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Antifungal susceptibility profile of clinical *Fusarium* spp. isolates identified by molecular methods

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***Objectives*: To analyse the susceptibility pattern of a collection of *Fusarium* clinical isolates.**

***Methods*: The antifungal susceptibility pattern of 67 isolates of *Fusarium* was analysed. Strains were identified by morphological and molecular methods by means of sequencing elongation factor α.**

***Results and conclusions*: Six different species were identified. *Fusarium solani* was the most frequently isolated, followed by *Fusarium oxysporum*, *Fusarium proliferatum* and *Fusarium verticilloides*. Amphotericin B was the only drug with *in vitro* activity (range: 0.015–32 mg/L). The rest of the antifungals tested (itraconazole, voriconazole, ravuconazole, posaconazole and terbinafine) showed very poor activity against *Fusarium*, confirming the multiresistant nature of this genus.**

Keywords: elongation factor α, antifungal resistance, emerging moulds

Introduction

Fusarium is a ubiquitous fungus widely distributed in soil, plants and different organic substrates. *Fusarium* species are important as plant pathogens causing different diseases and being responsible for important economic loss. During recent years, they have been increasingly associated with humans and now represent the second most frequent mould causing invasive fungal infections in immunosuppressed patients associated with high morbidity and mortality rates.[1,2]

The genus currently contains over 100 species. The most common pathogens are *Fusarium solani* and *Fusarium oxysporum* although other species have been reported as aetiological agents of human infection.[3–5] Identification to species level of *Fusarium* has been based on the study of their morphological characteristics. However, isolates involved in human infections usually do not produce the characteristic morphology that allows its identification.[6] Thus, recognition of *Fusarium* to species level is a laborious and time-consuming task only reserved to trained mycologists.[7] In order to solve this issue, molecular techniques have been developed to identify this genus.[8,9]

Fusarium spp. are resistant *in vitro* to many of the antifungal compounds licensed to treat fungal infections, and among them, *F. solani* is considered the most resistant. However, some data pointed out that the resistance could be species and even isolate dependent.[10] The management of fusariosis is not well defined. Antifungals alone or in combination together with other measures such as surgical intervention or colony stimulating growth factors have been used to treat these infections.[11] However, the mortality rate exceeds 75% in disseminated infections and an ominous outcome is expected without the recovery of the immunosuppression of the host.[12]

The susceptibility or resistance to antifungal agents may not predict the individual clinical outcome of *Fusarium* infections, but it is well-known that some kind of association between high MICs and poor response to antifungal treatment exists.[13,14] Therefore, the susceptibility profile of *Fusarium* spp. could be valuable as an aid to choose the best antifungal therapy. In addition, since susceptibility could be specific to one species, definitive identification at the species level by molecular methods may have clinical usefulness for the management of *Fusarium* infections.

The aim of this study is to analyse the activity *in vitro* of different antifungal compounds against a panel of clinical strains of *Fusarium* identified by a reference molecular technique,[9] consisting of partial sequencing of the translation elongation factor-1α (EF1α) gene.

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Materials and methods

Strains

A total of 67 clinical isolates of *Fusarium* spp. were included in this study. The isolates were obtained from a variety of clinical sources. Twenty-four strains were isolated from skin or nails, 16 from ocular samples, 13 from respiratory sites, 7 from blood cultures, 1 from urine, 1 from pericardial fluid and 5 of unknown origin. Each isolate was obtained from a different patient. The isolates were sent to the Mycology Reference Laboratory of National Centre for Microbiology of Spain during 2001–2007 for identification and susceptibility testing.

Morphological identification

The strains were subcultured in different media to ascertain their macroscopic and microscopic morphology. The media included malt extract agar (2% malt extract) (Oxoid S.A., Madrid, Spain), potato dextrose agar (Oxoid S.A.), oat meal agar (Oxoid S.A.) and potassium chloride agar (ClK, Oxoid S.A.).

All media were incubated at 30°C except for ClK agar, which was incubated at room temperature with cycles of 12 h of light followed by 12 h of dark.

PCR and DNA sequencing of EF1α region

Moulds were cultured in GYEP medium (0.3% yeast extract, 1% peptone, Difco, Soria Melguizo S.A., Madrid, Spain) with 2% glucose (Sigma-Aldrich Quimica, Madrid, Spain) for 24–48 h at 30°C. Genomic DNA was isolated using an extraction procedure described previously.[15]

DNA segments comprising a region of the EFα region were amplified with primers EF1 (5′-ATGGGTAAGARGACAAGAC-3′) and EF2 (5′-GGARGTACCAGTSATCATGTT-3′),[8] in a GeneAmp PCR System 9700 (Applied Biosystems). The reaction mixtures contained 0.5 μM of each primer, 0.2 μM of each deoxynucleoside triphosphate, 5 μL of PCR 10× buffer (Applied Biosystems, Madrid, Spain), 2.5 U of *Taq* DNA polymerase (Amplitaq; Applied Biosystems) and 25 ng of DNA in a final volume of 50 μL. The samples were amplified in a GeneAmp PCR System 9700 (Applied Biosystems) by using the following cycling parameters: one initial cycle of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 47°C and 2 min at 72°C, with one final cycle of 5 min at 72°C. The reaction products were analysed in a 0.8% agarose gel.

Sequencing reactions were done with 2 μL of a sequencing kit (BigDye Terminator cycle sequencing, ready reaction: Applied Biosystems), 1 μL of the primers (EF1 and EF2) and 3 μL of the PCR product in a final volume of 10 μL.

Sequences analysis

Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene; DNAstar, Inc., Madison, WI, USA). Sequence analysis was performed by comparison of the DNA sequences with EFα sequences of *Fusarium* spp. strains (with accession nos: DQ246834, DQ247188, AY337433, AY337436, AF008480, AY337437, AJ543560, AJ543570, DQ295140, DQ295141, DQ295142 and DQ246834) obtained from the GenBank database (http://www.ncbi.nih.gov/GenBank/).

Phylogenetic analysis

All phylogenetic analyses were conducted with InfoQuest FP software, version 4.50 (BIORAD Laboratories, Madrid, Spain). The methodology used was maximum parsimony clustering. Phylogram stability was assessed by parsimony bootstrapping with 2000 simulations.

Antifungal susceptibility testing

Microdilution testing was performed following the CLSI reference method,[16] with the following minor modifications: (i) RPMI 1640 was supplemented with glucose to reach a 2% concentration; and (ii) inoculum size was between 1×10^5 and 5×10^5 cfu/mL. Inocula were prepared by means of counting spores in a haemocytometer.[17–19] *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains.[16]

The antifungal agents used in the study were amphotericin B (range 16–0.03 mg/L) (Sigma-Aldrich Química), itraconazole (range 8–0.015 mg/L) (Janssen S.A., Madrid, Spain), voriconazole (range 8-0.015 mg/L) (Pfizer S.A.), ravuconazole (range 8–0.015 mg/L) (Bristol-Myers Squibb, Princeton, NJ, USA), posaconazole (range 8–0.015 mg/L) (Schering-Plough Research Institute, Kenilworth, NJ, USA) and terbinafine (range 16–0.03 mg/L) (Novartis, Basel, Switzerland). The endpoint was the antifungal concentration that produced a complete inhibition of visual growth at 48 h.

Results

Identification of Fusarium to species level

All isolates were identified to genus level by means of observation of morphology characteristics.[6,20] The morphological identification of *F. solani, F. oxysporum* and *Fusarium verticilloides* is straightforward. However, the proper identification of other species such as *Fusarium proliferatum, Fusarium reticulatum* or *Fusarium equiseti* requires sequencing of EFα. In any case, the confirmation of species was obtained by means of maximum parsimony analysis of the EFα sequences. Figure 1 shows a rooted cladogram with a sample of clinical isolates. All clades in the tree had bootstrap values of 100 which support the use of EFα as correct target for molecular identification of *Fusarium* spp. In order to facilitate the visualization of the cladogram, some isolates were not included in Figure 1, but the species distribution and the bootstrap values were identical. Among 67 clinical strains, 22 were *F. solani*, 14 *F. oxysporum*, 14 *F. proliferatum*, 13 *F. verticilloides*, 3 *F. equiseti* and 1 *F. reticulatum*.

Antifungal susceptibility testing

The geometric means (GMs) and ranges of the MICs of antifungal agents are shown in Table 1. In all experiments performed, MICs for quality control strains were in the expected range.

Amphotericin B was the most active agent against *Fusarium* spp., its GM MIC being 1.15 mg/L. The numbers of isolates for which MICs of amphotericin B were ≥2 mg/L differed depending on the species: 12 out of 22 (54.6%) *F. solani*, 9 out of 14 (64.3%) *F. proliferatum*, 4 out of 13 (30.8%) *F. verticilloides* and 1 out of 14 (7.1%) *F. oxysporum* had MICs ≥2 mg/L.

Azole drugs and terbinafine had high MICs for most *Fusarium* spp. (Table 1). Only 12.1% of strains tested with posaconazole had MICs of ≤1 mg/L, dropping to 4.5% for itraconazole and voriconazole.

Regarding species, based on our *in vitro* data, the most resistant isolates in this study were *F. solani* for which GM MICs, of

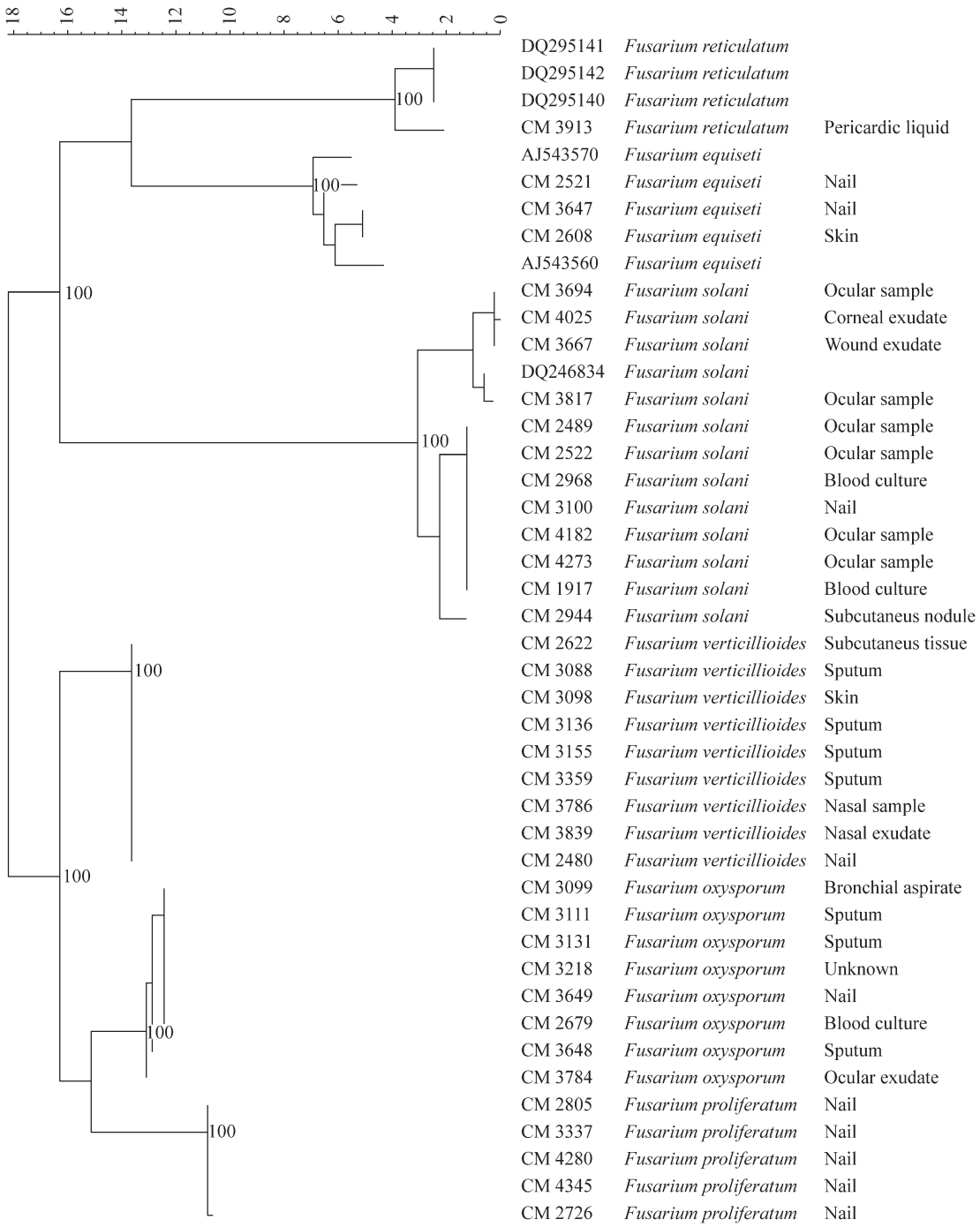

Figure 1. Phylogenetic tree of the subset of isolates included in the study obtained by using maximum parsimony phylogenetic analyses and 2000 bootstrap simulations based on EF1α sequences.

itraconazole, voriconazole, ravuconazole, posaconazole and terbinafine were >8 mg/L, but where the GM MIC of amphotericin B was 1.33 mg/L.

Discussion

Identification of moulds to species level by classical methods[6] is a cumbersome and time-consuming task. The expertise required is only available in reference laboratories, and even in them, at least five working days are required to identify a mould isolate to a species level by means of morphology observation. New rapid methods are needed in order to accomplish the identification on time to be useful for clinical management of the patient. Rapid molecular methods are being developed and they will probably replace the classical ones in the near future. In the meantime, proper identification of clinical isolates together with an antifungal susceptibility profile of them can help provide better treatment for patients infected with moulds. One of the main advantages of molecular methods is their sensitivity and

Table 1. Antifungal susceptibility results of clinical isolates of *Fusarium* spp.: MIC GMs and ranges

	MIC (mg/L)											
	amphotericin B		itraconazole		voriconazole		ravuconazole		posaconazole		terbinafine	
Fusarium spp.	GM	range	GM	range	GM	range	GM	range	GM	range	GM	range
F. solani (22)	1.33	0.5–8	16	16–16	14	4–16	16	16–16	16	16–16	29.96	16–32
F. verticilloides (13)	1.53	0.5–32	10.44	1–16	8	1–16	8.98	2–16	3.23	0.25–16	3.23	1–32
F. oxysporum (14)	0.78	0.12–2	11.31	1–16	4	0.5–16	8	1–16	4.63	0.06–16	10.77	0.5–32
F. proliferatum (14)	1.56	1–4	16	16–16	9.28	4–16	15.23	8–16	11.89	2–16	3.62	1–32
F. equiseti (3)	0.79	0.5–1	16	16–16	4	4–4	16	16–16	2	2–2	10.08	4–16
F. reticulatum (1)	0.015		16		1		1		1		0.25	
Total (67)	1.15	0.015–32	13.7	1–16	8	0.5–16	11.89	1–16	7.43	0.06–16	8.79	0.25–32

specificity, being fully discriminative even for closely related species. The majority of molecular methods are PCR-based techniques and use either specific probes or universal primers that are normally directed to conserved regions of the ribosomal DNA gene, particularly to the internal transcribed spacer (ITS) regions.[3,21,22]

In the case of *Fusarium* spp., sequencing of ITS analysis is considered unreliable for identification of strains because they contain two paralogous, discrepant ITS sequence types, which are a potential source of confusion.[23,24] Other genes have been used for the identification of *Fusarium* spp. and EFα has shown optimal results.[9,24,25] In this study, we have performed molecular identification with EFα and we have been able to differentiate among all the species analysed, as shown in Figure 1.

Up to now, over 15 species of *Fusarium* have been reported to cause infections in humans and animals, the most frequent aetiological agents being *F. solani* and *F. oxysporum*, but other species such as *F. verticilloides, Fusarium chlamydosporum*, *Fusarium dimerum*, *Fusarium napiforme*, *Fusarium nygamai*, *F. proliferatum* and *Fusarium sacchari* have also been reported in several cases of human infections.[5,23,26] In our collection, 46% of the strains belonged to species relatively infrequent as *F. proliferatum* (14 isolates), *F. verticilloides* (13), *F. equiseti* (3) and *F. reticulatum* (1). No other species encountered in other studies and related with human clinical samples were identified in this study. This fact could have been due to ecological reasons but it might be also due to not all fungi isolated for human sources being sent to a reference laboratory.

In this study, no activity *in vitro* of azoles drugs and terbinafine was detected against most of the isolates of *Fusarium* (Table 1). Amphotericin B has been the only drug that has shown activity *in vitro* against all the *Fusarium* species analysed with GM of 1.15 mg/L. Susceptible strains to this drug, with MICs of <2 mg/L were found: 13 out of 14 (92.9%) *F. oxysporum* isolates, 9 out of 13 (69.3%) *F. verticilloides*, 10 out of 22 (45.5%) *F. solani*, 5 out of 14 (35.7%) *F. proliferatum* and all isolates of *F. equiseti* and *F. reticulatum*. Azor *et al.*[27] have recently described the antifungal susceptibility profile of 50 clinical and environmental isolates of *F. solani*. MIC results were similar to those obtained in this work, amphotericin B being the most active drug.

Optimal treatment for *Fusarium* spp. has not yet been established. At best, response rates to antifungals such as lipid amphotericin B, voriconazole or posaconazole have ranged between 45% and 48%.[11,28,29] Kontoyiannis *et al.*[30] have analysed the impact on neutrophil recovery in the outcome of fusariosis. They concluded that this is the most important predictor of outcome. Other works have found similar results.[11,28,29] Taking into consideration that recovery from neutropenia is the most important factor, it would be better to treat these infections with the antifungal showing the highest *in vitro* activity against the isolate. In that way, we could gain enough time to enable the patient to recover a normal immune status. However, antifungal patterns regarding species could not be established by the data obtained and therefore we cannot make recommendations based on the identification of the isolate. However, as some isolates had lower MICs of amphotericin B, voriconazole and posaconazole, antifungal susceptibility testing could identify those isolates and help in the treatment of the patients.

Thus, as the susceptibility profile is isolate dependent, antifungal susceptibility testing should be performed for any *Fusarium* involved in an invasive fungal infection.

In summary, morphological and molecular identification of *Fusarium* species is cumbersome and should be restricted to laboratories with the required experience. An alternative for laboratories without the necessary experience could be the identification to genera level, but any strain isolated from a suspected invasive fungal infection should be identified to species level and its antifungal susceptibility profile determined. From a practical point of view, we have to bear in mind that *Fusarium* species is a multiresistant microorganism, as this work has demonstrated with a collection of clinical strains conclusively identified by molecular methods. However, as there are isolates with lower MICs, a joint effort should be initiated in order to determine if there is any kind of correlation among outcome of the patient, species identification and antifungal susceptibility profile of the isolate.

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Transparency declarations

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