A2 method, which is the standard protocol of the Clinical and Laboratory Standards Institute (CLSI) [12], with the lowest concentration of antibiotics without visible growth on the microplate of the Yeast-like Fungus DP Eiken Kit (Eiken Chemical, Tokyo, Japan): micafungin (MCFG), caspofungin (CPFG), amphotericin B (AMPH-B), itraconazole (ITCZ) and voriconazole (VRCZ) [12,13]. The breakpoint of each antifungal agent for *Aspergillus* was determined using the EUCAST Antifungal Agents Breakpoint tables for interpretation of minimum inhibitory concentrations (MICs) v. 8.0 [14]. For the epidemiological cut-off value (ECV) have been prescribed in this study as follows; 0.5 μg/mL was considered for CPFG [15]. The quality control was ensured by concurrent testing with the strain of *A. fumigatus* ATCC MYA-3626, which is a recommended strain of CLSI [12].

Results

A total of 42 strains of *Aspergillus* were genetically identified (≥99% identity) based on the sequence analysis of PCR amplicons from clinical specimens (Table 1). They consisted of 27 *A. fumigatus*, 7 *Aspergillus niger*, 3 *Aspergillus terreus*, 2 *Aspergillus lentulus*, and an each of *A. oryzae, Aspergillus tubingensis* and *Aspergillus felis*. To verify whether the results identified by MALDI-TOF MS could match the results by sequencing analysis, all isolates were simultaneously analysed by MALDI-TOF MS. As showin in Table 1, 38 out of 42 isolates (90.5 %) were mached as same spiceses of the genus *Aspergillus*. However, 2 specimens from cryptic species of *A. fumigatus* (*A. lentulus* and *A. felis*) showed very low score values (below 1.7) in MALDI-TOF MS analysis. Of note, these were not registered in the MALDI Biotyper standard library, and were unable to be correctly identified as its species. The score values for *A. tubingensis*, one of cryptic species of *A. niger*, were also below 1.772 and it was identified incorrectly as *A. niger.*

Table 1. Sequence analysis of Aspergillus isolates and application with MALDI-TOF MS

Section	Species	No. of isolates confirmed with PCR	No. of matched isolates with TOF MS (%)
Fumigati	*Aspergillus fumigatus*	27	27 (100)
	Aspergillus lentulus	2	0 (0)
	Aspergillus felis	1	0 (0)
Nigeri	*Aspergillus niger*	7	7 (100)
	Aspergillus tubingensis	1	0 (0)
Terrei	*Aspergillus terreus*	3	3 (100)
Flavi	*Aspergillus oryzae*	1	1 (100)
total		42	38 (90.5)

We further addressed those drug susceptibilities in isolates that was incorrectly identified using MALDI-TOF MS. Results of the drug susceptibilities for *Aspergillus* section *Fumigati* were indicated in Table 2. For CPFG (ECV: 0.5 μg/mL), the obtained MIC values of cryptic species for *A. fumigatus* ranged to above 4 μg/mL (*A. lentulus*) and 2 μg/mL (*A. felis*), whereas no resistance to CPFG was noted in *A. fumigatus*. For AMPH-B, 2 specimens of *A. lentulus* were resistant. For ITCZ, all specimens were ranged lower than breakpoint MIC. However, for VRCZ, the obtained MIC values even in two isolates of *A. fumigatus* ranged over the breakpoint. Furthermore, in each isolate of *A. lentulus* and *A. felis*, the obtained MIC value was 4 or 8 μg/mL [13-17].

Discussion

Recently, application of MALDI-TOF MS allowed accurate identification for pathogens at species levels and it is possible to discriminate them at strain levels including relatively genus *Aspergillus* [6,16,17] as well as common fungi (e.g., *Candida* spp. and *Cryptococcus* spp.) [18-20]. In this study, we demonstrated that the identification for *Aspergillus* with MALDI-TOF MS could obtain a high concordance rate (90.5 %), compared with the genetic identifications using PCR. However, some strains belonging to cryptic species remain not to be discriminated in this protocol. As the reason of this limitation in cryptic *Aspergillus* species, we raised that taxonomic references of those species were absent in the MALDI Biotyper standard library. Since cryptic *Aspergillus* species have emerged in clinical settings worldwide and they have high possibility to be resistant to azole and/or polyene antifungal agents, more *Aspergillus* spp. including cryptic species should be resistered into the library of MALDI-TOF MS [5].

Aspergillus felis showed the lowest score values in our anlaysis, and others also failed the identification [21]. *A. lentulus* and *A. tubingensis* were also unable to be identified and *A. tubingensis* was incorrectly identified as *A. niger*. Notablely, *A. lentulus* and *A. felis* showed the high MIC value for VRCZ (Table 2), and these results appeared to be consistent with the findings of previous studies [21,22]. Thus, we should consider further to perform drug resistance testing as

confirmation for its susceptibilities, when the obtained score value (SV) is less than 1.7 or the pathogen identification failed based on the results of MALDI-TOF MS.

In conclusion, reconfiguration of database of MALDI-TOF MS in house is still required. As rare *Aspergillus* spp. including cryptic species have potencies of various antifungal resistance, additional drug susceptibility testing should be considered in poor yields with MALDI-TOF MS analysis.

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Conflict of Interest: None

Table 2 Antifungal susceptibility for Aspergillus section Fumigati

Aspergillus fumigatus											
	minimum inhibitory concentration (microgram/mL)										
	<0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8
MCFG	21	6									
CPFG						22	5				
AMPH-B							15	12			
ITCZ						10	16	1			
VRCZ						13	10	2	1	1	
Aspergillus lentulus											
	minimum inhibitory concentration (microgram/mL)										
	<0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8
MCFG	1	1									
CPFG										2	
AMPH-B									2		
ITCZ							2				
VRCZ										1	1
Aspergillus felis											
	minimum inhibitory concentration (microgram/mL)										
	<0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8
MCFG		1									
CPFG									1		
AMPH-B								1			
ITCZ								1			
VRCZ											1

Abbreviations: MCFG, micafungin; CPFG, caspofungin; AMPH-B, amphotericin-B; ITCZ, itraconazole; VRCZ, voriconazole.

References

1. Lin SJ, Schranz J, Teutsch SM (2001) Aspergillosis case-fatality rate: systematic review of the literature. *Clin Infect Dis* 32: 358–366. [crossref]
2. Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann JW, et al. (2002) Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N Engl J Med* 347: 408–415.
3. Arendrup MC (2014) Update on antifungal resistance in Aspergillus and Candida. *Clin Microbiol Infect* 20 Suppl 6: 42–48. [crossref]
4. Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE, Melchers WJ (2010) Azole resistance profile of amino acid changes in Aspergillus fumigatus CYP51A based on protein homology modeling. *Antimicrob Agents Chemother* 54: 2425–2430.
5. Alastruey-Izquierdo A, Alcazar-Fuoli L, Cuenca-Estrella M (2014) Antifungal susceptibility profile of cryptic species of Aspergillus. *Mycopathologia* 178: 427–433.
6. Alanio A, Beretti JL, Dauphin B, Mellado E, Quesne G, Lacroix C, et al. (2011) Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for fast and accurate identification of clinically relevant Aspergillus species. *Clin Microbiol Infect* 17: 750–755.
7. Tanaka K, Waki, H, Ido Y, Akita S, Yoshida Y, Yoshida T (1988) Protein and polymer analysis up to m/z 100000 by laser ionization time-of flight mass spectrometry. *Rapid Communication in Mass Spectromeroty* 2: 151–153.
8. O'Donnell K, Sutton DA, Fothergill A, McCarthy D, Rinaldi MG, Brandt ME, et al. (2008) Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the Fusarium solani species complex. *J Clin Microbiol* 46: 2477–2490.
9. Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol* 61: 1323–1330. [crossref]
10. Hong SB, Go SJ, Shin HD, Frisvad JC, Samson RA (2005) Polyphasic taxonomy of Aspergillus fumigatus and related species. *Mycologia* 97: 1316–1329. [crossref]
11. Tominaga M, Lee YH, Hayashi R, Suzuki Y, Yamada O, Sakamoto K, et al. (2006) Molecular analysis of an inactive aflatoxin biosynthesis gene cluster in Aspergillus oryzae RIB strains. *Appl Environ Microbiol* 72: 484–490
12. Clinical and Laboratory Standards Institute (2008) Reference method for broth dilution antifungal susceptibility testing for filamentous fungi; approved standard. Document M38-A2. Wayne, PA: Clinical and Laboratory Standards Institute.

13. Toyotome T, Fujiwara T, Kida H, Matsumoto M, Wada T, Komatsu R (2016) Azole susceptibility in clinical and environmental isolates of Aspergillus fumigatus from eastern Hokkaido, Japan. *J Infect Chemother* 22: 648–650.

14. European Committee on Antimicrobial Susceptibility Testing (2015) Breakpoint tables for interpretation of MICs Version.

15. Espinel-Ingroff A, Fothergill A, Fuller J, Johnson E, Pelaez T, Turnidge J (2011) Wild-type MIC distributions and epidemiological cutoff values for caspofungin and Aspergillus spp. for the CLSI broth microdilution method (M38-A2 document). *Antimicrob Agents Chemother* 55: 2855–2859.

16. Cassagne C, Ranque S, Normand AC, Fourquet P, Thiebault S, Planard C, et al. (2011) Mould routine identification in the clinical laboratory by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *PLoS One* 6: e28425.

17. Lau AF, Drake SK, Calhoun LB, Henderson CM, Zelazny AM (2013) Development of a clinically comprehensive database and a simple procedure for identification of molds from solid media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol* 51: 828–834.

18. Marklein G, Josten M, Klanke U, Muller E, Horre R, Maier T, et al. (2009) Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. *J Clin Microbiol* 47: 2912–2917.

19. Rosenvinge FS, Dzajic E, Knudsen E, Malig S, Andersen LB, et al. (2013) Performance of matrix-assisted laser desorption-time of flight mass spectrometry for identification of clinical yeast isolates. *Mycoses* 56: 229–235. [crossref]

20. Tarumoto N, Sakai J, Kodana M, Kawamura T, Ohno H, Maesaki S (2016) Identification of Disseminated Cryptococcosis Using MALDI-TOF MS and Clinical Evaluation. *Med Mycol J* 57: E41–46.

21. Barrs VR, van Doorn TM, Houbraken J, Kidd SE, Martin P, et al. (2013) Aspergillus felis sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One* 8: e64871. [crossref]

22. Tamiya H, Ochiai E, Kikuchi K, Yahiro M, Toyotome T, Watanabe A, et al. (2015) Secondary metabolite profiles and antifungal drug susceptibility of Aspergillus fumigatus and closely related species, Aspergillus lentulus, Aspergillus udagawae, and Aspergillus viridinutans. *J Infect Chemother* 21: 385–39.

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