Less-Frequent *Fusarium* Species of Clinical Interest: Correlation between Morphological and Molecular Identification and Antifungal Susceptibility[▽]

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Forty-eight *Fusarium* isolates morphologically identified as belonging to seven species of clinical interest (i.e., *Fusarium chlamydosporum*, *Fusarium dimerum*, *Fusarium incarnatum*, *Fusarium napiforme*, *Fusarium nygamai*, *Fusarium proliferatum*, and *Fusarium sacchari*) were characterized molecularly by the analysis of the sequences of the TUB region of the β -tubulin gene. *F. chlamydosporum* and *F. dimerum* were the most genetically heterogeneous species. A high degree of correlation between the morphological and molecular identification was shown among the isolates studied. A table with the key morphological features for the identification of these *Fusarium* species is provided. The antifungal susceptibilities of the *Fusarium* isolates to 11 antifungal drugs were tested; terbinafine was the most active drug against all the species tested with the exception of *F. incarnatum*, for which amphotericin B was the most active.

The most frequent species causing fusariosis are *Fusarium solani*, *Fusarium oxysporum*, and *Fusarium verticillioides* (1, 16, 47), but several other species are also found to cause human infections, although less frequently. Some of these species are *Fusarium chlamydosporum*, *Fusarium dimerum*, *Fusarium incarnatum*, and the following other species that are included into the *Gibberella fujikuroi* species complex: *Fusarium napiforme*, *Fusarium nygamai*, *Fusarium proliferatum*, and *Fusarium sacchari* (30, 31). These species have been associated with different types of infection, in particular with keratomycoses and other ocular infections (10) and with disseminated infections in immunocompromised patients (2, 6, 17, 20, 23, 24, 26, 39, 41, 43, 44). The real incidence of these species is unknown since they are poorly known and laboratorians and clinical microbiologists are not generally aware of their possible presence in human infections.

Since the species of *Fusarium* are generally resistant to all the available antifungal drugs (40), it could be considered that speciation of *Fusarium* is necessary only for epidemiological purposes. However, some in vitro data concerning particular species seem to be very promising and deserve to be investigated clinically. For instance, *F. verticillioides* isolates were susceptible to posaconazole and terbinafine and *Fusarium thapsinum* isolates to terbinafine (4). The identification of fusaria to the species level is not easy, and in numerous clinical cases the etiological agent is reported as being a *Fusarium* sp. However, several recent studies have demonstrated the usefulness of molecular methods for the

identification of those *Fusarium* species that are difficult to distinguish morphologically (1, 4, 47). In recent years, the in vitro antifungal susceptibilities of the most frequent species of *Fusarium* have been evaluated (1, 3, 4, 40, 47), but only a few isolates of the less-common species have been studied. The objectives of our study were (i) to evaluate the correlation between the morphological and the molecular identification of less-frequent *Fusarium* species isolates received by our laboratory and (ii) to determine the antifungal susceptibilities of isolates representative of those less-common *Fusarium* species of clinical interest identified molecularly.

MATERIALS AND METHODS

Isolates and morphological identification. We included 48 *Fusarium* isolates in the study (Table 1). Twenty-nine of these isolates, which were mainly from clinical sources, were sent to our laboratory from different clinical centers for identification purposes. Those isolates were identified following the conventional morphological criteria (25, 28, 30). Briefly, to study the microscopic and colony features, the isolates were usually subcultured on potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI) and on oatmeal agar (OA; 30 g oat flakes, 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g KH_2PO_4 , 15 g agar, 1 liter tap water), incubated at 25°C in the dark. Sporodochia can be detected by examining the cultures under a stereoscopic microscope, usually after 7 days of incubation. The microscopic features were examined by making direct wet mounts with lactic acid from cultures on PDA and OA after 7 to 10 days of incubation. Growth rates were obtained from colonies on PDA at 25°C after 4 and 10 days of incubation in the dark. In addition, 19 reference strains from the Centraalbureau voor Schimmelcultures (CBS) and the Agricultural Research Service (ARS/NRRC) were used for a comparison.

Molecular study. For the phylogenetic analysis, we sequenced the TUB region of the β -tubulin gene, which has proven to be highly phylogenetically informative in different molecular studies of the genus *Fusarium* (33, 34, 35). For DNA extraction, amplification, and sequencing, we followed the procedures previously described by Gilgado et al. (13), with some modifications. We used the primers TUB-F (9) and T22 (33) with an annealing temperature of 55°C, and the PCR products were purified using a GFX PCR DNA kit (Pharmacia Biotech, Cerdanyola, Spain). We included five reference sequences retrieved from GenBank corresponding to *F. nygamai*, *F. napiforme*, *F. sacchari*, *Fusarium subglutinans*, and *F. proliferatum*, respectively, since they are the only TUB sequences of the species included in the study that are available in the database (Table 1). The

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TABLE 1. Isolates included in this study and their origins

Species and isolate ^a	Origin	GenBank TUB
<i>F. chlamydosporum</i>		
FMR 7232	Clinical, Argentina	FM878927
FMR 9683	Environmental, Spain	FM878934
FMR 9684	Environmental, Spain	FM878940
NRRL 32521	United States	FM878935
NRRL 34012	United States	FM878936
NRRL 34019	United States	FM878937
NRRL 34022	United States	FM878938
NRRL 34033	United States	FM878939
<i>F. dimerum</i>		
FMR 6320	Clinical, Brazil	FM878630
FMR 9042	Clinical, France	FM878631
FMR 9105	Clinical, India	FM878632
FMR 9580	Clinical, India	FM878633
FMR 9584	Clinical, India	FM878629
FMR 9590	Clinical, India	FM878634
CBS 115623	Unknown	FM878635
<i>F. incarnatum</i>		
FMR 4404	Clinical, Chile	FM878926
FMR 4403	Clinical, Chile	FM878941
FMR 9096	Clinical, India	FM878928
FMR 9097	Clinical, India	FM878929
FMR 9098	Clinical, India	FM878930
FMR 9099	Clinical, India	FM878931
FMR 9100	Clinical, India	FM878932
FMR 9101	Clinical, India	FM878933
CBS 131.73	Bahamas	FM878942
<i>F. napiforme</i>		
CBS 673.94		FM878607
NRRL 25196	United States	FM878605
NRRL 25201	United States	FM878606
		U34483 ^b
<i>F. nygamai</i>		
CBS 140.95	Clinical, Egypt	FM878613
CBS 675.94	<i>Striga hermonthica</i> , Sudan	FM878614
NRRL 13448	United States	FM878624
NRRL 22106	United States	FM878625
NRRL 25312	United States	FM878626
NRRL 25449	United States	FM878627
NRRL 25450	United States	FM878628
		U34481 ^b
<i>F. proliferatum</i>		
CBS 620.80	<i>Sitobion avenae</i> , United Kingdom	FM878609
FMR 4382	Clinical, United States	FM878610
FMR 4387	Clinical, United States	FM878611
FMR 5124	Clinical, Spain	FM878612
FMR 7239	Clinical, Spain	FM878615
FMR 7377	Clinical, Spain	FM878616
FMR 9036	Clinical, France	FM878617
FMR 9435	Clinical, United States	FM878621
FMR 9436	Clinical, United States	FM878622
		U34471 ^b
<i>F. sacchari</i>		
CBS 223.76	<i>Saccharum officinarum</i> , India	FM878608
FMR 9102	Clinical, India	FM878618
FMR 9103	Clinical, India	FM878619
FMR 9106	Clinical, India	FM878620
FMR 9578	Clinical, India	FM878623
		U34469 ^b
<i>F. subglutinans</i>		U34472 ^b

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^b Accession numbers of these sequences were retrieved from GenBank.

sequences were aligned using the ClustalX (version 1.8) computer program (46), followed by manual adjustments with a text editor. The phylogenetic analysis was performed using PAUP* version 4.0b10 (45). Maximum parsimony trees were obtained after 100 heuristic searches by using a random sequence addition and

tree bisection-reconnection branch-swapping algorithms, collapsing zero-length branches, and saving all minimal-length trees (MulTrees).

Antifungal susceptibility study. We evaluated the in vitro activity of 11 antifungal drugs against 48 *Fusarium* isolates (Table 2), which were grown on PDA plates and incubated at 25°C for 7 days. The tests were performed by using a microdilution reference method (8), with some modifications. The inocula were adjusted to a final concentration of 4×10^3 to 5×10^4 conidia/ml with a hemocytometer and verified by quantitative colony counts on PDA plates. The antifungal agents tested included amphotericin B (AMB), albaconazole (ABC), voriconazole (VRC), itraconazole (ITC), ravuconazole (RVC), terbinafine (TBF), ketoconazole (KTC), posaconazole (PSC), micafungin (MFG), fluconazole (FLC), and flucytosine (5-FC). MFG, FLC, and 5-FC were diluted in sterile distilled water and the others in dimethyl sulfoxide. Final drug concentrations ranged from 64 to 0.12 µg/ml for FLC and 5-FC, from 128 to 0.25 µg/ml for MFG, and from 16 to 0.03 µg/ml for the rest. The MIC endpoints for AMB, TBF, and the triazoles ABC, ITC, PSC, RVC, and VRC were defined as the lowest concentration that produced complete inhibition of growth (MIC₀). For FLC, KTC, 5-FC, and MFG, the endpoint was defined as the lowest concentration that produced 50% inhibition of growth (MIC₅₀). *Paeclomyces variotii* ATCC 36257 was included as a quality control strain. The tests were performed twice on two different days and, in those cases where the results did not coincide, they were repeated a third time. For those strains, the MIC was considered the mode of the three MICs.

RESULTS

Correlation between morphological and molecular identification. The 29 *Fusarium* isolates received for identification were morphologically identified as *F. chlamydosporum* ($n = 3$), *F. dimerum* ($n = 6$), *F. proliferatum* ($n = 8$), and *F. incarnatum* ($n = 8$). For four of the isolates, it was difficult to differentiate between *F. sacchari* and *F. subglutinans*. However, based on the presence of septate microconidia, we tentatively identified the four isolates as being *F. sacchari*. The formation of sporodochia was variable according to each isolate, but when present, sporodochia were observed as cushion-like or slimy masses on the agar surface, at least in primary cultures on PDA or OA after 7 to 10 days. A molecular comparison with reference strains confirmed the identification of our isolates. Table 3 summarizes the most relevant morphological features useful for distinguishing these species, which can help those laboratories that have no molecular facilities for the identification of clinical isolates.

Molecular study. With the primers we used we were able to amplify and sequence a fragment of 378 bp. Parsimony analysis of the data set yielded 225 phylogenetic trees of 157 steps in length. Three main clades with 100% bootstrap support were obtained (Fig. 1). The first of these comprised five different subclades, each representing the different species with the respective reference sequences. The first two included the isolates of *F. nygamai* ($n = 7$) and *F. napiforme* ($n = 3$), respectively. The *F. sacchari* ($n = 5$) subclade was genetically more distant; the fourth one grouped all isolates to *F. proliferatum* ($n = 9$), and the most distant subclade included only the reference sequence of *F. subglutinans*. The four species presented a very low intraspecific variability, showing only one haplotype each, with the exception of *F. sacchari*, which showed two haplotypes. The second main clade grouped the isolates of *F. incarnatum* ($n = 9$) and those of *F. chlamydosporum* ($n = 8$). These two species were clearly genetically differentiated and showed more genetic variability than those previously mentioned, with six and five haplotypes, respectively. Finally, the third main clade included the isolates morphologically identified as *F. dimerum*

TABLE 2. Activities of conventional and new antifungal drugs against isolates of seven *Fusarium* species of clinical interest

Antifungal agent	MIC ($\mu\text{g/ml}$) for indicated species (no. of isolates tested)															
	<i>F. chlamydosporum</i> (8)		<i>F. dimerum</i> (7)		<i>F. incarnatum</i> (9)		<i>F. napiforme</i> (3)		<i>F. nygamai</i> (7)		<i>F. proliferatum</i> (9)		<i>F. sacchari</i> (5)		Total (48)	
	Range	GM	Range	GM	Range	GM	Range	GM	Range	GM	Range	GM	Range	GM	Range	GM
ABC	4–32	11.31	4–16	8.83	8–32	16	4	4	8–16	9.75	8–16	10.08	4–8	6.06	4–32	9.79
AMB	1–4	1.54	1–2	1.64	0.5–2	0.93	2–4	3.18	2–8	3.28	2–8	3.7	1–2	1.52	0.5–8	1.94
ITC	32	32	32	32	32	32	32	32	32	32	32	32	32	32	32	32
KTC	4–32	8.72	1–32	3.62	2–4	2.72	8	8	16–32	21.53	8–32	21.77	2–32	18.38	1–32	8.98
PSC	0.5–32	2.38	32	32	1–32	2.94	32	32	32	32	32	32	2	2	0.5–32	9.94
RVC	1–16	7.34	4–16	8.83	8–16	9.33	2–4	3.18	4–16	8.83	8–32	20.16	4–8	5.28	1–32	8.98
TBF	0.125–1	0.27	0.125–2	0.91	0.5–32	10.08	0.06–0.25	0.12	0.125–2	0.34	0.125–1	0.29	0.125–0.25	0.14	0.06–32	0.6
VRC	1–4	2.83	2–8	4	2–4	3.7	1–2	1.59	4–8	6.56	8–16	9.33	2–4	3.03	1–16	4.24

($n = 7$), the most phylogenetically distant and heterogeneous species, which included three subclades with a total of six different haplotypes.

Antifungal susceptibility study. The results of the susceptibility tests are shown in Table 2. TBF was the most active drug, showing a total geometric mean (GM) MIC of 0.60 $\mu\text{g/ml}$ against all the species studied. One exception was *F. incarnatum*, which had a GM MIC of 10.08 $\mu\text{g/ml}$. AMB was the second most active drug, with a total GM MIC of 1.94 $\mu\text{g/ml}$. For this drug, *F. proliferatum* (GM MIC of 3.70 $\mu\text{g/ml}$), *F. nygamai* (GM MIC of 3.28 $\mu\text{g/ml}$), and *F. napiforme* (GM MIC of 3.18 $\mu\text{g/ml}$) were more resistant than the other species, whereas *F. incarnatum* showed the lowest GM MIC (0.93 $\mu\text{g/ml}$). Although VRC showed poor activity against all the strains tested, it was the third most active antifungal drug, with a total GM MIC of 4.24 $\mu\text{g/ml}$. PSC showed very variable results. Although it showed very high MICs in general, it exhibited significant activity for the majority of the strains of *F. chlamydosporum*, *F. incarnatum*, and *F. sacchari*. The rest of the azoles tested (RVC, ABC, ITC, and KTC) also showed high MICs for all the strains. FLC, 5-FC, and MFG were not active against any of the isolates (data not shown).

DISCUSSION

The use of the internal transcribed spacer rRNA gene sequences has proven to be useful for the identification of numerous fungal pathogens. However, this marker has been used only for the recognition of species complexes in the genus *Fusarium* (5). In contrast, β -tubulin has proven to be a good marker for species identification within the *Gibberella fujikuroi* species complex, although it is not very useful for other species because of paralogous or duplicated divergent alleles (5, 32). In general, we have obtained a high degree of correlation between the morphological and molecular identification of those clinical isolates that we have received for identification. The only species that was difficult to identify was *F. sacchari*. This is a species morphologically close to *F. subglutinans* (10, 25), and even Nelson et al. (28) considered them synonyms. *Fusarium sacchari*, *F. subglutinans*, and other related species constitute a morphologically similar group of species that can be differentiated practically only by the use of mating tests or molecular markers (25). Although several human infections have been attributed to *F. subglutinans* (10, 25), the identification of the case isolates is questionable. None of the clinical

isolates included in this study was molecularly identified as *F. subglutinans* (Fig. 1). Our study confirmed that *F. chlamydosporum* and, especially, *F. dimerum* represent complexes of species (37), as has occurred in other more common species of *Fusarium*, such as *F. solani*, *F. oxysporum*, and *F. verticillioides* (3, 4, 32, 33, 35, 36). It would be interesting to do further studies of the phylogeny of *F. chlamydosporum* and *F. dimerum*, which have been associated with severe human infections (10, 25).

TBF showed good activity against all the species tested, which is in agreement with previous studies where this drug worked well in vitro against different *Fusarium* species (4, 22). Although there is no history of this drug in clinical cases caused by the species included in the present study, it was successfully used in two cases of onychomycosis by *F. oxysporum* (12, 18). Moreover, Gupta et al. (18) reported that onychomycosis infections caused by *Fusarium* species show response to this drug. Recently, the successful treatment of a disseminated cutaneous *F. proliferatum* infection using liposomal AMB and TBF was reported (29). This combination therapy was also effective in the treatment of a disseminated *F. oxysporum* infection (42).

AMB and VRC, the second and third most active drugs, respectively, are the drugs recommended for treating fusariosis (11). In fact, the use of AMB has shown good results in different clinical cases of systemic infection involving some of the species included in this study, such as *F. chlamydosporum* (23), *F. sacchari* (17), *F. nygamai* (24), *F. dimerum* (26), and *F. proliferatum* (6). Regardless of the antifungal used, for fusariosis neutrophil recovery is fundamental for resolving the infection, as demonstrated in practically all the cases. Numerous AMB failures also exist. For example, *F. napiforme* showed resistance to this drug in the only published case of disseminated infection caused by this species (27), as did *F. proliferatum* (44) and *F. dimerum* (2). VRC has demonstrated efficacy in the treatment of disseminated fusariosis, but most of the isolates involved in such infections were not identified to the species level (38). Recently, a stem cell transplant recipient with an invasive infection by *F. dimerum* (7) was also successfully treated with VRC. Despite the high MICs showed by PSC, this drug proved to be active in several invasive infections caused by *F. proliferatum* (21) or *F. solani* (19). The inactivity of FLC, 5-FC, and MFG against *Fusarium* isolates demonstrated in our study has already been reported by other authors (4, 14, 40).

TABLE 3. Key morphological features of seven *Fusarium* species of clinical interest^a

Species	Key distinctive features
<i>F. chlamydosporum</i>	Colonies were whitish with rose to bright pink areas ^b Colony diam exceeded 30 mm ^b Conidiogenous cells from aerial mycelium were usually polyphialides Microconidia were abundant, usually with 0 to 2 septates, obovoid, or fusiform, usually single on the phialidic opening Macroconidia were rarely produced and only on sporodochia, with 3 to 5 septates, slightly falcate Chlamydospores were abundant, usually in chains, pale brown, roughened
<i>F. dimerum</i>	Colonies were light orange to apricot Colony diam was less than 30 mm Conidiogenous cells from aerial mycelium were commonly monophialides Microconidia were absent Macroconidia were on the aerial mycelium, mostly with 0 to 1 septates, strongly curved, often pointed at both ends Chlamydospores were sparse, single, or in short chains, smooth
<i>F. incarnatum</i>	Colonies were pale orange to salmon Colony diam exceeded 30 mm Conidiogenous cells from the aerial mycelium were monophialides, but commonly polyphialides Microconidia were 0-septate scarce, commonly with 3 to 5 septates, fusiform to falcate, single or in pairs on the phialidic opening Macroconidia were rarely produced on sporodochia Chlamydospores were sparse, single or in chains, smooth
<i>F. napiforme</i>	Colonies were white, becoming vinaceous to violet Colony diam exceeded 30 mm Conidiogenous cells from aerial mycelium were monophialides Microconidia were abundant, with 0 to 1 septates, usually obovoid, pyriform or napiform conidia also present, in false heads or in short chains Macroconidia were usually on sporodochia, mostly with 5 septates, falcate to straight Chlamydospores were sparse, usually in chains or clusters, pale brown, smooth
<i>F. nygamai</i>	Colonies were white becoming vinaceous to violet Colony diam exceeded 30 mm Conidiogenous cells from aerial mycelium were monophialides and polyphialides Microconidia were abundant, usually 0-septate, oval, in false heads or in short chains Macroconidia were only on sporodochia, with 3 to 5 septates, straight to slightly curved Chlamydospores were mostly abundant, single, in chains or in clusters, subhyaline, smooth or roughened
<i>F. proliferatum</i>	Colonies were white becoming vinaceous to violet Colony diam exceeded 30 mm Conidiogenous cells from aerial mycelium were monophialides and polyphialides Microconidia were abundant, usually 0-septate, clavate (some pyriform conidia present in old cultures), in long chains Macroconidia were only on sporodochia, with 3 to 5 septates, straight to slightly curved Chlamydospores were absent
<i>F. sacchari</i>	Colonies were white becoming vinaceous to violet Colony diam exceeded 30 mm Conidiogenous cells from aerial mycelium were monophialides and polyphialides Microconidia were abundant, usually with 0 to 2 septates, oval and slender, in false heads, or single or in pairs on the phialidic opening Macroconidia were rarely produced, only on sporodochia, with 3 to 4 septates, falcate Chlamydospores were absent

^a A detailed explanation and illustrations of the microscopic features included in this table have been published by de Hoog et al. (10), Guarro and Gené (15), and Leslie and Summerell (25).

^b On PDA at 25°C after 4 days.

In conclusion, we have demonstrated in this study that several *Fusarium* species of clinical interest can be identified through a detailed morphological exam. This fact can be very important for those clinical laboratories with no molecular

facilities. However, there are still some species that are difficult to identify morphologically, such as *F. sacchari* or *F. thapsinum* (4), and in these cases molecular identification is mandatory. This is the first in vitro study that tests numerous strains of

FIG. 1. One of the 225 most parsimonious trees obtained from heuristic searches based on TUB sequences. Bootstrap support values are indicated at the nodes. CI, consistency index; HI, homoplasy index; RI, retention index; asterisks, accession numbers of sequences retrieved from GenBank.

less-common clinical species of *Fusarium* and demonstrates the good in vitro activity of TBF. However, in vivo studies are needed to elucidate the potential clinical usefulness of this drug.

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