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Molecular Identification of *Aspergillus* Species Collected for the Transplant-Associated Infection Surveillance Network[▽]

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A large aggregate collection of clinical isolates of aspergilli ($n = 218$) from transplant patients with proven or probable invasive aspergillosis was available from the Transplant-Associated Infection Surveillance Network, a 6-year prospective surveillance study. To determine the *Aspergillus* species distribution in this collection, isolates were subjected to comparative sequence analyses by use of the internal transcribed spacer and β-tubulin regions. *Aspergillus fumigatus* was the predominant species recovered, followed by *A. flavus* and *A. niger*. Several newly described species were identified, including *A. lentulus* and *A. calidoustus*; both species had high in vitro MICs to multiple antifungal drugs. *Aspergillus tubingensis*, a member of the *A. niger* species complex, is described from clinical specimens; all *A. tubingensis* isolates had low in vitro MICs to antifungal drugs.

The genus *Aspergillus* is classified into eight subgenera, and each subgenus is subdivided into several sections that include many related species (12). As this classification scheme is unique to this genus and could be complex to a nontaxonomist seeking to identify species within this genus, it was proposed that species within the sections *Fumigati*, *Flavi*, *Nidulantes*, *Usti*, and *Terrei* be reported as a "species complex," for instance, "*Aspergillus fumigatus* species complex" (5). For the identification of isolates to the species complex level, as well as for the placement of a species within a complex, mycologists have historically relied on characterization of macroscopic and microscopic features. However, recent studies have demonstrated that the identification of different species within each of the *Aspergillus* species complexes is problematic because of the overlapping morphological features of these organisms (5).

Molecular studies have revealed the presence of several cryptic *Aspergillus* species among isolates identified as a single morphospecies (4, 16). For instance, *A. lentulus* was described in 2005 as a new species within the *A. fumigatus* complex; isolates were initially recovered from patients from one medical center in the United States. Subsequently, this species was isolated from patients in other geographical regions of the world and from environmental samples (4, 10, 18). Recently, another cryptic species, *A. calidoustus* (*A. ustus* complex), was described from isolates originally identified as *A. ustus*; *A. calidoustus* is genetically distinct and can grow at higher temperatures, a feature that was distinct from that of *A. ustus* (16). Interestingly, members of both of these newly described species have high MICs to several antifungal drugs, including azoles (4, 16). These studies and others have employed comparative DNA sequencing-based methods to achieve species identification of isolates within the species complex. The International Society for Human and Animal Mycology-sponsored *Aspergillus* Working Group has recommended the use of a comparative sequencing-based identification method that uses the ribosomal internal transcribed spacer (ITS) region for identification to the species complex level and a protein-coding locus, such as the β-tubulin region, for the identification of species within the complex for the identification of *Aspergillus* species (5).

We hypothesized that the molecular analysis of aspergilli collected from the Transplant-Associated Infection Surveillance Network (TRANSNET) would provide a more accurate description of species within the *Aspergillus* species complex and reveal cryptic species that cannot be identified by the use of morphological methods alone. The present study was designed to characterize the morphologically identified *Aspergillus* isolates by the use of a two-step molecular format that included comparative sequence analyses of the ITS and the β-tubulin regions. In addition, we tested the susceptibilities of selected *Aspergillus* isolates to amphotericin B (AMB), itraconazole (ITZ), voriconazole (VRZ), and posaconazole (POS).

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MATERIALS AND METHODS

Fungal isolates. TRANSNET, which is made up of 24 transplant centers throughout the United States, conducted prospective surveillance for invasive fungal infections in hematopoietic stem cell and solid organ transplant recipients from 2001 to 2006 (13). *Aspergillus* isolates recovered from transplant recipients with proven or probable invasive aspergillosis (IA) on the basis of modified criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (3) were identified by morphological methods, when possible, by the participating center. Available isolates were sent to the Fungus Reference Unit at the Centers for Disease Control and Prevention, where these identities were confirmed by morphology.

Comparative sequencing-based identification. Two hundred sixteen *Aspergillus* isolates, representing one isolate per patient, were available for molecular analysis. Two *Aspergillus* isolates recently identified as *A. calidoustus* (strains IFI04-0143 and IFI04-0142) by molecular methods were also included for analyses (16). These isolates were stored frozen at −70°C until use. The molecular identification scheme was as follows: (i) the initial classification of all the TRANSNET aspergilli by comparative analyses of the ITS sequence to achieve an *Aspergillus* species complex-level identification and (ii) the identification of the species within each species complex by use of the β-tubulin region (5).

Aspergillus isolates were thawed, subcultured on Sabouraud dextrose agar (Becton Dickinson, Sparks, MD), and visually checked for purity before molecular characterization.

Genomic DNA was extracted from aspergilli grown for 48 h on Sabouraud dextrose agar plates by using a DNeasy tissue kit (Qiagen, Valencia, CA). Universal fungal primers directed to the ITS1-5.8S-ITS2 and the β-tubulin regions were employed to amplify DNA from all *Aspergillus* isolates, as described previously (8, 9). The resultant PCR amplicons were purified by using an ExoSAP-IT enzyme system (USB Corporation, Cleveland, OH), according to the manufacturer's instructions. Sequencing of both strands (with the same primers used for PCR amplification) was performed with a BigDye Terminator (version 1.1) cycle sequencing kit (Applied Biosystems). All cycle sequencing reactions were performed on a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) by using an initial denaturation at 96°C for 5 s, followed by 30 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The products were purified with an Agencount CleanSEQ system (Beckman Coulter, Beverly, MA), dried, resuspended in 0.1 mM EDTA, and run on a 3730 DNA analyzer (Applied Biosystems) using of the protocols supplied by the manufacturer. The resultant nucleotide sequences were edited by using the Sequencher program (Gene Codes Corporation, Ann Arbor, MI) and aligned by using the program CLUSTAL W. Gene sequences derived from the ITS1-5.8S-ITS2 and the β-tubulin regions of all the *Aspergillus* isolates were compared with sequences in the GenBank database to identify isolates to the species complex level and to the species level within the complex.

Antifungal susceptibility testing. Susceptibilities to AMB, ITZ, VRZ, and POS were determined by using the CLSI M38A broth microdilution method for 23 newly described isolates and/or previously unrecognized *Aspergillus* isolates (7). The MIC was defined as the lowest concentration of the respective drug that resulted in a 100% growth reduction compared to the level of growth of the isolate in a drug-free control culture.

RESULTS

Comparative sequence analyses of the ITS regions of the 218 *Aspergillus* isolates in the GenBank sequence database revealed the following species distribution: 147 (67.4%) isolates belonged to the *A. fumigatus* complex, 29 (13.2%) to the *A. flavus* complex, 19 (8.7%) to the *A. niger* complex, 11 (7.4%) to the *A. terreus* complex, 6 (2.7%) to the *A. ustus* complex, 5 (2.3%) to the *A. versicolor* complex, and 1 to the *A. nidulans* complex.

Of the 147 *A. fumigatus* complex isolates, 139 (93.9%) were *A. fumigatus* (100% sequence identity to ATCC 1022 ex type), 4 (2.7%) were *A. lentulus* (100% sequence identity to type isolate FH5), 3 (2.0%) were *A. udagawae* (100% sequence identity to *Neosartorya udagawae* CBS 154.89), and 1 was *Neosartorya pseudofischeri* (100% sequence identity to isolate NRRL 20748). The ITS and β-tubulin sequences of all of the *A. flavus* ($n = 29$) and *A. terreus* ($n = 11$) isolates were 100% identical to the ITS and β-tubulin sequences of *A. flavus* ATCC 20043 and *A. terreus* ATCC 1012. Of the 19 *A. niger* complex isolates, 6 were identified as *A. tubingensis* (100% identity to *A. tubingensis* NRRL 4875) and 13 were identified as *A. niger* sensu stricto (100% identity to *A. niger* CBS 101699 and 99% sequence identity to isolate NRRL 363). Comparative sequence analyses of the β-tubulin regions of the six *A. ustus* complex isolates revealed 99 to 100% identity with *A. calidoustus* type isolate CBS 121601, and therefore, these isolates were reidentified as *A. calidoustus*. Of the five *A. versicolor* complex isolates, three isolates were identified as *A. versicolor* (99 to 100% sequence identity with the sequence of isolate NRRL 4791). Two isolates had a β-tubulin sequence identity of 100% with the β-tubulin sequence of *A. sydowii* NRRL 4768 and were therefore assigned to the respective species. The β-tubulin sequence of the *A. nidulans* isolate had 100% identity with *Emericella quadrilineata* isolate NRRL 4992 (anamorph, *Aspergillus tetrazonus*).

Table 1 describes the species identities of 23 selected uncommon aspergilli, brief patient and culture characteristics, and the patterns of susceptibility of these isolates to AMB, ITZ, VRZ, and POS. All four *A. lentulus* isolates were recovered from center E, while there was no center-specific recovery for any of the other aspergilli. Three of four *A. lentulus* isolates had AMB MICs of >1 μg/ml and VRZ MICs of >2 μg/ml. Two of three *A. udagawae* isolates had AMB MICs of >1 μg/ml and VRZ MICs of >2 μg/ml. All six *A. calidoustus* isolates had ITZ, VRZ, and POS MICs of ≥4 μg/ml. All other aspergilli had low MICs to the antifungals tested.

DISCUSSION

The present study was undertaken to identify by molecular methods *Aspergillus* isolates recovered from a multicenter prospective surveillance study of invasive fungal infections among transplant patients. The results of the study demonstrate that of the isolates received for analysis in this study, *A. fumigatus* remains the predominant etiological agent recovered from clinical samples, followed by *A. flavus* and *A. niger*. A good correlation between morphology and comparative sequencing-based methods was found by employing the ITS regions to identify aspergilli to the species complex level. Thus, this study indicates that clinical microbiology laboratories can continue to use morphological methods to accurately establish a species complex-level classification. According to the proposal of the International Society for Human and Animal Mycology *Aspergillus* Working Group, these isolates should then be reported as members of the particular species complex with an indication that such a complex may contain one or many species (5).

In contrast, morphological methods performed poorly in identifying species within each *Aspergillus* species complex; comparative sequencing-based identification by use of the β-tubulin protein-encoding locus clearly distinguished species within the *A. fumigatus*, *A. niger*, and *A. versicolor* complexes. Furthermore, comparative sequence analyses revealed the identity of one isolate as *E. quadrilineata*. This isolate was originally identified as *Emericella nidulans* by morphological methods. *E. quadrilineata* is morphologically similar to *E. nidu-*

TABLE 1. Isolate identity, type of IA, and antifungal susceptibilities of rare aspergilli recovered in this study

Strain no.	Transplant type	Site of isolation[a]	Molecular identity	Center	MIC μg/ml			
					AMB	ITZ	VRZ	POS
IFI03-0026	Heart	Hip (biopsy specimen)	*A. calidoustus*	A	0.5	>8	4	>8
IFI01-0058	Kidney/pancreas	Lung	*A. calidoustus*	A	1	>8	8	>8
IFI03-0056	HSCT[b]	Lung	*A. sydowii*	B	1	0.5	2	0.5
IFI03-0059	HSCT	Lung	*A. calidoustus*	B	0.5	>8	4	>8
IFI02-0227	HSCT	Lung	*A. tubingenesis*	C	0.25	1	1	0.06
IFI05-0038	HSCT	Lung	*A. tubingenesis*	D	0.125	1	1	0.25
IFI05-0048	HSCT	Lung	*A. sydowii*	E	1	0.25	1	0.25
IFI04-0143[c]	HSCT	Lung (BAL fluid)	*A. calidoustus*	E	1	>8	8	>8
IFI04-0137	HSCT	Lung	*A. udagawae*	E	1	0.25	2	0.25
IFI04-0142[c]	HSCT	Skin	*A. calidoustus*	E	1	>8	4	>8
IFI06-0001	HSCT	Lung	*A. lentulus*	E	2	0.5	4	0.25
IFI05-0046	HSCT	Lung	*A. lentulus*	E	2	0.5	2	0.25
IFI06-0011	HSCT	Lung (sputum)	*A. lentulus*	E	0.5	0.25	1	0.25
IFI06-0014	HSCT	Lung	*A. lentulus*	E	2	0.5	4	0.25
IFI02-0149	HSCT	Skin	*N. pseudofischeri*	F	0.125	0.25	2	0.5
IFI04-0005	HSCT	Lung	*A. tubingenesis*	G	0.125	1	1	0.5
IFI03-0138	Heart	Lung	*A. udagawae*	G	2	0.25	0.5	0.125
IFI06-0129	HSCT	Lung (sputum)	*A. tubingenesis*	H	0.125	0.5	0.5	0.25
IFI06-0126	HSCT	Blood	*E. quadrilineata*	H	0.5	0.25	0.5	0.25
IFI02-0093	HSCT	Skin lesion	*A. tubingenesis*	I	0.125	0.25	0.5	0.06
IFI02-0091	Liver	Lung (sputum)	*A. udagawae*	I	2	2	2	0.25
IFI03-0055	Lung	Lung	*A. calidoustus*	J	1	>8	4	>8
IFI04-0017	HSCT	Lung	*A. tubingenesis*	K	0.125	0.5	1	0.25

[a] The site of isolation from the lung includes cultures of biopsy specimens or bronchoalveolar lavage (BAL) fluid, unless otherwise indicated.
[b] HSCT, hematopoietic stem cell transplant.
[c] Previously identified as *A. calidoustus* (17).

lans, and this has resulted in misidentification when morphological methods alone are used for identification (17). Distinguishing these two species may be important, as *E. quadrilineata* differs from *E. nidulans* in their susceptibilities to AMB; *E. quadrilineata* appears to have a low in vitro MIC to AMB, while *E. nidulans* has a higher MIC to this drug (17). Similar to the finding of Verweij et al., we found that the *E. quadrilineata* isolate had an in vitro AMB MIC of 0.5 μg/ml and had low MICs to all azoles tested. Although they were not tested in this study, *E. quadrilineata* appears to have a propensity to have echinocandin MICs higher than those of *E. nidulans*, and this difference may be important in therapeutic decision making (17). It must be pointed out that IA due to *E. quadrilineata* appears to be rare; in the past, this organism has been reported to have been the cause of IA in a patient with chronic granulomatous disease and in one patient with central nervous system disease (17).

Four species within the *A. fumigatus* species complex, *A. fumigatus*, *A. lentulus*, *A. udagawae*, and *N. pseudofischeri*, were recovered in this study. *Aspergillus fumigatus* was found to make up 94% of the available isolates, suggesting that this species remains the predominant etiological agent of IA in transplant patients. *Aspergillus lentulus* has recently been described within the *A. fumigatus* species complex and has been recovered from patients in the United States (4), Japan (18), South Korea (10), Australia (11), and Spain (1, 2). In contrast to the clear global distribution of *A. lentulus*, all the *A. lentulus* isolates detected in this surveillance study were recovered from a single transplant center (center E). Interestingly, *A. lentulus* was first discovered and described as a new species after it was recovered from several patients who received hematopoietic stem cell transplants in this center in 1995 (4). The reason for this geographic clustering is not immediately clear; but it may include true differences in geographic distribution due to environmental variables, a common-source exposure within that transplant center, other clinical selection variables, and/or differences in culturing methods and diagnostics that can affect isolate recovery. A larger and more geographically diverse prospective study using standardized culture methods may be necessary in order to describe the true geographic distribution of this recently recognized species.

Previous studies have demonstrated that *A. udagawae*, *N. pseudofischeri*, and *A. lentulus* (members of the *A. fumigatus* species complex) have high MICs to multiple antifungal drugs (1, 4, 18). Similarly, the results from this study show that *A. udagawae* and *A. lentulus* have high MICs to AMB and VRZ, while the one isolate of *N. pseudofischeri* had a low MIC to all the antifungals. In contrast, all three species had low MICs to POS. Given the small number of samples ($n = 8$), it will be important to test more isolates to better understand the in vitro antifungal susceptibility patterns of the different species within the section *Fumigati*.

In this study, all the morphologically identified *A. ustus* isolates were identified (by molecular methods) as the newly described species *A. calidoustus* (16). Two *Aspergillus* isolates that were included in this study were identified as *A. calidoustus* (isolates IFI04-0143 and IFI04-0142) by molecular methods in a prior study by Varga and coworkers (16). We confirmed the findings presented in that previously published report that *A. calidoustus* isolates had high MICs to ITZ, VRZ, and POS (16). Another *Aspergillus* species, *A. sydowii*, is a known opportunistic fungal pathogen of corals, but it has been infrequently isolated from human cases of onychomycosis (15) and peritonitis (6). Here we report the isolation of *A. sydowii* from

two cases of IA (one proven and one probable); both of these isolates had low in vitro MICs to all antifungals tested.

For the first time, we found *A. tubingensis* to be a cause of IA in humans. All *A. tubingensis* isolates had low in vitro MICs to the antifungal drugs tested. *Aspergillus tubingensis* belongs to the *A. niger* complex (black aspergilli) and is commonly found on plant products and in processed foods, such as coffee, grapes, and cereals (14). This species is morphologically indistinguishable from *A. niger* and can be reliably identified only by molecular methods (14).

In summary, over 10% of the isolates associated with IA in transplant recipients were found to be cryptic species; molecular identification methods were essential in distinguishing these species. Because several of these species, including *A. lentulus* and *A. calidoustus*, have high in vitro MICs to antifungal agents, clinical studies on patient outcomes and larger epidemiologic analyses are warranted.

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