UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

Estudio de la resistencia a los antifúngicos en hongos patógenos humanos

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

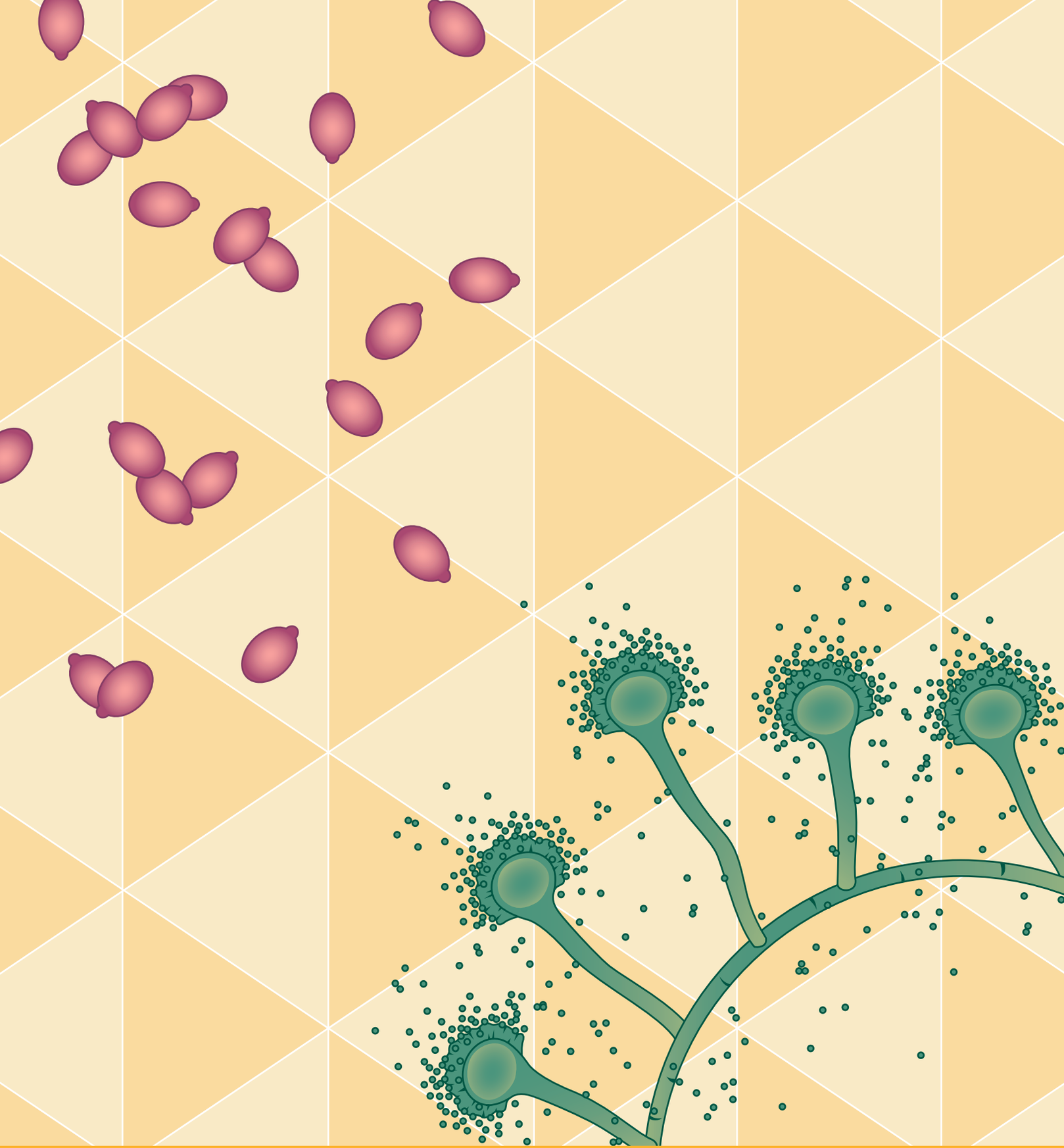
PRESENTADA POR

Olga Rivero Menéndez

Directores

Ana Alastruey Izquierdo
Manuel Cuenca Estrella

Madrid



OLGA RIVERO MENÉNDEZ

ESTUDIO DE LA RESISTENCIA A LOS ANTIFÚNGICOS EN HONGOS PATÓGENOS HUMANOS

Directores:

Dra. Ana Alastruey Izquierdo

Dr. Manuel Cuenca Estrella

Universidad Complutense de Madrid
Facultad de Ciencias Biológicas
Madrid, 2019

UNIVERSIDAD COMPLUTENSE MADRID

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

DEPARTAMENTO DE GENÉTICA, FISIOLOGÍA Y MICROBIOLOGÍA

TESIS DOCTORAL

Estudio de la resistencia antifúngica en hongos patógenos humanos

Memoria para optar al grado de doctor presentada por:

OLGA RIVERO MENÉNDEZ

Laboratorio de Referencia e Investigación en Micología

Instituto de Salud Carlos III

Madrid, 2019

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

DEPARTAMENTO DE GENÉTICA, FISIOLOGÍA Y MICROBIOLOGÍA

TESIS DOCTORAL

Estudio de la resistencia antifúngica en hongos patógenos humanos

Memoria para optar al grado de doctor presentada por:

OLGA RIVERO MENÉNDEZ

Laboratorio de Referencia e Investigación en Micología

Instituto de Salud Carlos III

Madrid, 2019

Directores:

Dra. Ana Alastruey Izquierdo

Científico Titular

Dr. Manuel Cuenca Estrella

Profesor de Investigación

UNIVERSIDAD COMPLUTENSE MADRID

DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD DE LA TESIS PRESENTADA PARA OBTENER EL TÍTULO DE DOCTOR

D./Dña. Olga Rivero Menéndez,
estudiante en el Programa de Doctorado de Microbiología y Parasitología,
de la Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid, como autor/a de la tesis presentada para la obtención del título de Doctor y titulada:

Estudio de la resistencia antifúngica en hongos patógenos humanos

y dirigida por: Dra. Ana Alastruey Izquierdo y Dr. Manuel Cuenca Estrella

DECLARO QUE:

La tesis es una obra original que no infringe los derechos de propiedad intelectual ni los derechos de propiedad industrial u otros, de acuerdo con el ordenamiento jurídico vigente, en particular, la Ley de Propiedad Intelectual (R.D. legislativo 1/1996, de 12 de abril, por el que se aprueba el texto refundido de la Ley de Propiedad Intelectual, modificado por la Ley 2/2019, de 1 de marzo, regularizando, aclarando y armonizando las disposiciones legales vigentes sobre la materia), en particular, las disposiciones referidas al derecho de cita.

Del mismo modo, asumo frente a la Universidad cualquier responsabilidad que pudiera derivarse de la autoría o falta de originalidad del contenido de la tesis presentada de conformidad con el ordenamiento jurídico vigente.

En Madrid, a 11 de julio de 2019

Fdo.: _____

Esta DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD debe ser insertada en la primera página de la tesis presentada para la obtención del título de Doctor.

Dña. Ana Alastruey Izquierdo, Científico Titular del Laboratorio de Referencia e Investigación en Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda,

D. Manuel Cuenca Estrella, Profesor de Investigación, Subdirector General de Servicios Aplicados, Formación e Investigación del Instituto de Salud Carlos III,

CERTIFICAN:

Que la presente memoria titulada **"Estudio de la resistencia a los antifúngicos en hongos patógenos humanos"** que presenta la Graduada en Biotecnología y Máster en Microbiología y Parasitología **Dña. Olga Rivero Menéndez** para obtener el grado de Doctora ha sido realizada bajo nuestra dirección, autorizándola para su presentación al Tribunal Calificador.

Madrid, 11 de julio de 2019.

Fdo. Ana Alastruey Izquierdo

Directora de la tesis doctoral

Fdo. Manuel Cuenca Estrella

Director de la tesis doctoral

A mis padres

A Javi Uceda

Hay esperanza en la deriva.

AGRADECIMIENTOS

Mentiría si dijese que me enfrento a la escritura de esta página, la última que redacto de mi tesis doctoral, sin haber pensado con mucho mimo antes en quién debe figurar en ella. A lo largo de estos cuatro años, tan bonitos como duros, ha sido mucha la gente que ha dedicado parte de su tiempo a aligerar el peso de la carga que ser un doctorando conlleva, y lo menos que puedo hacer es dedicarles unas líneas en las que se refleje lo agradecida que me siento de que formen parte de mi vida. Por desgracia, la brevedad nunca ha sido mi fuerte, aunque creo que la ocasión no merece ser parca en palabras.

En primer lugar, quiero dar las gracias a mis directores de tesis, Ana y Manolo. Gracias por darme la oportunidad de iniciar una carrera investigadora, por vuestro tiempo y por guiar mi trabajo. Gracias, Ana, por tu disponibilidad y por ser siempre tan resolutiva cuando surgen adversidades. Pero, sobre todo, gracias a ambos por la confianza depositada en mí, ya que me ha permitido sacar fuerzas de flaqueza en los momentos más difíciles. Si algo he aprendido a nivel personal durante el transcurso de mi tesis doctoral es que soy mucho más fuerte de lo que jamás habría imaginado.

No quisiera continuar sin agradecerle a Emilia, nuestra jefa de servicio, su cariño durante estos años. Gracias por siempre tener un momento para escucharme, aconsejarme, animarme y apoyarme. Tú y tu mente crítica habéis sido piezas indispensables para mi desarrollo, tanto profesional como personal.

El Laboratorio de Referencia e Investigación en Micología está lleno de personas mágicas, las cuales han conseguido que levantarse por las mañanas para ir a trabajar sea un placer si es para pasar tiempo con ellas. Creo que será difícil volver a encontrar un ambiente con semejante calidad humana en el que trabajar.

No podría empezar esta ronda de agradecimientos sin hacerlo por las Rocíos. En primer lugar, Ro, mi hermana, mi pilar fundamental durante todos estos años. Qué bueno conocerte en el máster, qué bien que aquel curso en Braganza terminase de unirnos, qué felicidad que la vida nos pusiese en el mismo laboratorio a hacer la tesis, qué suerte la mía de haber podido contar contigo sin condición. No puedo admirar más tu pasión por esto que hacemos, tu incansable motivación, tu inteligencia y tu tesón. Me considero afortunada de haber aprendido tanto de ti, y espero seguir haciéndolo durante mucho tiempo. Te quiero con locura, amiga.

Todo predoc tiene, durante su etapa de formación, un postdoc al que admirar y del que aprender. La mía ha sido Ro Rodas. Gracias por todo lo que me enseñas, por toda tu ayuda en los momentos más críticos, por estar ahí día y noche. Si como investigadora vales oro, como persona eres un diamante.

A Leticia, que ha sido mi “pareja de baile” en varias de las etapas más divertidas en el laboratorio, y que se ha volcado en mi formación siempre que la he necesitado. Gracias por tu cariño y tu empatía, por entenderme y por hacerme reír. Entre koalis todo es más fácil.

A Charo, de la que he aprendido prácticamente todo lo que sé de buenas prácticas en el laboratorio. Nunca podré agradecerte suficiente lo "pesada" que has sido conmigo, porque gracias a eso puedo decir que sé trabajar en un laboratorio con propiedad. Gracias de corazón por tu apoyo, por las confidencias y por todo lo que me has enseñado acerca de los hongos filamentosos.

Gracias a Nuria, por convertirse en una buena amiga y por todas las risas y vivencias compartidas aquí y al otro lado del charco. ¡Te quiero, swimmer! A Clara, a Haroldo, a Cris Armentia, a Macamoa y a Sue, por los grandes momentos dentro y fuera del laboratorio. Trabajar con vosotros ha sido un regalo.

A Irene, que es la hermana pequeña que siempre quise tener. Vas a ser una doctoranda de las que dejan huella, no tengo pruebas pero tampoco dudas. Gracias por tu cariño. A Juan Carlos y Narda, que recogen mi testigo en el grupo de investigación de Ana. Sois increíbles, y os deseo lo mejor en este etapa que acabáis de comenzar. Gracias por vuestra inestimable ayuda en estos últimos meses. Y por último, a Jose, con quien he creado un vínculo que sé que es para toda la vida. Te quiero mucho, gracias por aparecer cuando más te necesitaba. Tus abrazos han sido imprescindibles para encarar este año.

Todos los agradecimientos que podría dedicar a la plantilla de investigadores principales del laboratorio se quedarían cortos. Alicia, Laura, María José y, en especial, Óscar, muchísimas gracias por ser tan cercanos y siempre estar dispuestos a ayudar. Ha sido un honor poder aprender de vuestra infinita sabiduría todo este tiempo. A Teresa y a Cris Rueda, por siempre tener una palabra de ánimo preparada en los momentos más difíciles.

A Susana y a Cris Carballo, por sacar adelante el peso de la rutina de este laboratorio de forma incansable. ¡Sois unas heroínas! Gracias por siempre ser tan buenas y comprensivas con los estudiantes. Y a Gemita, a la que nunca hemos dejado de echar de menos.

Me gustaría también recordar a toda esa gente con la que, en algún momento, he colaborado o compartido trabajo. Gracias a Horacio, a Gema y a las dos Lauras, personas sin las que llevar a cabo mis investigaciones hubiese sido más complicado. Gracias a Luismi por tantos grandes momentos. Pero, sobre todo, gracias a mi Patri. Su estancia en nuestro laboratorio fue la etapa de trabajo más feliz, y a la vez más extenuante, que he vivido, y ha dado lugar a una amistad preciosa. Gracias por ser como eres, Petsi. Te quiero muchísimo.

I would also like to say a big thank you to all the people working at the Manchester Fungal Infection Group, especially to Dr. Paul Bowyer and Dra. Sara Gago, who were nothing but kind to me during my research stay in their lab. It was a true honour to work with you. Manchester will always be part of my heart!

Quiero continuar estos agradecimientos mencionando a mis amigos. Esa gente que de forma incondicional ha estado ahí, a pesar de muchas veces no entender muy bien "por qué trabajo tanto".

Cue, Jar, Clara, gracias por vuestro ánimo constante. Son muchos años juntas, pero quiero que sean un millón más. Sois mi mejor motivo para volver a casa.

A Luci y Guada, por ser las flores que siempre quise en mi jardín. Vuestro saber estar en el mundo me inspira cada día. Os quiero y os admiro a partes iguales. A Dani y a Jorge, porque sin vuestro cariño y todo lo que me hacéis reír, esto hubiese sido insoportable. Siempre seréis los hombres de mi vida. Os adoro.

A Santi, por ser el mejor amigo que se puede tener. A Isa, por siempre estar. A Arantxa y María, por haber pasado de ser compañeras de piso a un apoyo imprescindible durante muchos años. A Carlota y Ángela, porque me iría con ellas al fin del mundo. A Aroa y Diego, porque a pesar de la distancia, nunca nos faltará tiempo que dedicarnos. A Vir, por su dulzura. A Elena, por haber vivido conmigo estos últimos meses de locura al pie del cañón. A Laura e Irati, por ser los mejores descubrimientos de este año. A Julio, por ser capaz de leer mi mente y diseñar la portada de tesis que quería.

En estos casos, siempre se deja para el final a las personas más especiales. Por supuesto, en el primer lugar de los puestos de honor se encuentra mi familia. Mamá, papá, gracias por ser mi modelo a seguir y por vuestro apoyo en cada segundo de este largo camino. Si he llegado hasta aquí es, por encima de todo, gracias a vosotros. Gracias por haber empleado todo vuestro esfuerzo en hacerme como soy, en concienciarme de lo verdaderamente importante. Gracias por nunca haber cortado mis alas y siempre haber confiado en mí. Ojalá que todo lo que haga en esta vida os haga sentiros orgullosos de mí. Os quiero más que a nada en el mundo.

A mis tíos Manolo y Marisa, dos de mis principales referentes. Gracias también a mi tía Loli y a mi tío Juan, por siempre tener palabras de aliento preparadas.

Y, por supuesto, gracias, Ruben. Gracias por tu cariño incansable, tu ánimo constante, tu paciencia y tu aguante en lo que ha durado el viaje en cohete que ha sido esta etapa. Gracias por nunca tirar la toalla, ni dejar que la tirase yo. Este logro lo hemos conseguido a medias.

Por último, me gustaría dedicar esta tesis doctoral a la memoria de mi amigo Javi Uceda, al que no hay un solo día que no eche de menos. Gracias por cuidar de mí, y por mandarme fuerza en los peores momentos. Sé que, estés donde estés, te sientes muy orgulloso de mí. Siempre te llevaré conmigo.

ÍNDICE

ABREVIATURAS

A

ADN Ácido desoxirribonucleico

AFLP Polomorfismos en longitud de fragmentos amplificados
Amplified Fragment Length Polymorphism

AND Anidulafungina

AMB Anfotericina B

C

CL Colección de levaduras

CLSI Instituto de Estándares Clínicos y de Laboratorio
Clinical and Laboratory Standards Institute

CME Concentración Mínima Efectiva

CMI Concentración Mínima Inhibitoria

CNM Centro Nacional de Microbiología

CPF Caspofungina

E

ECOFF Punto de corte epidemiológico propuesto por EUCAST
Epidemiological Cut-Off

ECV Punto de corte epidemiológico propuesto por CLSI
Epidemiological Cut-off Value

EUCAST Comité Europeo de ensayos de sensibilidad a antibióticos
European Committee on Antibiotic Susceptibility Testing

F

FLC Fluconazol

G

GPI Glicosilfosfatidilinositol

H

HRM Curvas de fusión de alta resolución
High resolution melting

I

ISA Isavuconazol

ITC Itraconazol

ITS Espaciador transcrito interno
Internal Transcribed Spacer

M

MCF Micafungina

MGX Manogepix

MLP Tipado de longitud de microsatélites
Microsatellite length polymorphism

MLST Tipado multilocus de secuencias *Multilocus sequence typing*

MPC Concentración preventiva de mutantes *Mutant prevention concentration*

MSW Ventana de selección de mutantes *Mutant selection window*

O

OFM Olorofima

OMS Organización Mundial de la Salud

P

pb Pares de bases

PCR Reacción en cadena de la polimerasa *Polymerase Chain Reaction*

PSC Posaconazol

R

R Resistente

RAPD Amplificación aleatoria de ADN polimórfico *Randomly Amplified Polymorphic DNA*

S

S Sensible

SNP Polimorfismo de un solo nucleótido *Single Nucleotide Polymorphism*

s.s. *sensu stricto*

SSCP Polimorfismo conformacional de cadena sencilla *Single-Strand Conformation Polymorphism*

ST Secuencia Tipo

STR Microsatélites *Short Tandem Repeats*

syn. Synonym

T

TR Repetición en tándem *Tandem repeat*

U

UFC Unidades Formadoras de Colonia

V

VRC Voriconazol

W

WT Salvaje *Wild Type*

RESUMEN

Estudio de la resistencia a los antifúngicos en hongos patógenos humanos

Las mejoras en el diagnóstico, el tratamiento y el cuidado de pacientes inmunodeprimidos han incrementado su tasa de supervivencia, pero también han dado lugar al aumento progresivo de otras enfermedades, como las infecciones fúngicas invasoras. A pesar del avance que las herramientas para el diagnóstico de estas infecciones han experimentado, este sigue realizándose de forma tardía en muchas ocasiones, lo que con frecuencia conlleva el inicio de una terapia antifúngica empírica y profiláctica. Este uso generalizado de los antifúngicos es considerado una de las principales causas de la emergencia de la resistencia a estos compuestos entre un gran número de patógenos fúngicos. En los últimos años se ha observado un aumento en el número de cepas que han desarrollado resistencia secundaria entre las especies más prevalentes de los géneros *Candida* y *Aspergillus*, así como se han identificado especies que presentan una sensibilidad reducida intrínseca a los antifúngicos, como las especies crípticas de *Aspergillus* o aquellas pertenecientes a los géneros *Fusarium*, *Scedosporium*, *Lomentospora* o al orden Mucorales.

La resistencia antifúngica supone una amenaza para la salud humana, ya que los pacientes con infecciones provocadas por cepas resistentes tienen un mayor riesgo de sufrir fallo terapéutico. Por ello, en esta tesis dividida en cuatro capítulos se ha estudiado su presencia en cepas españolas, los principales mecanismos de resistencia y algunos de los aspectos más relevantes relacionados con su desarrollo, así como técnicas para su detección y la actividad de nuevas opciones terapéuticas en desarrollo que permitan mejorar su manejo clínico.

Los dos primeros capítulos se plantearon con el fin de profundizar en el conocimiento acerca de la adquisición de la resistencia secundaria en algunos de los patógenos fúngicos cuya incidencia se ha incrementado en distintos países de nuestro entorno, como *Candida glabrata* y las especies más frecuentes de *Aspergillus*.

En el primero, se constató el potencial de *C. glabrata* para adquirir y desarrollar resistencia a las equinocandinas, tanto en el ámbito clínico como en condiciones *in vitro* establecidas en el laboratorio. Estos compuestos son el tratamiento de primera línea para las candidiasis que provoca esta especie que, a su vez, exhibe una sensibilidad reducida al fluconazol. Además, se corroboró la necesidad de estudiar el perfil de sensibilidad a la anidulafungina y a la micafungina de forma paralela, conjuntamente con las mutaciones en los genes que codifican la enzima diana de estos antifúngicos, los *FKS*, para detectar la resistencia a equinocandinas en *C. glabrata*. A su vez, se concluyó que la presencia de alteraciones en el gen *MSH2* de cepas de esta especie, implicado en los mecanismos de reparación del ADN, no está asociada con el desarrollo de la resistencia antifúngica. Tampoco se encontró correlación entre genotipos específicos y las mutaciones en *MSH2*, el perfil de sensibilidad o el desarrollo de la resistencia en aislados clínicos de *C. glabrata* tras ser expuestos *in vitro* a micafungina y anidulafungina.

En el segundo, se estudiaron los principales mecanismos de resistencia a los triazoles presentes en las especies más prevalentes de *Aspergillus* en España, basados en la aparición de mutaciones en los genes *cyp51*. Esta clase de compuestos constituye la terapia de elección para tratar la aspergilosis invasiva, infección cuyo agente etiológico más frecuente es *Aspergillus fumigatus*, aunque cada vez es mayor el número de casos originados por otras especies del género, como *Aspergillus terreus* o *Aspergillus flavus*. El aislamiento de cepas resistentes a los triazoles se ha incrementado en todo el mundo y, aunque en nuestro país se ha descrito una baja proporción de cepas de *A. fumigatus* resistentes a estos antifúngicos, nunca se había estudiado el desarrollo de resistencia a los mismos en otras especies del género. A través del establecimiento de un programa de vigilancia de la resistencia antifúngica, se detectó un notable número de cepas de *A. fumigatus* resistentes a los triazoles, además de encontrar una serie de mutaciones no descritas en las cepas de *A. terreus* y *A. flavus* clasificadas como resistentes, lo que incita a continuar supervisando este fenómeno en España para poder plantear un plan de acción para su control.

El tercer capítulo de esta tesis comprende el desarrollo, la estandarización y la validación de una metodología basada en el análisis de curvas de fusión de alta resolución (HRM) para detectar de forma rápida y en un ensayo único las principales mutaciones en el gen *cyp51A* de A. *fumigatus* responsables de la resistencia a los azoles. Puesto que se ha descrito que estas alteraciones están asociadas a perfiles específicos de resistencia a esta clase de compuestos, el uso de esta herramienta permite el inicio temprano de una terapia antifúngica apropiada, algo fundamental para reducir la incidencia de este fenómeno.

La búsqueda de compuestos con capacidad antifúngica que posean nuevos mecanismos de acción es fundamental para reducir las limitaciones que tienen los antifúngicos en uso. Por ello, en el cuarto capítulo de esta tesis se estudió la actividad *in vitro* del manogepix y la olorofima frente a especies fúngicas cuyas opciones terapéuticas se encuentran limitadas en la actualidad debido a su resistencia intrínseca a los antifúngicos disponibles. Así, se demostró la eficacia del manogepix frente a las distintas especies de *Scedosporium*/*Lomentospora* y a las crípticas de *Aspergillus* más habitualmente aisladas, aunque solo fue moderadamente activo frente a *Fusarium solani* y *Alternaria alternata* y no mostró actividad frente a las especies de Mucorales. A su vez, la olorfima fue eficaz frente a todas las cepas de especies crípticas de *Aspergillus* estudiadas. Estos resultados aseguran futuros estudios que corroboren su buena actividad en modelos murinos de infección con el fin de ser aprobados para el uso clínico lo antes posible.

ABSTRACT

Research on antifungal resistance in human pathogenic fungi

Improvements in diagnostics and management of immunosuppressed patients have contributed not only to survival, but also to the increasing incidence of other diseases, such as invasive fungal infections. Despite the development and improvement of techniques for diagnosing these infections, diagnoses are often delayed, which leads to the start of empiric and prophylactic antifungal therapies. The extended use of antifungals is considered one of the main causes of the emergence of resistance among a great number of fungal pathogens. The number of strains developing secondary resistance among the most prevalent species of *Candida* and *Aspergillus* genera has increased. Also, species with decreased antifungal susceptibility have been identified, such as cryptic species of *Aspergillus* or those belonging to *Fusarium*, *Scedosporium* or *Lomentospora* genera, as well as Mucorales order.

Antifungal resistance poses a threat to human health, as therapeutic failure in patients infected with resistant strains is more probable than in those affected with susceptible ones. Therefore, in this doctoral dissertation, divided in four chapters, its presence among Spanish strains was investigated, as well as the main resistance mechanisms and some of the most relevant aspects related to its development. Besides, a technique for its detection was put forward and the activity of new therapeutics options in development was studied in order to improve its clinical management.

The first two chapters were proposed with the aim of gaining a deeper knowledge about how some of the fungal pathogens with a high incidence in several geographical areas, such as *Candida glabrata* or the main *Aspergillus* species, acquire secondary resistance to antifungals.

In the first one, *C. glabrata*'s ability to acquire and develop resistance to echinocandins, both in the clinical setting and under *in vitro* laboratory conditions, was proved. This antifungal class is considered the first-line treatment for candidiasis caused by this species which, at the same time, exhibits decreased susceptibility to fluconazole. Furthermore, the need of assessing both anidulafungin and micafungin's susceptibility profile, together with determining the presence of mutations in the *FKS* genes that encode the target enzyme for these antifungals, was confirmed in order to detect echinocandin resistance in *C. glabrata*. In addition to that, it was concluded that alterations in mismatch repair gene *MSH2* are not linked to the development of antifungal resistance. No correlation was found either between specific genotypes and *MSH2* mutations, the susceptibility profile or the development of resistance in *C. glabrata* clinical isolates after being *in vitro* exposed to micafungin and anidulafungin.

In the second chapter, the main mechanisms of azole resistance, based on the emergence of mutations in *cyp51* genes, among the most prevalent *Aspergillus* species in Spain were studied. These antifungals are the therapy of choice for treating invasive aspergilosis, caused mainly by *Aspergillus fumigatus*, although the number of cases produced by other species of this genus,

such as *Aspergillus terreus* or *Aspergillus flavus*, is growing. At the same time, the isolation of azole resistant *Aspergillus* strains has increased throughout the world. Even though a low rate of resistant strains of *A. fumigatus* has been detected in our country, the development of resistance in other species of the genus had never been studied. A considerable number of azole resistant strains of *A. fumigatus* was found when analysing the strains received within an established surveillance program on antifungal resistance, and some mutations that had never been described before were detected among *A. terreus* and *A. flavus* strains. Thus, azole resistance among *Aspergillus* species should be monitored in Spain in order to propose an action plan for its control.

The third chapter of this doctoral dissertation includes the development, standardization and validation of a methodology based in high resolution melting assay to detect the main mutations related to azole resistance *in Aspergillus fumigatus cyp51A* gene in a single and fast assay. As each one of these alterations is associated with different patterns of azole resistance, this screening tool allows early initiation of an appropriate antifungal therapy, which is essential to reduce the incidence of this phenomenon.

Current antifungals have several limitations, which makes clear the need to expand the search for new antifungal formulations with novel mechanisms of action. Thus, in the fourth chapter of this doctoral dissertation the *in vitro* activity of manogepix and olorofim against fungal species with limited therapeutic options, due to their intrinsic resistance to antifungals in use, was evaluated. Manogepix was effective against *Scedosporium*/*Lomentospora* species and the most frequently isolated cryptic species of *Aspergillus*, although it only exhibited moderate activity against *Fusarium solani* and *Alternaria alternata* and was ineffective against Mucorales. Olorofim was active against all cryptic species of *Aspergillus* studied. These results are encouraging for the development of future studies that confirm their activity in murine models of infection, which would eventually lead to their approval for clinical use.

1. Introducción

1. INTRODUCCIÓN

Esta tesis doctoral comprende el estudio en profundidad de la resistencia antifúngica en algunos de los patógenos humanos más habituales, por lo que en esta introducción se presenta el estado actual y las características más relevantes de las principales infecciones invasoras causadas por hongos y de los mecanismos de resistencia que estos desarrollan a los antifúngicos más importantes. Además, se describen las técnicas moleculares más utilizadas para detectar la resistencia antifúngica. Por último, se mencionan las nuevas moléculas con capacidad antifúngica que están siendo ensayadas frente a un amplio espectro de patógenos con el fin de comprobar su actividad para solventar las limitaciones que los antifúngicos actuales poseen.

1.1. Infección fúngica invasora: epidemiología y estimación de la prevalencia global

Los hongos son patógenos oportunistas capaces de causar infecciones que en individuos con un sistema inmune comprometido pueden dar lugar a enfermedades invasoras. Los pacientes susceptibles de contraerlas son, principalmente, aquellos que están en tratamiento quimioterápico o en estado crítico ingresados en unidades de cuidados intensivos, además de enfermos con neoplasias hematológicas y/o receptores de trasplantes de células madre hematopoyéticas o de órganos sólidos (Kriengkauykiat *et al.*, 2011).

El aumento de la población en riesgo de padecer infecciones fúngicas invasoras y, posiblemente, las mejoras alcanzadas en su diagnóstico y en la identificación del agente etiológico que las provoca han contribuido al notable incremento de su incidencia en los últimos años, constituyendo un problema de salud pública en todo el mundo (**Figura 1**). Las micosis invasoras pueden ser provocadas por una gran diversidad de hongos ya que, aunque más del 90% de la mortalidad vinculada a las enfermedades fúngicas es ocasionada por especies de los géneros *Candida*, *Aspergillus*, *Cryptococcus* y *Pneumocystis* (Brown *et al.*, 2012; Bongomin *et al.*, 2017), en los últimos años se ha observado un incremento de patógenos emergentes capaces de producir infecciones, como especies de *Fusarium*, *Scedosporium* o del orden Mucorales (Peman y Salavert, 2014).

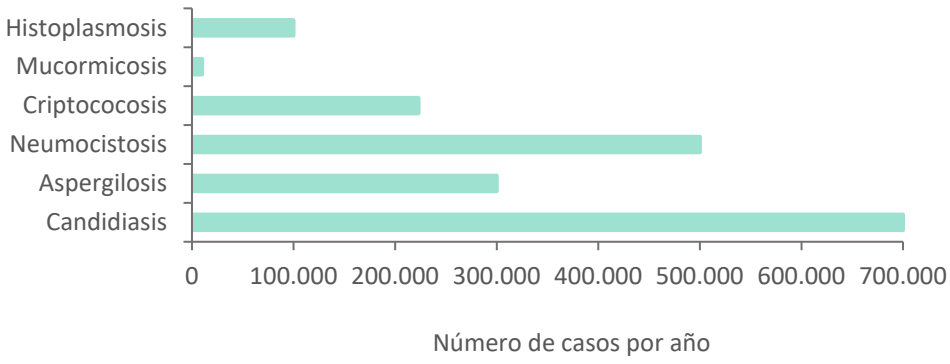


Figura 1. Estimación del número de casos anuales de las principales infecciones fúngicas invasoras (Bongomin *et al.*, 2017).

Las micosis invasoras están asociadas a elevados índices de morbi-mortalidad (Pagano *et al.*, 2011; Neofytos *et al.*, 2013; Klingspor *et al.,* 2015a; Peman y Quindos, 2016), estimándose que más de millón y medio de personas fallecen anualmente en el mundo por esta causa (Brown *et al.*, 2012).

1.1.1. Candidiasis invasora

Las especies del género *Candida* son comensales del ser humano, y se consideran miembros naturales de la microbiota del tracto gastrointestional y vaginal, de las mucosas y de la piel. Además de ser capaces de provocar infecciones orofaríngeas o vaginales, son los principales patógenos oportunistas fúngicos en infecciones invasoras, consecuencia de un aumento de la colonización fúngica y/o de defectos en las defensas del huésped. Las candidiasis invasoras afectan principalmente a pacientes que han sufrido un ingreso hospitalario prolongado en la unidad de cuidados intensivos y a enfermos inmunodeprimidos. La candidemia se considera su forma más común de presentación, y es clasificada como la cuarta causa más habitual de infección en el torrente sanguíneo en Europa y Estados Unidos (Quindos, 2014). Sin embargo, el aislamiento de estas levaduras en hemocultivos supone menos del 70% de los casos de candidiasis invasora (Peman y Quindos, 2016), siendo también frecuentes, entre otras, las infecciones intraabdominales, peritoneales y en órganos profundos (Cervera, 2012; Pappas *et al.*, 2018).

La prevalencia de las infecciones invasoras causadas por las especies de *Candida* varía en todo el mundo: en Europa oscila entre los 2,2 y 11 casos por cada 100.000 habitantes (Bongomin *et al.*, 2017), siendo España, Reino Unido, Dinamarca, Rusia y Hungría los países con la prevalencia más alta (Klimko *et al.*, 2015; Sinko *et al.*, 2015; Pegorie *et al.*, 2017; Astvad *et al.*, 2018). En nuestro país, el último dato de incidencia anual de candidemia recogido en el estudio poblacional CANDIPOP es de 8,1 casos por cada 100.000 habitantes (Puig-Asensio *et al.*, 2014). En Estados Unidos y en países de otros continentes, como Brasil o Pakistán, es aún superior, fluctuando entre los 9,5 y los 21 casos por 100.000 habitantes (Cleveland *et al.*, 2015; Giacomazzi *et al.*, 2016; Jabeen *et al.*, 2017). En todos estos estudios, la prevalencia es especialmente elevada entre pacientes neonatos y ancianos. El estudio CANDIPOP recoge una incidencia de 96,4 casos por cada 100.000 habitantes entre pacientes menores de un año, y de 26,5 casos por cada 100.000 habitantes entre pacientes con más de 71 años (Puig-Asensio *et al.*, 2014). Las candidiasis invasoras están asociadas a índices de alta mortalidad, variando del 25% al 60% en pacientes con infección intraabdominal y del 30% al 50% en pacientes que sufren candidemia (Posch *et al.*, 2017), y siendo del 15-35% en adultos y del 10-15% en neonatos (Puig-Asensio *et al.*, 2014).

A pesar de que el género *Candida* comprende alrededor de 150 especies, solo unas pocas son capaces de causar infecciones oportunistas. De ellas, *Candida albicans* es la responsable de la mayor parte de las candidiasis invasoras (40-70% de los casos) (Presterl *et al.*, 2007; Pfaller *et*

al., 2011a; Puig-Asensio *et al.*, 2014; Klingspor *et al.*, 2015b), aunque en los últimos años se ha observado un cambio epidemiológico hacia otras especies del género como *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* y *Candida krusei* que producen, junto con *C. albicans*, el 95% de las infecciones invasoras (McCarty y Pappas, 2016; Pappas *et al.*, 2018). Además, *Candida auris* ha emergido recientemente en diferentes partes del mundo como patógeno causante de infecciones nosocomiales y brotes en centros hospitalarios (Chowdhary *et al.*, 2017). La prevalencia de estas especies diferentes a *C. albicans* varía según la localización geográfica, la enfermedad subyacente y la edad del paciente, y han sido descritas como agentes etiológicos de casi el 50% de las candidiasis invasoras en algunos estudios poblaciones recientes (Chen *et al.*, 2006; Das *et al.*, 2011; Bergamasco *et al.*, 2013; Guinea *et al.*, 2014; Castanheira *et al.*, 2016). En Estados Unidos y en países del centro y norte de Europa *C. glabrata* es la segunda especie más frecuente (Poikonen *et al.*, 2010; Orasch *et al.*, 2014; Cleveland *et al.*, 2015; Hesstvedt *et al.*, 2015; Astvad *et al.*, 2018), mientras que en España y en América Latina es la tercera más común por detrás *de C. parapsilosis* (Guinea *et al.*, 2014; Peman y Quindos, 2016; Da Matta *et al.*, 2017). *C. glabrata* es más habitual en ancianos con neoplasias hematológicas, al contrario que *C. parapsilosis*, más común en neonatos y lactantes y asociada al uso de catéteres intravenosos y a la nutrición parenteral (McCarty y Pappas., 2016; Ramos-Martinez *et al.*, 2017).

Otra de las principales diferencias entre estas especies y *C. albicans* es que presentan menor susceptibilidad a los antifúngicos disponibles y/o desarrollan resistencia antifúngica con más facilidad, lo que dificulta el abordaje de las infecciones que causan.

1.1.2. Aspergilosis invasora

Aspergillus es un género de hongos filamentosos saprófitos y ubicuos en la naturaleza que contiene 350 especies agrupadas en los subgéneros *Aspergillus*, *Circumdati*, *Fumigati*, *Nidulantes*, *Polypaecilum* y *Cremei* que, a su vez, comprenden 22 secciones (Samson *et al.*, 2014; Kocsubé *et al.*, 2016). Sin embargo, solo alrededor de 30 de estas especies son capaces de producir infecciones (Darling y Milder, 2018), denominadas de forma general como aspergilosis, que comprenden un amplio rango de manifestaciones clínicas, en función del equilibrio entre la capacidad patógena del hongo y el tipo de respuesta inmunitaria del huésped.

Las especies de *Aspergillus* producen un gran número de esporas que liberan al aire y que son inhaladas por las vías respiratorias de humanos y animales a diario. En individuos inmunocompetentes son eliminadas en su mayoría del tracto respiratorio por los mecanismos de aclaramiento mucocilial y de inmunidad innata, sin suponer ninguna complicación de relevancia clínica. En cambio, en pacientes inmunocomprometidos las esporas son capaces de colonizar al huésped y, si las hifas invaden el tejido pulmonar, pueden dar lugar a una infección pulmonar invasora con posterior diseminación a otros órganos, como el hígado, el bazo, el riñón, la piel, los huesos, el cerebro o el corazón, por invasión de los vasos sanguíneos en pacientes neutropénicos (Patterson, 2011). Además de la aspergilosis invasora, las manifestaciones de la

aspergilosis más habituales son la aspergilosis broncopulmonar alérgica y la aspergilosis pulmonar crónica.

La población en riesgo de padecer aspergilosis invasora incluye a individuos inmunodeprimidos en tratamiento quimioterapéutico o con corticoides, o que han sufrido un trasplante de células madre hematopoyéticas (Stevens *et al.*, 2000). Se estima que su prevalencia global es de 300.000 casos (Denning, 2015), siendo Egipto y Vietnam los países más afectados de entre aquellos donde se ha estudiado este dato (16 y 10,7 casos por cada 100.000 habitantes, respectivamente) (Beardsley *et al.*, 2015; Zaki y Denning, 2017). En Europa estos valores son algo inferiores, oscilando entre 1,8 y 10,4 casos por cada 100.000 habitantes (Bongomin *et al.*, 2017). En España se estiman 2,8 casos de aspergilosis invasora por cada 100.000 habitantes (Rodríguez-Tudela *et al.*, 2015). Esta infección está asociada a un alto índice de mortalidad que se relaciona con la dificultad que tiene realizar su diagnóstico por la rápida progresión de la enfermedad, motivo por el que se suele iniciar el tratamiento antifúngico de forma empírica aún sin tener un diagnóstico definitivo (Latgé, 1999).

Aspergillus fumigatus es el principal agente etiológico de las diferentes formas de aspergilosis (85% de los casos) (Daegenais y Keller, 2009), pero otras especies como *Aspergillus flavus*, *Aspergillus terreus* y *Aspergillus niger* también pueden provocar estas infecciones (Cannon *et al.*, 2009). En 2013 se publicó el primer estudio epidemiológico multicéntrico en hongos filamentosos en España (FILPOP), en el que *Aspergillus* fue el principal género identificado, siendo *A. fumigatus* la especie más frecuente (48,5% del total de cepas analizadas) seguida de *A. flavus* (8,4%), *A. terreus* (8,1%) y *A. niger* (6,5%) (Alastruey-Izquierdo *et al.*, 2013). Unos años más tarde, se realizó el estudio FILPOP2 en el que se obtuvieron resultados semejantes, identificando de nuevo *A. fumigatus* de forma mayoritaria (52,7% del total) y porcentajes similares del resto de especies principales de *Aspergillus*: *A. niger* (5,3%), *A. flavus* (5,1%) y *A. terreus* (4,7%) (Alastruey-Izquierdo *et al.*, 2018).

Además, los avances en las técnicas de identificación molecular han dado lugar a importantes cambios en la taxonomía de los hongos. Los análisis filogenéticos han puesto de manifiesto la existencia de *complejos de especies*, lo que ha dado lugar a la descripción de nuevas especies denominadas *crípticas* dentro de los principales géneros de patógenos fúngicos. En *Aspergillus* se han descrito especies crípticas que hasta entonces habían sido identificadas erróneamente y subestimadas al utilizar metodologías de identificación clásica, además de haberse reclasificado otras de ellas. Puesto que la secuenciación de la región espaciadora interna del ADNr (*internal transcribed spacer*, ITS) solo permite la clasificación de las cepas de *Aspergillus* a nivel de complejos de especies que agrupan las especies relacionadas, se deben utilizar otras dianas, como la β-tubulina o la calmodulina, para llegar a una correcta identificación a nivel de especie en este género (Balajee *et al.*, 2009a; Alastruey-Izquierdo *et al.*, 2012).

La primera especie críptica de este género en describirse en infecciones humanas fue *Aspergillus lentulus* (Balajee *et al.*, 2005a) seguida de *Aspergillus thermomutatus* (syn.

Neosartorya pseudofischeri) (Balajee *et al.*, 2005b). Ambas forman parte del complejo de *A. fumigatus* y son difíciles de distinguir por métodos clásicos. Unos años después se describió *Aspergillus calidoustus* en aislados que originalmente habían sido identificados como *Aspergillus ustus* (Varga *et al.*, 2008). Otras especies crípticas que han sido aisladas en infecciones humanas son *Aspergillus udagawae*, *Aspergillus fumigatiaffinis* y *Aspergillus hiratsukae* en el complejo de *A. fumigatus*, *Aspergillus alliaceus* y *Aspergillus tamarii* dentro del de *A. flavus*, *Aspergillus citrinoterreus*, *Aspergillus aureoterreus*, *Aspergillus carneus* y *Aspergillus hortai* como parte del de *A. terreus*, *Aspergillus tubingensis* y *Aspergillus awamori* del de *A. niger* o *Aspergillus insuetus* y *Aspergillus keveii* del de *Aspergillus ustus* (Gautier *et al.*, 2016). Su prevalencia en el ámbito clínico, según una serie de estudios prospectivos realizados en Estados Unidos, Brasil y España, varía entre un 11% y un 19% (Balajee *et al.*, 2009b; Alastruey-Izquierdo *et al.*, 2013; Alastruey-Izquierdo *et al.*, 2014; Negri *et al.*, 2014; Alastruey-Izquierdo *et al.*, 2018). Además, la relevancia de algunas de estas especies se ve incrementada debido a la reducida sensibilidad a los antifúngicos que presentan (Alastruey-Izquierdo *et al.*, 2014), hecho que se ha correlacionado con un peor pronóstico clínico (Zbinden *et al.*, 2012; Seroy *et al.*, 2018).

1.1.3. Infecciones invasoras causadas por otros hongos filamentosos

En los últimos años han aumentado el número de infecciones causadas por otros hongos, denominados emergentes, debido probablemente a los avances diagnósticos y a la mejora de las técnicas de identificación, además de a la creciente presión selectiva que ejercen las terapias antifúngicas disponibles, puesto que muchas de estas especies poco comunes presentan resistencia a los tratamientos utilizados en la práctica clínica (Lass-Florl y Cuenca-Estrella, 2017).

La mucormicosis es una infección fúngica oportunista causada por hongos ampliamente distribuidos en el medio ambiente que pertenecen al orden Mucorales, el cual incluye más de 50 géneros. De ellos, diez son capaces de producir enfermedad en humanos (Hoffmann *et al.*, 2013), cuya prevalencia está condicionada por su ubicación geográfica y su nicho ecológico. *Mucor* y *Rhizopus* son responsables del 65% de los casos, aunque otros géneros como *Rhizomucor*, *Cunninghamella*, *Apophysomyces*, *Saksenaea* o *Lichtheimia* también poseen especies que pueden ocasionar enfermedad invasora (Farmakiotis y Kontoyiannis, 2016). Las esporas que estos hongos liberan al aire son las causantes de infecciones rinocerebrales o pulmonares. La prevalencia de la mucormicosis es más elevada en países en desarrollo que en países desarrollados: en India es de 14 casos por cada 100.000 habitantes (Chakrabarti y Singh, 2014), mientras que en España es mucho menor, de 0,43 casos / 100.000 habitantes según un estudio realizado en el año 2005 (Torres-Narbona *et al.*, 2007), habiéndose observado un aumento en su incidencia en otros países europeos (Bitar *et al.*, 2009; Saegeman *et al.*, 2010). La mucormicosis tiene un pronóstico desfavorable con altas tasas de mortalidad (hasta del 80% en casos de infección pulmonar (Lin *et al.*, 2017) y hasta del 96% en casos de infección diseminada (Long y Koyfman, 2015)) debida a la respuesta muy limitada a los tratamientos

antifúngicos de las especies que la producen (Alastruey-Izquierdo *et al.*, 2009a; Skiada *et al.*, 2018).

Entre estos patógenos fúngicos emergentes de relevancia clínica también destacan aquellos pertenecientes a los géneros *Scedosporium* y *Fusarium*. La infección invasora causada por las especies de *Scedosporium* y por *Lomentospora prolificans* (anteriormente *Scedosporium prolificans*) se denomina scedosporosiosis. *Scedosporium apiospermum* es la especie de *Scedosporium* más frecuente en infecciones humanas, aunque realmente se trata de un complejo formado por cinco especies: *Scedosporium apiospermum sensu estricto*, *Scedosporium boydii*, *Scedosporium angustum*, *Scedosporium ellipsoideum* y *Scedosporium fusoideum* (Ramírez-García *et al.*, 2018). En un estudio realizado en Australia la incidencia de la scedosporiosis fue descrita como de un caso por cada 100.000 habitantes al año (Heath *et al.*, 2009), y en los estudios FILPOP y FILPOP2 *Scedosporium* fue el segundo género más frecuente, después de *Aspergillus* (Alastruey-Izquierdo *et al.*, 2013; Alastruey-Izquierdo *et al.*, 2018). Sus manifestaciones clínicas son múltiples, y a menudo son mortales por la ineficacia de los tratamientos antifúngicos en uso (Lackner *et al.*, 2014).

Aunque el género *Fusarium* está integrado por aproximadamente 300 especies (Al-Hatmi *et al.*, 2018), solo alrededor de 70 de ellas son capaces de producir infección en el humano y en los animales (Guarro, 2013), ya que se tratan principalmente de patógenos de plantas. La fusariosis invasora puede resultar fatal en individuos inmunocomprometidos, alcanzando tasas de mortalidad del 70% en sus formas diseminadas (Ortoneda *et al.*, 2002). En dos estudios realizados en varios centros médicos de Italia se describieron 15 casos de fusariosis invasora entre 11.802 pacientes con neoplasias hematológicas (Pagano *et al.*, 2006) y tres casos entre 1.249 pacientes receptores de trasplantes de células madre hematopoyéticas (Pagano *et al.*, 2007). Además, en un hospital de Brasil su incidencia se incrementó de los 0,86 casos por cada 1.000 admisiones entre los años 2000 y 2005 a los 10,23 casos / 1.000 admisiones entre 2006 y 2010 (Nucci *et al.*, 2013). Entre sus manifestaciones clínicas se incluyen la afectación pulmonar o sanguínea (Nucci *et al.*, 2003). Los complejos de especies *Fusarium solani* y *Fusarium oxysporum* son responsables del 50% y del 20% de los casos de fusariosis invasora, respectivamente, mientras que el resto de casos los producen las especies de los complejos de *Fusarium fujikuroi*, *Fusarium incarnatum-equiseti* y *Fusarium dimerum*, estos dos últimos de forma muy poco frecuente (Guarro, 2013; Al-Hatmi *et al.*, 2016a). Debido a la baja sensibilidad de estas especies a la terapia antifúngica (Al-Hatmi *et al.*, 2016b), el tratamiento de la fusariosis invasora no está bien definido.

Alternaria comprende cientos de especies que son saprófitas del suelo, aire y otros hábitats que son capaces de penetrar en el cuerpo a través de traumas o mediante su inhalación para originar infecciones cutáneas y subcutáneas, rinosinusitis u onicomicosis y, en menor frecuencia, alternariosis invasiva. Además, es uno de los principales géneros fúngicos causantes de alergia. Las especies que producen predominantemente afección clínica son *Alternaria alternata* y

Alternata infectoria (syn. *Lewia infectoria*) (Pastor y Guarro, 2008), las cuales son consideradas patógenos multiresistentes (Alastruey-Izquierdo y Cuesta *et al.*, 2011).

1.2. Tratamientos antifúngicos frente las infecciones fúngicas invasoras y su mecanismo de acción

En la actualidad existen tres clases principales de compuestos con actividad antifúngica autorizados para el uso clínico, los polienos, los azoles y las equinocandinas, los cuales actúan frente a una de las dos dianas principales en los hongos: el ergosterol, componente esencial de la membrana plasmática fúngica responsable de su fluidez y permeabilidad (Scorzoni *et al.*, 2017), y el β-glucano, polisacárido estructural que constituye más del 50% de la pared celular de los hongos (Campoy y Adrio, 2017) (**Figura 2**).

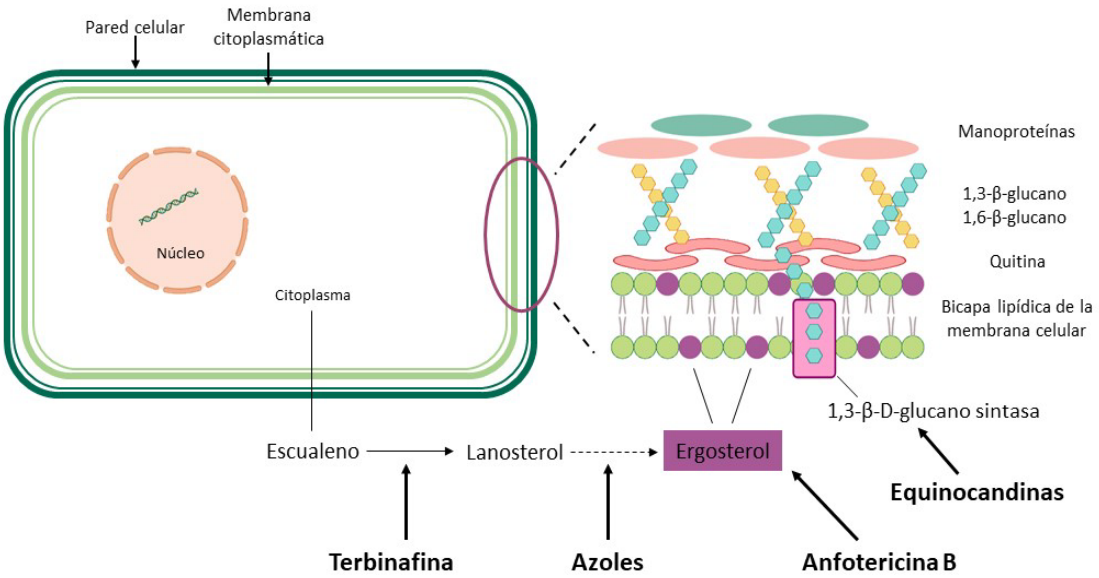


Figura 2. Mecanismos de acción de las diferentes familias de antifúngicos en uso clínico.

Los polienos son un grupo de compuestos naturales que actúan a nivel de la membrana plasmática del hongo. Aunque existen varios antifúngicos poliénicos, como la nistatina y la natamicina, el más utilizado es la anfotericina B (Oura *et al.*, 1955). El mecanismo de acción de la anfotericina B no está del todo claro, pero se basa en su unión al ergosterol, generando poros en la membrana plasmática fúngica que dan lugar a su disrupción y posterior pérdida del contenido citoplasmático, lo que resulta en la muerte celular (Brajtburg *et al.*, 1990). Además, se ha propuesto otro mecanismo de acción en el que está implicada la liberación de especies reactivas de oxígeno provocada por la inducción de estrés oxidativo y por peroxidación lipídica de las células fúngicas, viéndose afectada la permeabilidad de su membrana plasmática (Mesa-Arango *et al.*, 2014). La anfotericina B es eficaz frente a especies de *Candida*, *Aspergillus* y

algunos Mucorales, entre otros géneros (Campoy y Adrio, 2017). Sin embargo, no es considerada en muchos casos como tratamiento de primera línea debido a su afinidad por el colesterol presente en la membrana de las células humanas, lo cual produce alta toxicidad y da lugar a numerosos efectos secundarios (Lemke *et al.*, 2005). Con el fin de minimizar este efecto tóxico, que afecta principalmente a los riñones, se han desarrollado una serie de reformulaciones que consisten en la combinación de la anfotericina B y lípidos en una proporción que mejore su margen terapéutico. Estas formas lipídicas comerciales son la anfotericina B liposomal, el complejo anfotericina B lípido y la anfotericina B en dispersión coloidal (Robinson y Nahata, 1999; Adler-Moore y Proffitt, 2008; Hamill, 2013).

La familia de los azoles incluye los imidazoles y los triazoles. El mecanismo de acción de estos compuestos también tiene por diana el ergosterol y está basado en la inhibición competitiva de una enzima fundamental en su ruta de biosíntesis, la 14 α-esterol demetilasa. Esta enzima pertenece a la familia de las proteínas citocromo P450 Cyp51 en hongos y P450 Erg11 en levaduras, y cataliza el paso del lanosterol a ergosterol mediante la eliminación de un grupo metil en la posición C-14. Cuando un azol se une a través del grupo nitrógeno presente en el anillo azólico al grupo hemo de este citocromo se bloquea el crecimiento de la membrana plasmática fúngica como causa de la toxicidad producida por la acumulación de esteroles metilados, lo cual conlleva la muerte celular (Vanden Bossche *et al.*, 1995; Sheehan *et al.*, 1999). Igual que ocurre con la anfotericina B, los azoles presentan afinidad, en mayor o menor medida en función del antifúngico usado, por las enzimas del citocromo P450 de las células mamíferas, dando lugar a toxicidad y relevantes interacciones con otros medicamentos concomitantes (Nivoix *et al.*, 2008a).

Los triazoles son activos frente a un gran número de especies fúngicas, y la posibilidad de ser administrados por vía oral los convierte en imprescindibles como tratamiento prolongado o profilaxis (Allen *et al.*, 2015). El fluconazol es efectivo contra las infecciones superficiales y sistémicas producidas por *Candida* y *Cryptococcus*, aunque *C. glabrata* y *C. krusei* muestran sensibilidad disminuida a este antifúngico (Lewis, 2011; Nett y Andes, 2016). El itraconazol es eficaz contra las distintas formas de aspergilosis, y también, aunque en menor medida, contra las de candidiasis (Patterson, 2005). Además, es el tratamiento de elección frente a enfermedades causadas por hongos dimórficos, como la histoplasmosis, la coccidiomicosis o la blastomicosis (Chang *et al.*, 2017). El voriconazol muestra una actividad similar a la del fluconazol frente a las especies de *Candida* y *Cryptococcus* (Girmenia, 2009), y es el tratamiento principal contra la aspergilosis invasora, ya que aumenta la supervivencia y disminuye los efectos secundarios que la anfotericina B genera (Herbrecht *et al.*, 2002), y contra las infecciones causadas por especies de *Fusarium* y de *Scedosporium*, aunque su actividad frente a algunas de estas es limitada (Lackner *et al.*, 2012). A su vez, no es efectivo frente a especies del orden Mucorales ni protege contra las enfermedades que ocasionan. Esta limitación es resuelta por el posaconazol, que mejora de esta forma el espectro de acción del voriconazol. Esto le convierte en una buena profilaxis en pacientes inmunodeprimidos frente a infecciones producidas por casi

todos los hongos y levaduras (Cornely *et al.*, 2007), aunque no suele usarse como tratamiento principal debido a su pobre penetración en el sistema nervioso central (Girmena, 2009). El compuesto más reciente dentro de la familia de los triazoles es el isavuconazol, el cual presenta actividad frente a las mismas especies fúngicas que el posaconazol. Puesto que presenta una efectividad similar, una mejor farmacocinética y menor toxicidad que el voriconazol, se usa de forma cada vez más habitual como tratamiento frente a la aspergilosis invasora y la mucormicosis (Maertens *et al.*, 2016; Marty *et al.*, 2016).

Las equinocandinas constituyen la familia de antifúngicos, compuesta por la caspofungina, la micafungina y la anidulafungina, que actúa comprometiendo la integridad de la pared celular fúngica. Lo hace mediante el bloqueo no competitivo de la enzima 1,3-β-D-glucano sintasa, que pertenece al complejo de proteínas responsables de la producción de polímeros de 1,3-β-glucano y 1,6-β-glucano, componentes fundamentales de esta pared sin los cuales se debilita y se produce la lisis celular (Wiederhold y Lewis, 2003). La 1,3-β-D-glucano sintasa está constituida por dos subunidades, la Fks y la Rho1, siendo la Fks la parte activa de la enzima codificada por tres genes parálogos, *FKS1*, *FKS2* y *FKS3* (Perlin, 2007). La ventaja más destacable de las equinocandinas es que no producen toxicidad ni interacciones con otros fármacos, puesto que las células mamíferas carecen de pared celular y su diana existe únicamente en los hongos (Andes, 2013). Son el tratamiento de elección frente a las candidiasis invasoras (Pappas *et al.*, 2016), pero no son efectivas frente a Mucorales, *Cryptococcus* u otros hongos dimórficos. Tienen baja biodisponibilidad oral, lo que hace que su administración tenga que ser intravenosa, y se han descrito casos de fallo cardiaco en pacientes en tratamiento con esta clase de antifúngicos (Stover *et al.*, 2014).

1.3. Determinación de la sensibilidad antinfúngica

El estudio de la sensibilidad de los patógenos fúngicos permite valorar su respuesta *in vitro* a los antifúngicos de uso clínico con el fin de detectar cepas resistentes y determinar el mejor tratamiento para erradicar las infecciones que causan.

Actualmente existen dos métodos de referencia estandarizados para la evaluación de la sensibilidad antifúngica, desarrollados por el *Clinical and Laboratory Standards Institute* estadounidense (CLSI) (Rex, 2008a; Rex, 2008b) y por el *European Committee on Antimicrobial Susceptibility Testing* (EUCAST) (EUCAST, 2017a; EUCAST, 2017b). Estas dos técnicas guardan una serie de similitudes y de diferencias (**Tabla 1**), pero sus resultados se consideran comparables (Cuenca-Estrella y Rodríguez-Tudela, 2010). Ambas determinan las concentraciones mínimas inhibitorias (CMIs) de los antifúngicos frente a las cepas mediante la valoración del crecimiento de estas en placas de microdilución que contienen diluciones seriadas de cada antifúngico en comparación con un pocillo control sin fármaco, definiéndose la CMI como la concentración mínima de antifúngico necesaria para inhibir el crecimiento de un hongo. Sin embargo, la inhibición de algunas especies con determinados antifúngicos, como ocurre con

los hongos filamentosos y las equinocandinas, es solo parcial y el hongo es capaz de producir hifas muy cortas y ramificadas. En esos casos se determina la concentración mínima efectiva (CME), que se define como la concentración de antifúngico más baja a la que se observa un crecimiento aberrante del hongo con respecto a su crecimiento control en el mismo medio pero sin fármaco (Kurtz *et al.*, 1994).

Tabla 1. Diferencias existentes entre los métodos de microdilución de CLSI y EUCAST para la determinación de la sensibilidad antifúngica (adaptada de Alastruey-Izquierdo *et al.*, 2015).

Factor	**CLSI**	**EUCAST**
Contenido en glucosa en el medio	0,2%	2%
Densidad del inóculo	0,5 – 2,5 x 10^3 UFC/mL * 0,4 – 5 x 10^4 UFC/mL **	1 – 5 x 10^5 UFC/mL
Visual	Visual	Espectrofotométrica (L)
Fondo de placa multipocillo	En forma de U	Plano
Determinación de la CMI de la anfotericina B en levaduras #	100% inhibición	90% inhibición

* Levaduras; ** Hongos filamentosos; # Según CLSI M27-A3 y EUCAST 7.3.1.

La interpretación de los resultados de los estudios de sensibilidad *in vitro* para su extrapolación al ámbito clínico se realiza de acuerdo a los puntos de corte clínicos, basados en la combinación de la distribución de las CMIs, las dosis de los antifúngicos administradas, la respuesta clínica y parámetros farmacocinéticos y farmacodinámicos. Gracias a estos, las cepas se pueden categorizar como sensibles (existe una alta probabilidad de que el fármaco sea apropiado para el tratamiento del paciente), intermedias o sensibles dependientes de dosis (los pacientes requieren una dosis superior del fármaco para alcanzar una respuesta clínica exitosa) o resistentes (no se recomienda el uso del fármaco para el tratamiento del paciente por alto riesgo de fallo terapéutico) (Alastruey-Izquierdo *et al.*, 2015). Existen puntos de corte clínicos para ambos métodos para los antifúngicos más comúnmente utilizados como tratamiento frente a varias especies de levaduras y hongos filamentosos (**Tablas 2 y 3**) pero no para patógenos fúngicos emergentes o menos habituales, en cuyo caso se utilizan los puntos de corte epidemiológicos para definir las distintas poblaciones.

Los puntos de corte epidemiológicos, denominados ECV por CLSI y ECOFF por EUCAST, se establecen de forma previa al punto de corte clínico mediante el estudio de la distribución de las CMIs de una gran colección de cepas de una misma especie fúngica frente a un antifúngico *in vitro*. Permiten diferenciar la población salvaje o sin mecanismos de resistencia adquiridos de aquella con mecanismos de resistencia para un antifúngico determinado (Espinel-Ingroff y Turnidge, 2016).

Existen otras metodologías que permiten determinar la sensibilidad antifúngica *in vitro*, como los comerciales Etest (bioMérieux), Vitek (bioMérieux) y Sensititre YeastOne (Trek Diagnostic System) o las placas multipocillo de agar suplementados con antifúngico (Guinea *et al.*, 2019; Meletiadis *et al.*, 2019). Además, se ha propuesto recientemente un método, basado en el análisis comparativo de los espectros de masas de los aislados fúngicos al ser expuestos a diferentes concentraciones de antifúngico, que posibilitaría la aplicación de la técnica de espectrometría de masas MALDI-ToF para determinar el perfil de resistencia de los hongos (Vella *et al.*, 2013; Saracli *et al.*, 2015; Vella *et al.*, 2017; Paul *et al.*, 2018).

Tabla 2. Puntos de corte clínicos para las principales especies de *Candida* según los métodos del EUCAST y CLSI.

		Punto de corte clínico (mg/L)									
		C. albicans		*C. glabrata*		*C. krusei*		*C. parapsilosis*		*C. tropicalis*	
Antifúngico	Metodología	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >
AMB	EUCAST	1	1	1	1	1	1	1	1	1	1
	CLSI	-	-	-	-	-	-	-	-	-	-
FLC*	EUCAST	2	4	0,002	32	-	-	2	4	2	4
	CLSI	-	16	32**	32	-	-	2	4	2	4
ITC	EUCAST	0,06	0,06	EI	EI	EI	EI	0,12	0,12	0,12	0,12
	CLSI	-	-	-	-	-	-	-	-	-	-
VRC	EUCAST	0,06	0,25	EI	EI	EI	EI	0,12	0,25	0,12	0,25
	CLSI	0,12	0,5	-	-	0,5	2	0,12	1	0,12	0,5
PSC	EUCAST	0,06	0,06	EI	EI	EI	EI	0,06	0,06	0,06	0,06
	CLSI	-	-	-	-	-	-	-	-	-	-
CPF	EUCAST	-	-	-	-	-	-	-	-	-	-
	CLSI	0,25	1	0,12	0,25	0,25	0,5	2	4	0,25	0,5
MCF	EUCAST	0,015	0,015	0,03	0,03	EI	EI	0,002	2	EI	EI
	CLSI	0,25	0,5	0,06	0,12	0,25	0,5	2	4	0,25	0,5
AND	EUCAST	0,03	0,03	0,06	0,06	0,06	0,06	0,002	4	0,06	0,06
	CLSI	0,25	0,5	0,12	0,25	0,25	0,5	2	4	0,25	0,5

AMB: anfotericina B; FLC: fluconazol; ITC: itraconazol; VRC: voriconazol; PSC: posaconazol; CPF: caspofungina; MCF: micafungina; AND: anidulafungina. * EUCAST ha propuesto un punto de corte clínico del fluconazol para otras especies: S ≤ 2 mg/L, R >4 mg/L; ** Sensible dependiente de dosis.

Tabla 3. Puntos de corte clínicos para las principales especies de *Aspergillus* según el método del EUCAST.

	Punto de corte clínico (mg/L)							
Antifúngico	*A. fumigatus*		*A. flavus*		*A. terreus*		*A. nidulans*	
	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >
AMB	1	2	EI	EI	-	-	-	-
ITC	1	2	1	2	1	2	1	2
VRC	1	2	EI	EI	EI	EI	EI	EI
PSC	0,12	0,25	EI	EI	0,12	0,25	EI	EI
ISA	1	1	EI	EI	1	1	0,25	0,25
CPF	EI	EI	EI	EI	EI	EI	EI	EI
MCF	EI	EI	EI	EI	EI	EI	EI	EI
AND	EI	EI	EI	EI	EI	EI	EI	EI

EI: Evidencia insuficiente para establecer puntos de corte clínicos.

1.4. Resistencia a los antifúngicos

En los últimos años, el uso generalizado de los antifúngicos como profilaxis y como terapia empírica o dirigida ha dado lugar a la emergencia de resistencias a estos fármacos entre los patógenos fúngicos más habituales, lo que incrementa el riesgo para la salud humana que suponen las infecciones que provocan. Este fenómeno ocurre a causa de la presión selectiva que ejercen los antifúngicos, la cual facilita la selección de cepas que desarrollan diferentes mecanismos de resistencia para sobrevivir y reproducirse en su presencia. Además, se ha observado un cambio en la epidemiología de los hongos, y cada vez son más frecuentes las infecciones causadas por especies intrínsecamente resistentes a un antifúngico o multirresistentes (Pfaller y Diekema, 2004), como es el caso de *C. auris* (Lockhart *et al.*, 2017).

El desarrollo de la resistencia antifúngica es un complejo proceso en el que están involucrados el huésped y su estado inmunitario, el antifúngico elegido para realizar la terapia y múltiples factores microbianos del patógeno que produce la enfermedad (White *et al.*, 1998; Brown y Netea, 2012), y es una de las principales causas de fallo terapéutico en las infecciones fúngicas invasoras (Cowen *et al.*, 2014). Esta falta de respuesta al tratamiento se da con mayor frecuencia en pacientes inmunodeprimidos (Ben-Ami *et al.*, 2008), y la terapia también puede verse comprometida en casos en los que el antifúngico no sea capaz de penetrar en una concentración terapéutica en la zona donde se encuentra la infección, contribuyendo así al desarrollo de la resistencia por parte de esa cepa expuesta a concentraciones subóptimas de fármaco (Shields *et al.*, 2014; Jensen *et al.*, 2015; Grau *et al.*, 2015).

La resistencia a los antifúngicos puede ser definida tanto desde un punto de vista microbiológico como clínico. La resistencia microbiológica de un hongo está asociada a la baja sensibilidad *in vitro* de este frente a un antifúngico, y se determina mediante los puntos de corte establecidos para cada patógeno fúngico. En función de las características particulares de cada microorganismo, la resistencia microbiológica se puede subdividir en: (i) resistencia primaria o intrínseca, la cual ocurre en cepas de una misma especie que son resistentes a un antifúngico de forma espontánea sin haber sido previamente expuestas al fármaco; y (ii) resistencia secundaria o adquirida, que se desarrolla después de la exposición a los antifúngicos y que puede ser debida a alteraciones fenotípicas o genotípicas que se pueden manifestar de forma estable o transitoria (Pfaller, 2012).

La resistencia antifúngica clínica es aquella que ocurre cuando hay crecimiento o ausencia de inhibición de un hongo en el foco de infección del paciente, aunque en el mismo existan concentraciones terapéuticas de un fármaco con actividad *in vitro* frente al agente etiológico de la infección. En este caso, la resistencia depende del antifúngico, de los factores relacionados con el paciente o de ambos, más que del microorganismo que produce la infección.

1.4.1. Resistencia a los polienos

La resistencia a la anfotericina B es poco habitual, y suele estar asociada a patógenos fúngicos que muestran resistencia intrínseca a estos fármacos, como las especies de *Scedosporium*, *Fusarium*, Mucorales y algunas de las crípticas de *Aspergillus*, además de la sensibilidad disminuida que presentan *A. terreus*, *A. flavus* o *Aspergillus nidulans* (Lass-Florl *et al.*, 2005; Hadrich *et al.*, 2012). Algunas cepas de *C. auris* también presentan resistencia intrínseca a anfotericina B (Lockhart *et al.*, 2017). A pesar de que se han descrito de forma puntual aislados clínicos de especies de *Candida* con resistencia secundaria a este antifúngico (Krcmery *et al.*, 1997; Hull *et al.*, 2012), la resistencia adquirida a este compuesto en especies sensibles es infrecuente y la principal resistencia secundaria en *Candida* y *Aspergillus* se desarrolla a azoles y equinocandinas.

1.4.2. Resistencia a los azoles

Puesto que los azoles son la única opción entre los antifúngicos como tratamiento y profilaxis oral frente a las infecciones fúngicas invasoras, la emergencia de su resistencia supone un grave problema a nivel global. En diversos estudios epidemiológicos se ha descrito resistencia adquirida en especies de *Aspergillus* y de *Candida* sensibles y resistencia intrínseca en especies emergentes (Alastruey-Izquierdo *et al.*, 2014; Lackner *et al.*, 2014; Al-Hatmi *et al.*, 2016; Chowdhary *et al.*, 2017; Skiada *et al.*, 2018).

1.4.2.1 Resistencia a los azoles en *Candida* spp.

Las cepas de *C. albicans* que causan candidiasis invasoras, en especial candidemias, son más sensibles a fluconazol que cepas de otras especies del género que originan estas infecciones (Diekema *et al.*, 2012; Guinea *et al.*, 2014; Ying *et al.*, 2016), las cuales están asociadas a una mayor mortalidad. *C. glabrata* presenta una baja sensibilidad a los triazoles, en especial al fluconazol, lo que ha dado lugar al aumento de su prevalencia como causante de infecciones en pacientes en tratamiento o profilaxis con compuestos de esta clase (Guinea *et al.*, 2014). Las cepas de *C. tropicalis* que ocasionan infecciones están asociadas a niveles variables de resistencia a fluconazol, mientras que las de *C. parapsilosis* son resistentes a este triazol de forma más esporádica (Whaley *et al.*, 2016). Existen varios mecanismos de resistencia a azoles en *Candida*, entre los cuales se incluyen los que involucran cambios en la enzima diana, codificado por la proteína Erg11 (Kanafani y Perfect, 2008). Se han descrito numerosas mutaciones puntuales en ella asociadas a resistencia a fluconazol, las cuales dan lugar a una diana alterada que reduce su afinidad o incluso pone en peligro la unión del antifúngico (Morio *et al.*, 2010; Grossman *et al.*, 2015; Tan *et al.*, 2015). A su vez, en *C. albicans* se ha observado la sobreexpresión de *ERG11*, aunque de forma poco frecuente, debida a mutaciones en el factor de transcripción *UPC2* (Silver *et al.*, 2004; Flowers *et al.*, 2012). Este aumento en la expresión de

ERG11 también ha sido detectada en otras especies de *Candida* (Vandeputte *et al.*, 2005; Rogers *et al.*, 2006; Navarro-Rodriguez *et al.*, 2019). La pérdida de función del gen *ERG3* también ha sido asociada con el desarrollo de resistencia a azoles en *C. albicans* (Vale-Silva *et al.*, 2012; Morio *et al.*, 2012), y se ha descrito un mecanismo que implica alteraciones genómicas, como la recombinación mitótica, la conversión génica y la formación de un isocromosoma, que confiere resistencia mediante el incremento de copias de *ERG11* (Selmecki *et al.*, 2006). Sin embargo, el mecanismo más común de resistencia en *Candida* es la inducción de bombas de flujo, que da lugar a un descenso de la concentración del antifúngico en la célula fúngica. La regulación de las bombas de flujo está codificada en las especies de este género por los genes *MDR* o *CDR* de las familias de transportadores MFS (*major facilitator superfamily*) y ABC (*ATP-binding cassette*), respectivamente (Franz *et al.*, 1998; Borst *et al.*, 2005; Sanguinetti *et al.*, 2005). También se han descrito factores de transcripción que regulan la expresión de estos transportadores en función de la exposición al antifúngico en algunas especies, como *TAC1* y *MRR1* en *C. albicans* y *PDR1* en *C. glabrata*. Las mutaciones de ganancia de función en estos genes pueden conferir resistencia antifúngica al dar lugar a la sobreexpresión de los transportadores (Coste *et al.*, 2006; Vermitsky *et al.*, 2006; Morschhauser *et al.*, 2007).

1.4.3. Resistencia a los azoles en *Aspergillus* spp.

La resistencia a azoles entre las especies de *Aspergillus*, fundamentalmente en *A. fumigatus*, ha sido descrita a nivel mundial (Rivero-Menendez *et al.*, 2016). La emergencia de esta resistencia está vinculada al uso masivo de esta familia de compuestos para el tratamiento prolongado de las aspergilosis (Dannaoui *et al.*, 2001; Howard *et al.*, 2009; Camps *et al.*, 2012a) pero también se sospecha que está relacionada con el empleo de fungicidas azólicos para la protección de cosechas en el campo (Verweij *et al.*, 2009). Europa es el conteniente donde se ha descrito con mayor frecuencia la resistencia a azoles en cepas tanto clínicas como ambientales de *A. fumigatus*, con prevalencias desde 1,1% a 28% en función del país donde se haya desarrollado el estudio (Rivero-Menendez *et al.*, 2016). En España esta resistencia es poco frecuente. Se detectó un 1,8% de aislados resistentes a al menos un azol de pacientes con aspergilosis invasora probable o aspergiloma ingresados entre 1999 y 2011 en un centro hospitalario (Escribano *et al.*, 2013), aunque en el estudio FILPOP no se encontró resistencia secundaria en las cepas de *A. fumigatus* procedentes de 29 hospitales de todo el país (Alastruey-Izquierdo *et al.*, 2013).

También se han descrito casos de otras especies de *Aspergillus* con valores de CMI elevados frente a azoles, como *A. terreus*, *A. flavus* o *A. niger* (Zoran *et al.*, 2018; Choi *et al.*, 2019; Perez-Cantero *et al.*, 2019), además de algunas de las especies crípticas (Alastruey-Izquierdo *et al.*, 2014).

En *Aspergillus*, las principales dianas de los azoles son las proteínas Cyp51. La mayoría de las especies de este género posee dos isoenzimas Cyp51, Cyp51A y Cyp51B (Mellado *et al.*, 2001),

codificadas a su vez por los genes *cyp51A* y *cyp51B*. En *A. fumigatus* se ha demostrado que el crecimiento *in vitro* del hongo se ve afectado solo si los dos genes se bloquean a la vez (Mellado *et al.*, 2005; Hu *et al.*, 2007). *A. flavus* es la única especie de *Aspergillus* que se conoce hasta la fecha que posee una tercera proteína Cyp51, la Cyp51C, la cual guarda similitud con Cyp51A. El mecanismo de resistencia a azoles más frecuente de este género fúngico está asociado a modificaciones en esta diana, principalmente en Cyp51A. Se han descrito varios polimorfismos de un solo nucleótido (*single nucleotide polymorphisms*, SNPs) en el gen *cyp51A* que dan lugar a alteraciones en la secuencia aminoacídica de su proteína, las cuales son responsables de un aumento en las CMIs y pueden producir resistencia a uno o varios azoles, en función de la posición en la que se produzca el cambio, mediante la reducción de la eficiencia de la unión del ligando o compuesto azólico a la enzima.

Las mutaciones puntuales más comunes descritas en *A. fumigatus* cuya relación con la resistencia a azoles ha sido corroborada incluyen aquellas en la glicina en la posición 54 (G54), asociadas a resistencia cruzada a itraconazol y posaconazol (Diaz-Guerra *et al.*, 2003; Nascimento *et al.*, 2003), en la metionina en la posición 220 (M220), resistentes a itraconazol y posaconazol y con sensibilidad reducida y variable a voriconazol y a isavuconazol (Mellado *et al.*, 2004; Van Ingen *et al.*, 2015), en la glicina en posición 138 (G138), que causan resistencia simultánea a todos los azoles (Howard *et al.*, 2006; Wiederhold *et al.*, 2016), en la prolina 216 (P216), a la cual se le asocia resistencia a itraconazol y posaconazol al sustituirse por lisina (L) (Camps *et al.*, 2012a; Hagiwara *et al.*, 2014), y en la glicina 448 (G448), cuya modificación por serina (S) genera resistencia a voriconazol e isavuconazol, y baja sensibilidad frente a itraconazol y posaconazol (Bellete *et al.*, 2010; Pelaez *et al.*, 2012). Además, se han descrito de forma ocasional otras mutaciones puntuales en Cyp51A que podrían tener relación con el desarrollo de resistencia, aunque no se ha demostrado su implicación exacta en la disminución de la sensibilidad de las cepas que las poseen (Howard *et al.*, 2009; Bueid *et al.*, 2010; Alanio *et al.*, 2011; Albarrag *et al.*, 2011; Escribano *et al.*, 2011; Mortensen *et al.*, 2011; Camps *et al.*, 2012a; Bader *et al.*, 2013; Prigitano *et al.*, 2014).

Todas estas modificaciones puntuales en *cyp51A* se describen de forma general en cepas aisladas de pacientes que han estado sometidos a terapia prolongada con azoles. En estos casos, la cepa sensible a azoles que provoca la infección, tras un tratamiento con fármacos azólicos, desarrolla un fenotipo resistente fruto de la presión selectiva ejercida por la medicación administrada al paciente (Howard *et al.*, 2009; Hagiwara *et al.*, 2014).

Además, se han descrito inserciones en tándem de un número variable de bases en la región promotora de *cyp51A* combinadas, en ocasiones, con alteraciones puntuales en el propio gen que producen resistencia a azoles. Estas dan lugar a un aumento en la expresión de *cyp51A* que contribuye al fenotipo de resistencia (Zhang *et al.*, 2017). La más habitual es la duplicación de 34 pares de bases en el promotor del gen junto a una sustitución de una leucina (L) por una histidina (H) en posición 98 de la proteína (TR_{34}/L98H), que está relacionada con resistencia

simultánea a todos los azoles (Mellado *et al.*, 2007). También es común la duplicación de 46 pares de bases en el promotor con las mutaciones de una tirosina (Y) por una fenilalanina (F) en la posición 141 y de una treonina (T) por una arginina (A) en posición 289 de la proteína (TR_{46}/Y121F/T287A), asociada a resistencia a voriconazol e isavuconazol y CMIs elevadas a itraconazol y posaconazol (Van der Linden *et al.*, 2013; Pelaez *et al.*, 2015; Wiederhold *et al.*, 2016). La mutación Y121F ha sido descrita en solitario también con un perfil de resistencia similar a cuando aparece en la combinación anterior (Lescar *et al.*, 2014). Otro mecanismo que se ha encontrado, aunque con menos frecuencia, consiste en una duplicación de 53 pares de bases en el promotor de la diana (TR_{53}), sin más modificaciones en el gen, que genera resistencia a todos los azoles (Hodiamont *et al.*, 2009; Mavridou *et al.*, 2015; Le Pape *et al.*, 2016). Estas tres modificaciones de la diana de los azoles se han descrito tanto en cepas clínicas como en cepas ambientales en todo el mundo (Garcia-Rubio *et al.*, 2017) y se han relacionado con el uso de fungicidas en el campo (Snelders *et al.*, 2012), habiéndose aislado en pacientes que no habían estado en terapia antifúngica anteriormente (Verweij *et al.*, 2007). Recientemente se ha descrito en una cepa clínica una repetición de 120 pares de bases en tándem en el promotor de *cyp51A* ligada a la combinación de mutaciones puntuales F46Y, M172V y E427K en la proteína que también produce resistencia a azoles (Hare *et al.*, 2019).

A pesar de que la resistencia a los triazoles en *A. fumigatus* es atribuida principalmente a las alteraciones en Cyp51A, se han descrito cepas resistentes en las que no se han encontrado mutaciones ni en su gen ni en su promotor (Arendrup *et al.*, 2010; Denning *et al.*, 2011). En estos aislados se evidencia la existencia de otros mecanismos de resistencia independientes de la alteración de la diana de los azoles, muchos de los cuales aún se encuentran en estudio. Uno de ellos se basa en la disminución de la concentración efectiva del azol mediante las bombas de flujo, los transportadores ABC y los MFS (Cannon *et al.*, 2009). Además, también se ha descrito la sobreexpresión de *cyp51B* en una cepa resistente sin ningún otro mecanismo aparente (Bueid *et al.*, 2013). Sin embargo, el papel de este gen en el desarrollo de la resistencia a azoles en *A. fumigatus* no se ha esclarecido aún, ya que nunca se han relacionado las mutaciones encontradas en él con este fenómeno.

También se ha observado que la acción de varios factores de transcripción en la síntesis de ergosterol puede comprometer la sensibilidad a azoles en esta especie, como una de las proteínas de unión al elemento regulador del esterol (*sterol regulatory element binding proteins*, SREBPs), la SrbA (Willger *et al.*, 2008), o HapE, cuya mutación P88L produce la sobreexpresión de *cyp51A*, que conlleva la reducción de su sensibilidad por esta clase de antifúngicos (Camps *et al.*, 2012b).

Los mecanismos de resistencia a azoles en otras especies del género *Aspergillus* han sido estudiados en menor profundidad. En *A. terreus* la modificación que se ha descrito con mayor frecuencia es la de la metionina en posición 217 (M217) en la proteína Cyp51A, la cual se ha encontrado combinada con otra sustitución del ácido aspártico en posición 344 (D344) de la

misma enzima cuando la metionina cambia a valina (M217V). Las mutaciones en la M217 han sido relacionadas con las que tienen lugar en la M220 en *A. fumigatus*, ya que ambas están vinculadas a un perfil de resistencia a itraconazol y posaconazol y de sensibilidad variable a voriconazol e isavuconazol (Arendrup *et al.*, 2012a; Zoran *et al.*, 2018). Sin embargo, no se ha demostrado aún que estas mutaciones sean las responsables de la disminución de la sensibilidad a los azoles. En *A. flavus* se han encontrado de forma esporádica una serie de mutaciones puntuales en las proteínas Cyp51A y Cyp51B en varias cepas con diferentes perfiles de sensibilidad reducida a azoles, aunque su papel en el desarrollo de la resistencia no ha sido comprobado (Krishnan-Natesam *et al.*, 2008; Sharma *et al.*, 2018). Algunas de ellas aparecían en aislados que también poseían alteraciones en Cyp51C, muchas de las cuales fueron descartadas como causantes del fenotipo de resistencia azólica al ser encontradas también en cepas sensibles a estos compuestos (Liu *et al.*, 2012; Paul *et al.*, 2015; Sharma *et al.*, 2018; Choi *et al.*, 2019). Actualmente, las sustituciones S196F, Y319H, A324P y V465M son las únicas en esta proteína que se sospechan responsables de la resistencia a azoles, junto a otros mecanismos estudiados, aunque solo se han detectado de forma aislada (Paul *et al.*, 2015; Sharma *et al.*, 2018). Por último, todos los cambios que se han encontrado en las proteínas Cyp51A y Cyp51B de aislados resistentes a azoles de *A. niger* también se detectaron en cepas sensibles (Iatta *et al.*, 2016; Hashimoto *et al.*, 2017; Perez-Cantero *et al.*, 2019), por lo que no se han podido relacionar directamente con la resistencia a estos compuestos.

En *A. flavus* y *A. niger* también se ha observado la sobreexpresión de los genes *cyp51* o de aquellos que codifican las bombas de flujo en cepas resistentes a uno o varios azoles, pudiendo ser la explicación de su baja sensibilidad (Paul *et al.*, 2015; Sharma *et al.*, 2018; Perez-Cantero *et al.*, 2019).

1.4.4. Resistencia a las equinocandinas

Se estima que un 60% de los pacientes que sufren candidemia son tratados con una equinocandina, ya que son el tratamiento de primera línea para las infecciones causadas por *Candida* y disminuyen considerablemente su mortalidad (Cleveland *et al.*, 2012; Andes *et al.*, 2012). Estas especies son generalmente sensibles a equinocandinas, aunque *Candida guilliermondii* o *C. parapsilosis* presentan CMIs intrínsecamente más elevadas a estos compuestos (Perlin, 2007; Garcia-Effron *et al.*, 2008a), y la incidencia de la resistencia a esta familia de antifúngicos en estudios poblacionales realizados es baja para la mayoría de las especies (Pfaller *et al.*, 2011a; Pfaller *et al.*, 2013; Guinea *et al.*, 2014; Morales-Lopez *et al.*, 2017; Mencarini *et al.*, 2018). Sin embargo, desde 2005 (Laverdiere *et al.*, 2006) el número de casos de resistencia adquirida a equinocandinas está aumentando, sobre todo en *C. glabrata*. El incremento de cepas resistentes está siendo particularmente relevante en áreas geográficas donde esta especie es más incidente, como el centro y el norte de Europa o Estados Unidos.

El mecanismo por el que las especies de *Candida* pueden ver reducida su sensibilidad a las equinocandinas se basa en la presencia de mutaciones, adquiridas o intrínsecas, en los genes *FKS* que codifican la subunidad catalítica de la enzima diana de estos compuestos, la 1,3-β-D-glucano sintasa. Estas alteraciones suelen tener lugar en dos regiones muy conservadas de estos genes, denominadas regiones calientes o *hot-spots* (Beyda *et al.*, 2012; Pham *et al.*, 2014a). A pesar de que la 1,3-β-D-glucano sintasa de todas las especies está codificada por tres genes *FKS*, todas las modificaciones causantes de resistencia se han descrito en el gen *FKS1*, salvo en *C. glabrata*, en la que se encuentran en *FKS1* y *FKS2* (Perlin, 2011). Esto se debe a que *FKS1* y *FKS2* son funcionalmente redundantes en esta especie, viéndose el crecimiento fúngico afectado solo en casos en los que ambos genes estén anulados (Katiyar *et al.*, 2012). En cambio, la disrupción del gen *FKS1* en *C. albicans* da lugar a la inhibición del crecimiento fúngico (Mio *et al.*, 1997). El gen *FKS3* no parece tener un papel relevante en el fenotipo de resistencia, aunque se ha descrito recientemente que los genes *FKS2* y *FKS3* pueden actuar como reguladores negativos de *FKS1* en *C. albicans*, aumentando su expresión y, por tanto, la resistencia a equinocandinas y la producción de β-glucano si están anulados (Suwunnakorn *et al.*, 2018).

Las alteraciones en los *FKS* reducen de 50 a 3000 veces la sensibilidad de la 1,3-β-D-glucano sintasa a las equinocandinas, en función de la posición de la sustitución aminoacídica que se produzca. Así, las modificaciones en los nucleótidos de los tripletes que codifican el primer y el quinto aminoácido del *hot-spot* 1 de Fks1, y de Fks2 en el caso de *C. glabrata*, que son una fenilalanina y una serina, respectivamente, están asociadas a un fenotipo de resistencia más pronunciado y a un perfil de CMIs a equinocandinas más elevadas que otras mutaciones, las cuales confieren una resistencia más débil, pudiendo las cepas que las poseen responder positivamente ante dosis más altas del fármaco (Garcia-Effron *et al.*, 2009a; Garcia-Effron *et al.*, 2009b; Desnos-Ollivier *et al.*, 2011; Arendrup *et al.*, 2012b; Fekkar *et al.*, 2013; Jensen *et al.*, 2014). Las mutaciones puntuales más frecuentes en *C. glabrata* ocurren en esos dos aminoácidos del *hot-spot* 1 de Fks2: el cambio de la serina en posición 663 de la proteína por una prolina (S663P) y la deleción de la fenilalanina en posición 659 (ΔF659) están relacionadas con altos valores de CMIs y con una elevada tasa de fallo terapéutico (Arendrup y Perlin, 2014). Generalmente, las cepas de *C. glabrata* con alteraciones en los genes *FKS* presentan CMIs altas para toda la familia de equinocandinas, dando lugar a su resistencia cruzada, aunque se han descrito cepas cuyas mutaciones solo les hacen desarrollar resistencia a uno de estos compuestos, manteniéndose sensibles al resto (Arendrup *et al.*, 2012b; Shields *et al.*, 2012; Jensen *et al.*, 2015; Bordallo-Cardona *et al.*, 2017).

Por otro lado, las especies *C. parapsilosis* y *C. guilliermondii* presentan alteraciones intrínsecas en los *hot-spots* de su gen *FKS1* (García-Effron *et al.*, 2008; Dudiuk *et al.*, 2017) (**Tabla 4**). Estas dan lugar a altos valores de CMIs a equinocandinas aunque, de forma ocasional, las infecciones causadas por cepas de estas especies con CMIs elevadas han sido tratadas con éxito (Pfaller *et al.*, 2005; Kale-Pradhan *et al.*, 2010; Fernandez-Ruiz *et al.*, 2014).

Tabla 4. Secuencias aminoacídicas de las regiones *hot-spot* de Fks de las principales especies de *Candida*. El número indica la posición que corresponde al primer aminoácido de cada *hot-spot* en las distintas especies. En magenta se marcan las posiciones asociadas con perfiles de resistencia a equinocandinas elevados, en verde aquellas asociadas a resistencia débil y en amarillo aquellas que confieren sensibilidad disminuida intrínseca (adaptada de Perlin, 2015).

Especie	Fks1		Fks2	
	hot-spot 1	*hot-spot* 2	*hot-spot* 1	*hot-spot* 2
C. albicans	641 FLTLSLRDP	1357 DWIRRYTL		
C. krusei	655 FLILSIRDP	1364 DWIRRYTL		
C. glabrata	625 FLILSLRDP	1340 DWIRRYTL	659 FLILSLRDP	1374 DWIRRYTL
C. guilliermondii	632 FMALSIRDP	1347 DWIRRYTL		
C. tropicalis	76* FLTLSIRDP	792* DWIRRYTL		
C. parapsilosis	652 FLTLSIRDA	1369 DWIRRYTL		

* Se requiere realizar la secuenciación completa de estos genes para confirmar la posición exacta del *hot-spot* en esta especie.

Se han detectado casos de cepas clínicas de *C. glabrata* resistentes a equinocandinas que también presentan resistencia a fluconazol, las cuales tienen un peor pronóstico (Pham *et al.*, 2014a; Farmakiotis *et al.*, 2014) y para las que ni siquiera las guías recogen recomendaciones para su tratamiento más allá del uso de anfotericina B (Pappas *et al.*, 2016). La explicación genética para la rápida emergencia de la multirresistencia en *C. glabrata* es desconocida, pero se cree que puede basarse en la plasticidad de su genoma haploide (Healey *et al.*, 2016a). No obstante, se baraja la posible implicación de los mecanismos de reparación del ADN en el aumento de cepas multirresistentes. Estos sistemas son los encargados de corregir los errores que la ADN polimerasa comete en la replicación del ADN, y se ha observado en humanos que defectos en los genes implicados en su correcto funcionamiento pueden estimular un incremento en la tasa de mutación del genoma (Hakem, 2008). Recientemente se ha descrito que las alteraciones en el gen *MSH2*, involucrado en la vía de reparación del desapareamiento de bases (*mismatch repair*), promueven la adquisición de la resistencia antifúngica en *C. glabrata* (Healey *et al.*, 2016b).

Por otro lado, la resistencia a equinocandinas en *Aspergillus* es un fenómeno muy poco estudiado. Esta familia de antifúngicos solo inhibe parcialmente el crecimiento de las especies de este género, por lo que se utiliza la CME como método para evaluar su actividad. El inconveniente de la CME es que su determinación es más complicada. Además, no existen puntos de corte clínicos para equinocandinas y los ECV/ECOFF existentes frente a especies de *Aspergillus* no son muy seguros para evaluar la posible resistencia de las cepas, pudiendo esta estar infravalorada, ya que se han descrito infecciones de brecha en pacientes recibiendo terapia con equinocandinas (Madureira *et al.*, 2007; Arendrup *et al.*, 2009).

1.5. Métodos moleculares para la detección de la resistencia antifúngica

La administración de una terapia antifúngica inadecuada para tratar una infección está asociada, además de a la aparición de resistencia, a un peor pronóstico clínico y a una mayor mortalidad (Ha *et al.*, 2012; Verweij *et al.*, 2015). Puesto que el inicio temprano de un tratamiento apropiado resulta crucial para el paciente, es necesario desarrollar nuevas técnicas que agilicen el proceso de caracterización de un aislado fúngico como sensible o resistente a los antifúngicos. En este contexto, se ha descrito que la detección de mutaciones puntuales en los genes que codifican las dianas de las principales familias de antifúngicos podría ser un mejor marcador de resistencia que la determinación de las CMIs (Shields *et al.*, 2012). Por ello, los sistemas basados en PCR cuantitativa o en tiempo real se consideran idóneos para detectar resistencia a través del estudio de estos genes de forma rápida y eficaz (Balashov *et al.*, 2005; Park *et al.*, 2005; Balashov *et al.*, 2006; Garcia-Effron *et al.*, 2008b; Xu *et al.*, 2010) ya que, además, pueden ser desempeñados a partir de muestras clínicas de forma directa sin necesidad de que el hongo sea cultivado, lo cual conlleva una reducción en el tiempo de detección (Denning *et al.*, 2011; Zhao *et al.*, 2013; Chong *et al.*, 2015; Chong *et al.*, 2016; Zhao *et al.*, 2016).

Una técnica derivada de la PCR cuantitativa que ha resultado útil para analizar las mutaciones en genes implicados en la resistencia a antifúngicos en *Candida* es el análisis de curvas de fusión de alta resolución (*high resolution melting*, HRM) (Loeffler *et al.*, 2000). Se trata de una PCR a tiempo real que, durante la amplificación de productos, intercala entre el ADN de doble cadena una molécula fluorescente con capacidad saturante. Después, los productos de PCR son desnaturalizados y rápidamente renaturalizados, variando su temperatura de desnaturalización en función de la longitud o la composición de nucleótidos de los productos, lo que provoca cambios en la fluorescencia. Este método de análisis permite determinar variables alélicas con éxito (Odell *et al.*, 2005), facilitando la distinción de las cepas sensibles de las resistentes y las mutaciones que provocan esta resistencia, además de ser útil también para otra serie de aplicaciones microbiológicas, como la identificación fúngica (Gago *et al.*, 2011).

A su vez, la metodología Luminex se ha usado para reconocer mutaciones puntuales que confieren resistencia a equinocandinas ya descritas en formato multiplex en *C. glabrata* con buena sensibilidad (Pham *et al.*, 2014b). A pesar de que procura buenos resultados en un tiempo reducido, es una técnica con un elevado coste y que, además, solo resulta útil cuando se lleva a cabo desde cultivo debido a su falta de sensibilidad en muestras clínicas.

1.6. Nuevos agentes antifúngicos en estudio

Los antifúngicos aprobados para uso clínico presentan múltiples limitaciones, entre las que se incluye su ineficacia para tratar las infecciones causadas por algunas especies fúngicas, lo que provoca elevadas tasas de mortalidad y el progresivo aumento de la resistencia a los mismos. También destacan su toxicidad cuando son administrados en altas dosis o en tratamientos

prolongados y su interacción con otros fármacos. Estos inconvenientes evidencian la necesidad de extender la búsqueda de formulaciones antifúngicas que, además de mejorar las ya existentes, posean nuevos mecanismos de acción que sean específicos de los patógenos para evitar la toxicidad en el humano, algo complejo debido a su grado de similitud (Denning y Bromley, 2015).

En la actualidad, existen una serie de nuevos agentes que se encuentran en desarrollo para tratar infecciones sistémicas. Algunos, como el manogepix o la olorofima, actúan frente a nuevas dianas, mientras que otros tienen los mismos mecanismos de acción que los antifúngicos ya existentes, como la síntesis de ergosterol o de β-glucano, pero poseen ciertas ventajas en comparación con los compuestos disponibles en clínica.

El manogepix (APX001A; E1210), actúa sobre la aciltransferasa Gwt1, catalizadora del paso de acilación del inositol en la ruta de biosíntesis del glicofosfatidilinositol (GPI), el cual se une a glicoproteínas que se transportan y anclan a la pared celular fúngica a través de él para participar en su ensamblaje. La inhibición de Gwt1 da lugar a defectos en el tráfico y anclaje a la pared de manoproteínas de tipo GPI, que son imprescindibles para mantener su integridad, además de para la adhesión, la filamentación y la patogenicidad del hongo. Asimismo, juegan un relevante papel en su protección frente a la detección por parte del sistema inmune del huésped que invaden (Tsukara *et al.*, 2003; McLellan *et al.*, 2012; Watanabe *et al.*, 2012). Gwt1 tiene una aciltransferasa homóloga en humanos, Pig-W, pero el manogepix no es capaz de inhibirla (Watanabe *et al.*, 2012).

La actividad de este agente ha sido demostrada tanto *in vitro* como en formulación oral, intravenosa e intraperitoneal para tratar modelos murinos con infecciones causadas por *C. auris*, *C. albicans* resistente a equinocandinas, *C. glabrata* resistente a equinocandinas y multirresistente, *A. fumigatus* resistente a azoles, *Scedosporium* / *Lomentospora*, *F. solani, Cryptococcus neoformans* y *Rhizopus delemar* (Hata *et al.*, 2011; Pfaller *et al.*, 2011b; Pfaller *et al.*, 2011c; Castanheira *et al.*, 2012; Wiederhold *et al.*, 2015; Arendrup *et al.*, 2018; Berkow *et al.*, 2018; Hager *et al.*, 2018; Shaw *et al.*, 2018; Zhao *et al.*, 2018a; Zhao *et al.*, 2018b; Alkhazraji *et al.*, 2019; Gebremariam *et al.*, 2019a; Lepak *et al.*, 2019; Pfaller *et al.*, 2019; Zhao *et al.*, 2019), siendo capaz de penetrar en sitios anatómicos de difícil acceso para otros antifúngicos (Hager *et al.*, 2018). Además, también ha mostrado buena actividad *in vitro* frente a otras especies de *Aspergillus* y *Candida*, aunque es ineficaz frente a *C. krusei*, *Fusarium*, *Coccidioides immitis* y *Coccidioides posadasii* (Miyazaki *et al.*, 2011; Pfaller *et al.*, 2011c; Pfaller *et al.*, 2011d; Castanheira *et al.*, 2012; Gebremariam *et al.*, 2019; Viriyakosol *et al.*, 2019). Tras los buenos resultados obtenidos en la fase I de ensayo clínico, el manogepix se encuentra actualmente en fase II.

La olorofima (F901318) forma parte de una nueva familia de antifúngicos denominada orotomidas. Su mecanismo de acción se basa en la inhibición de la dihidroorotato deshidrogenasa, una enzima oxidorreductasa que cataliza la conversión del dihidroorotato en

orotato en la ruta de biosíntesis de la pirimidina. La pirimidina es esencial para las células fúngicas, tanto para la síntesis de ADN y ARN como para la síntesis de la pared celular y de fosfolípidos (Garavito *et al.*, 2015), y se ha comprobado en modelos animales de infección con *A. fumigatus* (D'Enfert *et al.*, 1996), *C. albicans* (Noble y Johnson, 2005), *Histoplasma capsulatum* (Retallak *et al.*, 1999) o C*. neoformans* (de Gontijo *et al.*, 2014) que aquellos con mutaciones en su ruta de biosíntesis resultan menos virulentos. A pesar de que las células mamíferas también poseen una forma de esta enzima, la actividad de la olorofima parece ser específica frente a hongos (Oliver *et al.*, 2016), y su eficacia *in vitro* ha sido comprobada frente a un amplio espectro de patógenos fúngicos, como distintas especies de *Penicillium*, *Aspergillus* (incluidas cepas resistentes a azoles de *A. fumigatus*), *Fusarium* o *Scedosporium*, además de *H. capsulatum*, *C. posadasii*, *C. immitis* y dermatofitos (Oliver *et al.*, 2016; Buil *et al.*, 2017; Wiederhold *et al.*, 2017a; Biswas *et al.*, 2018a; Jorgensen *et al.*, 2018; Lackner *et al.*, 2018; Wiederhold *et al.*, 2018a). En cambio, no son activos frente a levaduras ni Mucorales (Oliver *et al.*, 2016; Jorgensen *et al.*, 2018). Su administración oral, intraperitoneal e intravenosa ha resultado eficaz en estudios *in vivo* en modelos murinos de infección con *A. fumigatus*, *A. flavus*, *Aspergillus nidulans*, *Aspergillus tanneri*, especies pertenecientes al complejo de *A. terreus*, *C. immitis* y *C. posadasii* (Oliver *et al.*, 2016; Negri *et al.*, 2017; Lackner *et al.*, 2018; Wiederhold *et al.,* 2018a; Seyedmousavi *et al.,* 2019). En la actualidad, este agente antifúngico ha completado la fase I de ensayo clínico con éxito y está en fase II como agente oral e intravenoso.

Aparte de la olorofima y el manogepix, existen otros compuestos con capacidad antifúngica en desarrollo que poseen una diana distinta a los fármacos disponibles. El VL-2397 es un sideróforo de origen natural que actúa como agente quelante de iones de aluminio, aunque se desconoce si este es su verdadero mecanismo de acción (Nakamura *et al.*, 2017a), y ha demostrado actividad frente a modelos murinos e invertebrados de candidiasis y aspergilosis invasora (Nakamura *et al.*, 2017b; Wiederhold *et al.*, 2017b).

Entre los nuevos fármacos que actúan contra las mismas dianas que los antifúngicos en uso clínico destacan la rezafungina (CD-101) y el ibrexafungerp (MK-3118; SCY-078) que, al igual que las equinocandinas, bloquean la 1,3-β-D-glucano sintasa (Jimenez-Ortigosa *et al.*, 2014; Krishnan *et al.*, 2017), aunque este último es un triterpeno que tiene una estructura diferente a la de esta familia de compuestos y actúa en un sitio distinto, lo que hace que la interacción enzima-droga sea diferente. Otros compuestos antifúngicos en desarrollo que comparten diana con los actualmente disponibles son VT-1129, VT-1161 y VT-1598, cuya especificidad por las enzimas del citocromo P450 de los hongos ha sido mejorada en detrimento de las de los mamíferos, y su actividad ha sido comprobada con éxito frente a un amplio rango de patógenos fúngicos (Wiederhold *et al.*, 2018b).

1.7. Técnicas de caracterización genotípica

La tipificación molecular engloba un conjunto de metodologías que permiten estudiar la epidemiología local de una enfermedad causada por un determinado agente infeccioso, proporcionando información acerca del origen de la infección y de su ruta de transmisión para, así, poder prevenirla y controlarla. También facilitan el estudio de la relación entre aislados con el fin de estudiar brotes. Además, entre muchas otras aplicaciones, posibilitan el análisis de la correlación existente entre el genotipo detectado y los distintos fenotipos asociados al patógeno, como el desarrollo de la resistencia al tratamiento, y permiten seguir la evolución de esta resistencia en los aislados secuenciales provenientes de pacientes.

El progreso y avance de la biología molecular ha permitido el desarrollo de varias técnicas genotípicas de tipificación de patógenos fúngicos, todas ellas basadas en la amplificación de ADN por PCR. En los años 90 surgieron la amplificación aleatoria de ADN polimórfico (*randomly amplified polymorphic DNA*, RAPD) (Lehmann *et al.*, 1992; Loudon *et al.*, 1993), el análisis de polimorfismos conformacionales de cadena sencilla (*single-strand conformation polymorphism analysis*, SSCP) (Walsh *et al.*, 1995) y la búsqueda de polimorfismos en la longitud de fragmentos amplificados (*amplified fragment length polymorphism*, AFLP) (Borst *et al.*, 2003; Lee *et al.*, 2004), métodos que, a pesar de tener utilidad, son poco reproducibles entre ensayos y difícilmente comparables entre laboratorios. Por ello, en los primeros años de los 2000 se desarrollaron dos métodos que solventaron estas limitaciones y que actualmente son los más usados en el campo de la micología, el análisis de secuencias de distintos genes (*multilocus sequence typing*, MLST) y el análisis de longitud de microsatélites (*microsatellite length polymorphism*, MLP).

El MLST se basa en el análisis de polimorfismos genéticos. Normalmente, se eligen para su estudio una serie de genes constitutivos de cada especie que, además de tener suficientes SNPs como para facilitar la diferenciación entre cepas, tengan suficiente secuencia de ADN común como para poder diseñar cebadores que permitan la amplificación de todos los aislados. Los amplicones de estos genes obtenidos por PCR son secuenciados y su secuencia comparada con una base de datos informatizada para caracterizar cada cepa según su perfil alélico (*sequence type*, ST). El ST se define a partir de la asignación de un número de alelo para cada variación de secuencia que se detecta de cada marcador. Esta técnica de genotipado es robusta y reproducible, y sus resultados son fáciles de interpretar. Resulta muy útil para tipificar las distintas especies de *Candida* (Bougnoux *et al.*, 2003; Dodgson *et al.*, 2003; Tavanti *et al.*, 2005; Jacobsen *et al.*, 2007), de *Scedosporium* (Bernhardt *et al.*, 2013) o de *Fusarium* (O'Donell *et al.*, 2010), aunque es poco discriminativa para diferenciar aislados de *A. fumigatus* (Bain *et al.*, 2007).

El MLP se caracteriza por la amplificación de microsatélites o regiones cortas repetidas (*short tandem repeats*, STR) constituidas por dos o hasta un máximo de seis nucleótidos repetidos en tándem a lo largo del genoma. Los microsatélites suelen encontrarse en regiones no codificantes

del ADN y están asociados a una alta tasa de mutación que depende de la especie estudiada. Para conocer la variación en el número de repeticiones de los STR se diseñan cebadores, uno de los cuales se marca con un fluoróforo, en sus regiones adyacentes conservadas para amplificarlos por PCR y estudiar su tamaño en relación a un estándar interno que se añade a las muestras antes de analizar su migración por electroforesis capilar. Esta técnica es reproducible, comparable entre laboratorios y posee un alto poder de discriminación entre cepas, aunque se requiere una tecnología específica para su estandarización y un conocimiento previo para analizar los resultados obtenidos con programas informáticos. Se han descrito múltiples STRs para las distintas especies de *Aspergillus* y *Candida* (Alanio *et al.*, 2017), aunque para algunas de ellas, como *C. glabrata*, no está definido cuáles resultan más discriminativos (Foulet *et al.*, 2005; Grenouillet *et al.*, 2007; Brisse *et al.*, 2009; Enache-Angoulvant *et al.*, 2010; Abbes *et al.*, 2012).

Además, la secuenciación de genomas completos también se está utilizado con la finalidad de estudiar la diversidad genética de una especie. Su poder de discriminación es superior al del resto de metodologías de tipado, permitiendo obtener resultados más precisos en estudios epidemiológicos o de dinámica de poblaciones (Sharma *et al.*, 2016; Lockhart *et al.*, 2017; Biswas *et al.*, 2017; Garcia-Rubio *et al.*, 2018; Carrete *et al.*, 2019). Sin embargo, el uso de esta técnica se ve limitado en la actualidad por su elevado coste y por la dificultad y complejidad del análisis de los resultados obtenidos.

2. Justificación y Objetivos

2. JUSTIFICACIÓN Y OBJETIVOS

El uso indiscriminado de antifúngicos para reducir las altas tasas de mortalidad a las que están asociadas las infecciones fúngicas invasivas es el principal factor que ha dado lugar a la emergencia de la resistencia a los mismos, constituyendo una amenaza para la salud pública. En los últimos años se ha descrito un aumento, por un lado, en el número de cepas que desarrollan resistencia secundaria al ser expuestas a los antifúngicos disponibles y, por otro, en el número de infecciones causadas por especies que presentan sensibilidad reducida de forma intrínseca.

Una de las especies cuya incidencia se ha incrementado notablemente es *Candida glabrata*, la cual exhibe de forma constitutiva valores de CMIs elevados al fluconazol. Las equinocandinas son, por tanto, el tratamiento de elección para las infecciones causadas por esta especie. Sin embargo, en la última década se ha detectado un aumento progresivo en el número de cepas clínicas con resistencia adquirida a esta clase de antifúngicos en varias áreas geográficas, restringiendo a un único antifúngico las opciones terapéuticas. Pese a que en España la tasa de resistencia a equinocandinas en *C. glabrata* es baja, es necesario estudiar en profundidad cómo se desarrolla este fenómeno para intentar desarrollar estrategias con el fin de minimizar su aparición.

La proporción de aislados clínicos con resistencia a los triazoles de las distintas especies de *Aspergillus*, en especial de *Aspergillus fumigatus*, también se está viendo gradualmente incrementada en todo el mundo, lo que compromete el uso de estos fármacos como tratamiento de primera línea para la aspergilosis invasora. Esto limita las alternativas terapéuticas para estas infecciones, puesto que las equinocandinas solo se recomiendan en terapias combinadas. La caracterización fenotípica y molecular de estas cepas y el estudio de los principales mecanismos de resistencia a los azoles en los genes *cyp51* de estas especies son fundamentales, ya que se ha descrito que las distintas mutaciones en estos genes están asociadas a perfiles de resistencia específicos. Además, el origen de algunas de estas alteraciones se ha relacionado con el uso prolongado de antifúngicos de esta clase, mientras que el de otras con el empleo de azoles utilizados como fungicidas en la agricultura. Por tanto, el conocimiento y la monitorización de la resistencia podrían ayudar a diseñar planes de acción y control que ayuden a evitar la propagación de este fenómeno.

Los antifúngicos que se utilizan en la actualidad tienen varias limitaciones. Además de generar resistencia secundaria de forma cada vez más frecuente, también pueden producir toxicidad y efectos secundarios, interaccionar con otros fármacos concomitantes o no alcanzar niveles terapéuticos en algunas localizaciones anatómicas del paciente. A su vez, existen especies multirresistentes que son muy difíciles de tratar con los compuestos actualmente disponibles. Entre estas destacan las especies de los géneros *Scedosporium*, *Lomentospora* y *Fusarium* y el orden Mucorales, además de algunas especies de *Aspergillus* denominadas crípticas. Por consiguiente, resulta imprescindible el desarrollo de nuevas formulaciones que reduzcan las restricciones anteriormente descritas y tengan actividad frente a especies

multirresistentes. Por tanto, dentro del marco de la resistencia antifúngica, se han planteado una serie de objetivos que ayuden a comprender el desarrollo de este fenómeno y a abordar algunas de las principales problemáticas que se le asocian:

Objetivo primero: Estudiar el desarrollo clínico y en el laboratorio de la resistencia a equinocandinas en *Candida glabrata* por medio de su caracterización molecular y del análisis de la correlación con su genotipo.

Objetivo segundo: Estudiar la identificación molecular, la sensibilidad antifúngica y el principal mecanismo de resistencia a los azoles de las especies de *Aspergillus* incluidas en un programa de vigilancia de la resistencia antifúngica.

Objetivo tercero: Desarrollar, estandarizar y validar una metodología de análisis de curvas de fusión de alta resolución para la detección de las principales mutaciones puntuales en el gen *cyp51A* asociadas a resistencia a azoles en *Aspergillus fumigatus*.

Objetivo cuarto: Evaluar la actividad antifúngica *in vitro* de dos nuevos compuestos frente a cepas multirresistentes mediante el uso de las metodologías de determinación de sensibilidad antifúngica desarrolladas por EUCAST y CLSI.

4.1. Valoración de la eficacia del manogepix frente a un conjunto de especies de hongos emergentes.

4.2 Valoración de la eficacia de la olorofima frente a especies crípticas de *Aspergillus*.

3. Capítulos

3.1. Capítulo 1

3.1. Capítulo 1. Caracterización molecular del desarrollo clínico y en condiciones de laboratorio de la resistencia a equinocandinas en *Candida glabrata*

Publicación original:

Rivero-Menendez O, Navarro-Rodriguez P, Bernal-Martinez L, Martin-Cano G, Lopez-Perez L, Sanchez-Romero I, Perez-Ayala A, Capilla J, Zaragoza O, Alastruey-Izquierdo A. 2019. Clinical and laboratory development of echinocandin resistance in *Candida glabrata*: molecular characterization. *Front Microbiol*, 10: 1585. doi: 10.3389/fmicb.2019.01585.

En los últimos años se ha observado una cambio epidemiológico en las especies capaces de causar infecciones fúngicas, aumentando el número de casos producidos por *Candida glabrata*. A su vez, se ha advertido un incremento en la tasa de aislados clínicos de esta especie resistentes a las equinocandinas. Esto supone un problema, ya que esta especie posee una sensibilidad disminuida al fluconazol.

Por ello, en este trabajo se estudió el mecanismo asociado a la resistencia a las equinocandinas, las mutaciones en las regiones *hot-spot* de los genes *FKS1* y *FKS2* que codifican su enzima diana 1-3-β-D-glucano sintasa. Se analizaron cinco pacientes con aislados secuenciales de *C. glabrata* resistentes a esta familia de antifúngicos. Para dos de estos pacientes se dispuso de aislados sensibles previos a estos, los cuales también fueron analizados. Las cepas resistentes poseían diferentes mutaciones en el *hot-spot* 1 de *FKS2*, mientras que las sensibles no tenían alteraciones. Las cepas fueron genotipadas usando las metodologías de MLST y MLP, concluyendo que los aislados sensibles y resistentes del mismo paciente guardaban relación genotípica. Esto indicó que la resistencia a equinocandinas pudo ser adquirida durante el tratamiento. Además, se encontraron aislados con distintas mutaciones en los genes *FKS* pero con el mismo genotipo en el mismo paciente. Al estudiar las alteraciones en el gen *MSH2*, no se observó ninguna asociación entre estas y la resistencia antifúngica o los genotipos de los aislados.

A su vez, también se investigó el desarrollo de resistencia *in vitro* tras la exposición a equinocandinas. Las cepas sensibles disponibles de los pacientes desarrollaron colonias con mutaciones en *FKS*, diferentes a las adquiridas *in vivo*, a partir de la exposición a una concentración de 0,06 mg/L de micafungina durante 48 horas. No se encontró correlación entre el desarrollo de resistencia y el genotipo de un conjunto de cepas sensibles después de ser expuestas a micafungina y anidulafungina y, tras calcular la concentración preventiva de mutantes y la ventana de selección de mutantes para ambas equinocandinas, se observó que se requieren concentraciones superiores a 2 mg/L de ambas equinocandinas para prevenir la aparición *in vitro* de colonias resistentes con alteraciones en los genes *FKS*.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 11 July 2019
doi: 10.3389/fmicb.2019.01585

Clinical and Laboratory Development of Echinocandin Resistance in *Candida glabrata*: Molecular Characterization

*Olga Rivero-Menendez[1], Patricia Navarro-Rodriguez[2], Leticia Bernal-Martinez[1,3], Gema Martin-Cano[1], Laura Lopez-Perez[1], Isabel Sanchez-Romero[4], Ana Perez-Ayala[5], Javier Capilla[2], Oscar Zaragoza[1,3] and Ana Alastruey-Izquierdo[1,3]**

[1] Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain, [2] Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Tarragona, Spain, [3] Spanish Network for the Research in Infectious Diseases (RD16CIII/0004/0003), Instituto de Salud Carlos III, Madrid, Spain, [4] Hospital Universitario Puerta de Hierro, Madrid, Spain, [5] Hospital Universitario 12 de Octubre, Madrid, Spain

OPEN ACCESS

Edited by:
Miguel Cacho Teixeira,
University of Lisbon, Portugal

Reviewed by:
Marie Desnos-Ollivier,
Institut Pasteur, France
Cheshta Sharma,
The University of Tennessee Health Science Center (UTHSC), United States
Dominique Sanglard,
Université de Lausanne, Switzerland

***Correspondence:**
Ana Alastruey-Izquierdo
anaalastruey@isciii.es

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 18 April 2019
Accepted: 25 June 2019
Published: 11 July 2019

Citation:
Rivero-Menendez O, Navarro-Rodriguez P, Bernal-Martinez L, Martin-Cano G, Lopez-Perez L, Sanchez-Romero I, Perez-Ayala A, Capilla J, Zaragoza O and Alastruey-Izquierdo A (2019) Clinical and Laboratory Development of Echinocandin Resistance in Candida glabrata: Molecular Characterization. Front. Microbiol. 10:1585. doi: 10.3389/fmicb.2019.01585

The pathogenic yeast *Candida glabrata* has become a public health issue due to the increasing number of echinocandin resistant clinical strains reported. In this study, acquisition and development of resistance to this antifungal class were studied in serial *C. glabrata* isolates from five patients admitted in two Spanish hospitals with a resistant profile against echinocandins associated with different mutations in hot-spot 1 of *FKS2* gene. For two of these patients susceptible *FKS* wild-type isolates obtained prior to resistant ones were also investigated. Isolates were genotyped using multilocus sequence typing and microsatellite length polymorphism techniques, which yielded comparable results. Susceptible and resistant isolates from the same patient had the same genotype, being sequence type (ST) 3 the most prevalent among them. Isolates with different *FKS* mutations but the same ST were present in the same patient. *MSH2* gene alterations were also studied to investigate their correlation with antifungal resistance acquisition but no association was found with antifungal resistance nor with specific genotypes. *In vitro* exposure to increasing concentrations of micafungin to susceptible isolates developed colonies carrying *FKS* mutations in agar plates containing a minimum concentration of 0.06 mg/L of micafungin after less than 48 h of exposure. We investigated the correlation between development of resistance and genotype in a set of susceptible strains after being *in vitro* exposed to micafungin and anidulafungin but no correlation was found. Mutant prevention concentration values and spontaneous growth frequencies after selection with both echinocandins were statistically similar, although *FKS* mutant colonies were more abundant after micafungin exposure ($p < 0.001$). Mutation S663P and F659 deletion were the most common ones found after selection with both echinocandins.

Keywords: *Candida glabrata*, echinocandins, antifungal resistance, *FKS*, *MSH2*, genotyping, anidulafungin, micafungin

INTRODUCTION

Infections caused by *Candida* species, extensively referred to as candidiasis, have been described as the most common fungal disease globally (Pappas et al., 2018). Although *Candida albicans* is the species causing the highest number of infections in clinical settings, an increasing prevalence of other *Candida* species has been reported in the last years, being *Candida glabrata* the second most common species isolated from invasive candidiasis in North America and central and northern countries in Europe (Pfaller et al., 2012; Asmundsdottir et al., 2013; Lortholary et al., 2014; Milazzo et al., 2014; Castanheira et al., 2016). In Spain, only *C. albicans* and *Candida parapsilosis* are more frequently isolated than *C. glabrata* from patients with fungemia (Guinea et al., 2014).

Echinocandins are the first line antifungal therapy against *C. glabrata* infections, as this species generally presents low susceptibility to azole drugs. Echinocandins non-competitively inhibit the 1–3-β-D-glucan synthase, which is responsible for the synthesis of β-glucan polymers that confer integrity to the fungal cell wall. Nevertheless, an ever-growing number of echinocandin resistant clinical isolates have been reported worldwide in the last years and population studies in the United States and Denmark have shown an increase in echinocandin resistance rate (Alexander et al., 2013; Farmakiotis et al., 2014; Vallabhaneni et al., 2015; Astvad et al., 2017), which is conferred by the presence of point mutations in specific regions (denominated as hot-spots) of *FKS* genes, which encode this enzyme's catalytic subunits (Katiyar et al., 2012).

FKS mutations have been reported to correlate with elevated *in vitro* minimal inhibitory concentrations (MICs) and clinical failure (Alexander et al., 2013), yet an explanation for this increase in echinocandin resistant strains has not been proved. Several possibilities are being studied, such as strains proneness to acquire resistance as an answer to echinocandin exposure (Bordallo-Cardona et al., 2017, 2018a,c; Shields et al., 2019), the existence of hidden reservoirs in the human body of echinocandin resistant *C. glabrata* isolates (Shields et al., 2014; Grau et al., 2015; Jensen et al., 2015; Healey et al., 2017) or molecular mechanisms like *MSH2* mutator phenotype (Delliere et al., 2016; Healey et al., 2016b; Byun et al., 2018; Hou et al., 2018; Singh et al., 2018; Bordallo-Cardona et al., 2019).

Multilocus sequence typing (MLST) and microsatellite length polymorphism (MLP) have been described as typing methodologies with high discrimination power (Dodgson et al., 2003; Foulet et al., 2005; Abbes et al., 2012) for assesing *C. glabrata* strain relatedness.

The objective of the present study was to investigate the antifungal susceptibility, molecular mechanisms of echinocandin resistance and strain relatedness of a series of *C. glabrata* sequentially isolated from patients admitted in two hospitals in Madrid; and also the potential development of echinocandin resistance of susceptible *C. glabrata* isolates collected from 2013 to 2017 after *in vitro* exposure to a range of micafungin and anidulafungin concentrations.

MATERIALS AND METHODS

Yeast Isolates: Patients and Identification

Eighteen *C. glabrata* strains sequentially isolated from five patients admitted in two centers (Hospital Universitario Puerta de Hierro and Hospital Universitario 12 de Octubre, both located in Madrid, Spain) were selected for showing a resistance profile against echinocandins. For two of these patients previous susceptible isolates were also available and analyzed. 89% (16/18) of them were obtained from blood cultures, while the two remaining were isolated from a catheter (one isolate from Patient 1) and from ascitic liquid (one isolate from Patient 2) (**Table 1**). For *in vitro* exposure to micafungin and anidulafungin assays, 14 *C. glabrata* strains collected from 2012 to 2017 from Hospital Universitario Puerta de Hierro, all isolated from blood cultures except one from ascitic liquid, were chosen for being susceptible to echinocandin drugs. All strains were isolated during routine diagnostic procedures at the hospitals and received at the Mycology Reference Laboratory of the Spanish National Centre for Microbiology. Isolates were characterized by morphological features and confirmed as *C. glabrata* by amplification and sequencing of their ITS1-5.8S-ITS2 regions (White et al., 1990). According to the Law 14/2007 of 3rd July on Biomedical Research and the Recommendation CM/Rec(2016)6 of the Committee of Ministers to member States on research on biological materials of human origin, no informed consent was required as no work was performed neither with samples of human origin nor with clinical data. The Mycology Reference Laboratory directly received fungal strains, isolated from the patients as routine diagnostic procedures in the hospital and referred to the National Centre for Microbiology according to routine procedures.

Antifungal Susceptibility Testing

Minimal inhibitory concentrations were determined and confirmed following EUCAST 7.3.1 reference method for yeasts[1]. Antifungals tested were anidulafungin (range 0.007–4 mg/L; Pfizer, Madrid, Spain), micafungin (range 0.004–2 mg/L; Astellas Pharma Inc., Tokyo, Japan), caspofungin (range 0.032–16 mg/L; Merck Sharp & Dohme, United Kingdom) and fluconazole (range 0.125–64 mg/L; Pfizer, Madrid, Spain).

Candida krusei ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains in all test performed. The optical density of the inoculated plates was determined after 24 and 48 h of incubation at 35°C in a humid atmosphere, and strains were classified as susceptible or resistant according to clinical breakpoints established by EUCAST for *C. glabrata*: MIC > 0.032 mg/L for micafungin, MIC > 0.064 mg/L for anidulafungin and MIC > 32 mg/L for fluconazole[2].

DNA Extraction and *FKS* Sequencing

Genomic DNA of all isolates was extracted using the phenol-chloroform method (Tang et al., 1992). Molecular

[1]http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_7_3_1_Yeast_testing__definitive.pdf (accessed April 8, 2019).
[2]http://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals/

TABLE 1 | *Candida glabrata* sequential isolates from five patients admitted in two hospitals in Madrid: isolation dates, anatomic sources, *FKS2* alterations, *in vitro* susceptibility to echinocandins and fluconazole performed by EUCAST and genotyping results by MLST and MLP.

Hospital	Patient	Strain	Isolation date	Anatomic source	*FKS 2* alteration	MIC EUCAST (mg/L)				Typing	
						ANF	CPF	MCF	FLC	MLP* (bp)	MLST (ST)
Hospital	1	CNM-CL9829	14/03/16	Blood culture	–	0.007	0.25	0.007	4	205-243-134-267-	ST3
Universitario Puerta		CNM-CL9835	17/03/16	Catheter	–	0.015	0.25	0.007	4	262-325	
de Hierro, Madrid,		CNM-CL9975	17/04/16	Blood culture	D666H	**0.125**	0.5	0.03	4		
Spain		CNM-CL9877	17/06/16	Blood culture	L664R	**0.125**	1	**0.06**	2		
		CNM-CL9889	07/07/16	Blood culture	L664R	**0.125**	1	**0.125**	2		
	2	CNM-CL9857	21/05/16	Blood culture	–	0.03	0.25	0.007	2	205-243-134-267-	ST3
		CNM-CL9883	23/06/16	Ascitic fluid	D666E	**0.25**	1	**0.06**	4	262-325	
		CNM-CL9897	17/07/16	Blood culture	S663P	**2**	>16	**>2**	32		
	3	CNM-CL9931	21/10/16	Blood culture	ΔF659	**2**	>16	**2**	2	187-251-122-270-	ST2
		CNM-CL9939	14/11/16	Blood culture	ΔF659	**2**	>16	**2**	**64**	265-296	
		CNM-CL9991	23/11/16	Blood culture	ΔF659	**2**	>16	**2**	**>64**		
	4	CNM-CL9932	21/10/16	Blood culture	S663P	**2**	>16	**2**	32	205-243-134-267-	ST3
		CNM-CL9981	12/11/16	Blood culture	S663P	**2**	>16	**>2**	32	262-325	
		CNM-CL9992	24/11/16	Blood culture	S663P	**2**	>16	**>2**	**64**		
Hospital	5	CNM-CL9646	25/02/15	Blood culture	D666N	**0.125**	0.5	**0.06**	**64**	237-236-128-270-	ST149
Universitario 12 de		CNM-CL9775	11/11/15	Blood culture	D666N	**0.125**	0.5	**0.06**	2	262-290	
Octubre, Madrid,		CNM-CL9988	16/11/16	Blood culture	D666N	**0.25**	2	**0.06**	2		
Spain		CNM-CL10047	16/01/17	Blood culture	ΔF659 + D666N	**>4**	16	**2**	**64**		

*CNM-CL, Yeast Collection of the Spanish National Center for Microbiology; ANF, anidulafungin; CPF, caspofungin; MCF, micafungin; FLC, fluconazole; MLP, Microsatellite Length Polymorphism; bp, base pairs; MLST, Multilocus Sequence Typing; ST, sequence type. In bold letters, MIC values that are above the EUCAST clinical breakpoints stablished for those antifungals. *MLP: ERG3-MTI-RPM2-GLM4-GLM5-GLM6.*

mechanisms of echinocandin resistance were studied by amplifying hot-spot regions 1 and 2 of *FKS1* and *FKS2* (Thompson et al., 2008; Zimbeck et al., 2010; Duran-Valle et al., 2012; Bizerra et al., 2013) as previously described with the following modifications: PCR reaction mixtures contained 25 ng of DNA, 0.2 μM of each primer, 0.2 μM of deoxynucleoside triphosphate (Roche, Spain), 5 μL of PCR 10× buffer (Applied Biosystems, Foster City, CA, United States), 2 mM of $MgCl_2$ (Applied Biosystems, Foster City, CA, United States), 5.2% DMSO and 2.5 U of Taq DNA polymerase (Applied Biosystems, Foster City, CA, United States) in a final volume of 50 μL. PCRs conditions used were set as previously described (Duran-Valle et al., 2012), with an annealing temperature of 52°C for hot-spot regions 1 and 2 of *FKS1*, 53°C for hot-spot region 1 of *FKS2* and 58°C for hot-spot region 2 of *FKS2*. PCR amplicons were purified using Illustra ExoProStar 1-step (GE Healthcare Life Science, United Kingdom), and were sequenced after by Sanger method with an ABI3730XLsequencer (Applied Biosystems, Foster City, CA, United States). DNA sequences were analyzed with DNAStar Lasergene 12 software (DNAStar Inc., United States), and queried against *FKS1* (GenBank number CAGL0G01034g) and *FKS2* (GenBank number CAGL0K04037g) sequences of the type strain CBS 138[3].

Assessment of Pooled Reservoir of Mixed Resistant Isolates

For two patients with isolates harboring two different *FKS* mutations, the possible coexistence of diverse populations within the same sample was studied by randomly isolating ten colonies from the original samples sent from the hospital for DNA extraction and *FKS* amplification and sequencing.

Genotyping by MLST

Six housekeeping gene loci (*FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP1*, and *URA3*) were studied for all isolates as previously described (Dodgson et al., 2003), with the following modifications: PCR reaction mixtures contained 25 ng of DNA, 1 μM of each primer, 0.05 μM of deoxynucleoside triphosphate, 5 μL of PCR 10× buffer, 2 mM of $MgCl_2$, and 2.5 U of Taq DNA polymerase in a final volume of 50 μL. 5.2% of DMSO was added only to amplify *NMT1*. PCR conditions were set as described, but with an annealing temperature of 62°C for *FKS* and *URA3*. DNA sequences obtained were compared to *C. glabrata* MLST database[4] to assign an allele number for each locus in order to define a sequence type (ST) or genotype according to the isolates' allelic profile.

Genotyping by MLP

Six short tandem repeat markers described for *C. glabrata* (*ERG3*, *MTI*, *RPM2*, *GLM4*, *GLM5*, and *GLM6*) (Foulet et al., 2005;

[3]http://www.ncbi.nlm.nih.gov/

[4]https://pubmlst.org/cglabrata/

Abbes et al., 2012) were amplified by PCR for all isolates using forward labeled primers as previously described (Foulet et al., 2005; Abbes et al., 2012; Duran-Valle et al., 2012) with the following modifications: *GLM5* was labeled with HEX and *GLM6* with NED fluorochromes. PCR reaction mixtures contained 20 ng of DNA, 0.2 μM of deoxynucleoside triphosphate, 2 μL of PCR 10× buffer, 2.5 mM of $MgCl_2$, 5 U of Taq DNA, 0.25 μM of *RPM2* and *ERG3* primers and 1 μM of *MTI*, *GLM4*, *GLM5*, and *GLM6* primers in a final volume of 20 μL. PCR program used from amplifying all markers consisted on an initial step of 5 min at 95°C, followed by 40 cycles of 95°C for 30 s, 57°C for 1 min and 72°C for 1 min, and an additional step of 7 min at 72°C. Amplicons were sized by capillary electrophoresis using Hi-Di formamide (Applied Biosystems, Foster City, CA, United States) and ROX 500 (Applied Biosystems, Foster City, CA, United States) as internal size standard, as described (Duran-Valle et al., 2012). Reactions were analyzed in duplicate, and fragment sizes were calculated using Peak Scanner software 1.0 (Applied Biosystems, Foster City, CA, United States).

MSH2 Sequencing

MSH2 gene of sequentially isolated strains from patients was amplified and sequenced as previously described (Healey et al., 2016b), and DNA sequences were queried against *MSH2* (Genbank number CAGL0I07733g) sequence of CBS 138 type strain.

In vitro Exposure to Growing Concentrations of Micafungin and Anidulafungin and Analysis of Generated Isolates

The potential development of micafungin and anidulafungin resistance of susceptible *C. glabrata* isolates was studied following a previously reported procedure (Bordallo-Cardona et al., 2017) with some modifications.

Adjusted inocula (2×10^9 to 4×10^9 CFU/mL) from overnight cultures in 7 mL of yeast extract-peptone-dextrose broth of these isolates were cultured on Sabouraud plates containing eight different echinocandin concentrations (from 0.015 to 2 mg/L). Two different sets of experiments were tested at first. Plates at all concentrations were stroked at once with 100 μL of inocula and checked for growth daily for up to 5 days at 35°C. For progressive exposure, the lowest concentration plate was inoculated and checked for growth after 24 h at 35°C. If isolates were observed, they were cultured on a plate containing the next twofold concentration. The procedure was repeated up to highest concentration available.

After exposure, mutant prevention concentration (MPC) and mutant selection window (MSW) were calculated for each isolate as previously reported (Zhao and Drlica, 2001; Drlica, 2003; Bordallo-Cardona et al., 2018c). Briefly, MPCs were defined as the lowest concentration that can totally inhibit fungal growth for each isolate after 5 days of incubation; for calculation purposes, MPC values that exceeded the highest concentration tested were transformed to the next dilution (i.e., if MPC >2 mg/L, it was changed to MPC = 4 mg/L). MSWs were defined as the range of concentrations between the MIC, obtained by EUCAST method, and the MPC for each isolate.

Spontaneous growth frequency was also calculated as the ratio of viable colonies growing on 2 mg/L echinocandin-containing plates and the initial inoculum stroked in them, as some plates containing lower concentrations did not allow the counting of individual colonies.

Micafungin and anidulafungin susceptibility of up to four isolates selected from each growing concentration was performed, and the hot-spot regions 1 and 2 of *FKS1* and *FKS2* genes were sequenced.

Statistical Analysis

All data obtained after *in vitro* exposure to echinocandins assays were compared using the Wilcoxon signed-rank test and the Fisher's exact test (IBM SPSS Statistics for Windows, version 22.0; United States), considering as statistically significant a *P*-value of <0.05.

RESULTS

In vitro Susceptibility and Determination of *FKS* Mutations of Sequentially Isolated Strains

Control strains were within the accepted ranges according to EUCAST QC ranges for all antifungals tested.

As shown in **Table 1**, a wide range of fluconazole MIC values was found among isolates tested. All strains isolated from Patients 3, 4, and 5 were echinocandin resistant, according to EUCAST breakpoints established for anidulafungin and micafungin. For Patients 1 and 2, echinocandin susceptible isolates were also available and analyzed. The first resistant isolate from Patient 1 was only resistant to anidulafungin.

All resistant isolates harbored an echinocandin resistance related mutation at hot-spot region 1 of *FKS2* gene. Mutations found were S663P ($n = 4$), D666N ($n = 4$), ΔF659 ($n = 4$), L664R ($n = 2$), D666E ($n = 1$), and D666H ($n = 1$). No mutations were found at *FKS1* nor at hot-spot region 2 of *FKS2*. Each mutation was related to a different echinocandin resistant profile, as S663P and ΔF659 showed higher MIC values than the rest of the isolates that harbored other mutations.

Isolates With Different *FKS* Mutations Can Be Present in the Same Patient

Resistant isolates with different *FKS2* mutations were found in the same patient in two cases (Patient 1: D666H and L664R; and Patient 2: D666E and S663P). *FKS2* gene sequencing of 10 randomly selected colonies from the original samples of those isolates sent from the hospital led to the same *FKS* mutation in all of them, so the absence of a mixed culture of resistant isolates was confirmed.

MLST and MLP Analysis

Three different STs were differentiated by MLST among the 18 isolates studied (**Table 1**). All isolates from the same patient had the same genotype. ST3 was found in three out of four patients of one hospital. The other two STs found were ST2 and a recently described ST149.

MLP methodology yield comparable results to those of MLST (**Table 1**).

MSH2 Gene Sequencing

All isolates from the same patient harbored the same SNPs in *MSH2* gene. Non-synonymous loss-of-function combined mutations V239L/A942T were found in all isolates coming from one patient, while the rest of isolates did not harbor any non-synonymous mutations in this gene.

Behavior of Echinocandin Susceptible *C. glabrata* Isolates From Patients 1 and 2 When *in vitro* Exposed to Growing Concentrations of Micafungin

In vitro exposure of echinocandin susceptible isolates CNM-CL9829, CNM-CL9835, and CNM-CL9857 from Patients 1 and 2 to micafungin generated *FKS* mutations that conferred echinocandin resistance after less than 48 h of incubation. Progressive exposure allowed the collection of isolates up to 2 mg/L, while colonies in direct exposure grew only up to 0.5 mg/L. All colonies obtained in plates containing 0.015 and 0.03 μg/mL were susceptible to micafungin and anidulafungin and had no mutations in hot-spot regions of *FKS* genes. All isolates growing from 0.06 μg/mL were resistant to both echinocandins and harbored the resistant related mutation S663P in *FKS2* gene (**Figure 1**).

Correlation Between Potential Development of Echinocandin Resistance After *in vitro* Exposure to Micafungin and Anidulafungin of Echinocandin Susceptible Isolates of *C. glabrata* and Their Genotype

Table 2 shows genotyping results by MLST and MLP, echinocandin susceptibility by EUCAST, MPC and MSW after *in vitro* direct exposure to micafungin and anidulafungin of fourteen echinocandin susceptible *C. glabrata* strains collected from 2012 to 2017 from Hospital Universitario Puerta de Hierro, total number of colonies analyzed per isolate (up to 4 colonies per concentration) after 5 days of incubation and *FKS* mutations found.

Four different STs were found among these strains. The most common one was ST3, found in half of the isolates (n = 7), followed by ST19 (29%, n = 4), ST149 (14%, n = 2), and ST6 (7%, n = 1).

Mutant prevention concentration values after anidulafungin and micafungin exposure differ widely between strains (**Table 2**), although no significant differences were found between the geometric mean of MPCs after anidulafungin exposure and after micafungin exposure after 5 days of incubation (2.44 mg/L versus 1.72 mg/L).

Geometric mean of spontaneous growth frequency for micafungin-containing plates had no significant difference with that for anidulafungin-containing plates (8×10^{-8} versus 4.1×10^{-8}; $p = 0.78$), and ranges were very similar for both of them (4.1×10^{-7} to 3.2×10^{-9} in the presence of micafungin and 3.7×10^{-7} to 5.3×10^{-9} in the presence of anidulafungin).

A total number of 296 and 258 isolates were analyzed after micafungin and anidulafungin exposure, respectively (**Figure 2**).

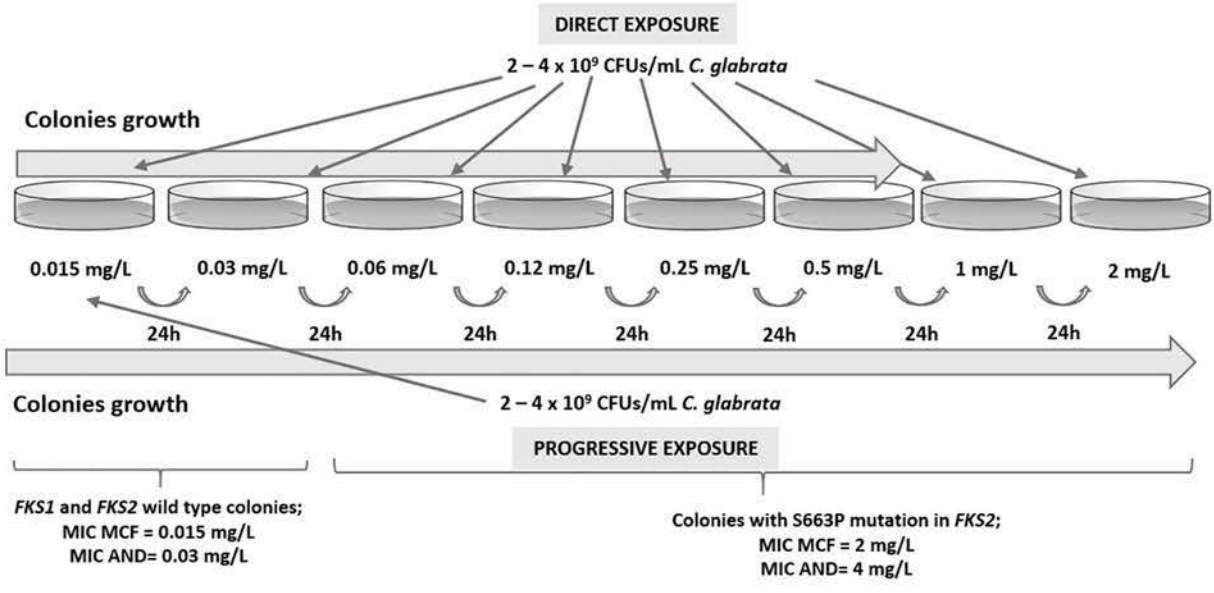


FIGURE 1 | Results from *in vitro* direct and progressive exposure to micafungin of susceptible *C. glabrata* isolates from Patients 1 and 2.

TABLE 2 | Isolation year, micafungin and anidulafungin MIC values, mutant prevention concentration (MPC), mutant selection window (MSW), total number of colonies analyzed and *FKS* alterations found in them after 5 days of incubation for each isolate, and their genotype by MLP and MLST.

Strain	Isolation year	Anidulafungin exposure					Micafungin exposure					MLP* (bp)	MLST (ST)
		MIC (mg/L)	MPC (mg/L)	MSW (mg/L)	Total no. isolates analyzed	*FKS* alteration	MIC (mg/L)	MPC (mg/L)	MSW (mg/L)	Total no. isolates analyzed	*FKS* alteration		
CNM-CL9210	2012	0.03	2	0.03–2	14	–	0.007	4	0.007–4	27	2-S663P	237-236-128-270-262-290	149
CNM-CL9215	2012	0.03	2	0.03–2	13	–	0.007	4	0.007–4	26	2-S663P	237-236-128-270-262-290	149
CNM-CL9269	2012	0.03	4	0.03–4	26	2-S663P	0.007	4	0.007–4	27	2-S663P	205-243-134-267-262-325	3
CNM-CL9332	2013	0.03	4	0.03–4	19	1-S629P; 2-F659Y	0.015	4	0.015–4	27	2-S663P	215-242-134-282-265-298	19
CNM-CL9342	2013	0.06	2	0.06–2	11	–	0.015	0.25	0.015–0.25	13	2-ΔF659	215-242-134-282-265-298	19
CNM-CL9392	2013	0.06	4	0.06–4	26	2-S663P	0.015	4	0.015–4	27	2-S663P	215-242-134-282-265-298	19
CNM-CL9555	2014	0.03	4	0.03–4	26	2-S663P	0.015	4	0.015–4	20	2-ΔF659	205-243-134-267-262-325	3
CNM-CL9571	2014	0.03	2	0.03–2	12	–	0.015	0.5	0.015–0.5	13	–	205-243-134-267-262-325	3
CNM-CL9780	2015	0.03	4	0.03–4	25	2-S663P	0.007	4	0.007–4	20	1-S629P; 2-ΔF659; 2-L662W	215-242-134-282-265-298	19
CNM-CL9785	2015	0.03	0.5	0.03–0.5	13	–	0.007	4	0.007–4	26	2-S663P	205-243-134-267-262-325	3
CNM-CL9862	2016	0.03	0.5	0.03–0.5	10	–	0.007	0.5	0.007–0.5	11	–	230-243-128-270-262-325	6
CNM-CL9906	2016	0.03	4	0.03–4	18	2-S663P	0.007	0.5	0.007–0.5	14	2-ΔF659	205-243-134-267-262-325	3
CNM-CL10190	2017	0.03	4	0.03–4	16	2-ΔF659	0.007	0.5	0.007–0.5	19	–	205-243-134-267-262-325	3
CNM-CL10194	2017	0.03	4	0.03–4	29	2-S663P	0.007	2	0.007–2	26	2-ΔF659; 2-S663P	205-243-134-267-262-325	3
Global GM		0.03	2.44		Total = 258		0.01	1.72		Total = 296			
Range		0.03–0.06	0.5–>2	0.03–>2			0.007–0.015	0.25–>2	0.007–>2				

*GM, geometric mean. FKS alteration: (1) mutation in FKS1 gene; (2) mutation in FKS2 gene. *MLP: ERG3-MTI-RPM2-GLM4-GLM5-GLM6.*

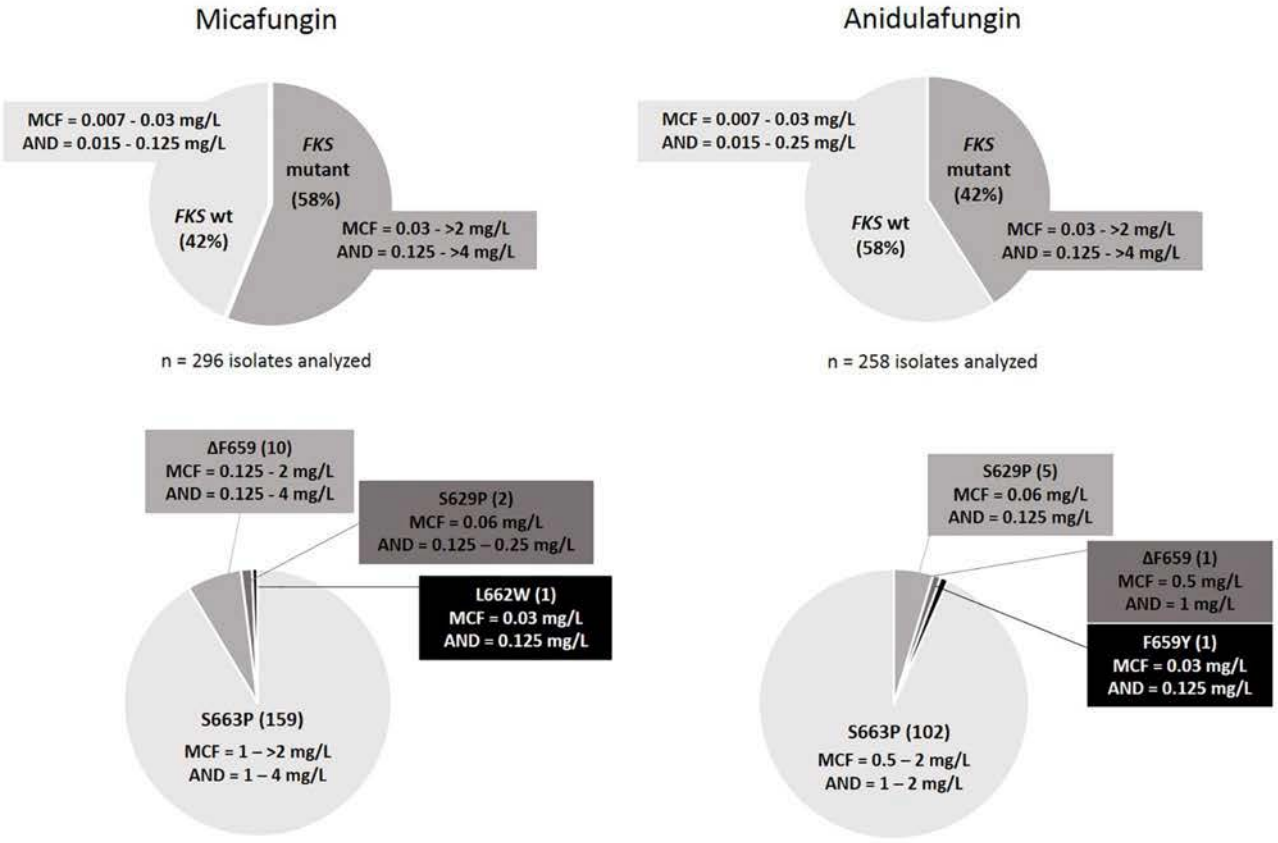


FIGURE 2 | *FKS* mutant and wild-type colony rates with their MIC ranges **(Top)**, and *FKS* mutations found with range of MIC values per mutation **(Bottom)** after *in vitro* exposure to micafungin and anidulafungin.

The lowest concentrations of these antifungals in which resistant colonies harboring *FKS* mutations were found were 0.06 mg/L and 0.12 mg/L, respectively, while they appeared up to the highest concentration tested in both cases, 2 mg/L. 58% of the isolates yielded in micafungin plates harbored *FKS* mutations related to echinocandin resistance, which was a significantly higher number than the 42% of the isolates that did so after anidulafungin exposure ($p < 0.0001$). The most prevalent mutation found was S663P (no significant differences were found among the frequency of occurrence of this substitution after exposure to both echinocandins: $n = 159$ from MCF plates and $n = 102$ from AND plates; 92.4 and 93.5% of *FKS* mutant isolates generated, $p = 0.8147$), followed by ΔF659 (its appearance rate was close to be significantly different after exposure to both antifungals: $n = 10$ from MCF plates and $n = 1$ from AND plates, $p = 0.0552$), F659Y ($n = 1$ from AND plates) and L662W ($n = 1$ from MCF plates) in hot-spot 1 from *FKS2* gene and S629P ($n = 5$ from AND plates and $n = 2$ from MCF plates) in hot-spot 1 from *FKS1* gene. All of these isolates were echinocandin resistant by EUCAST, and their *FKS* mutations and resistance were stable and reproducible after subculturing on antifungal-free plates. The same isolate could develop different *FKS* mutations after exposure. Out of the total number of isolates analyzed after micafungin and anidulafungin exposure, 1 and 19%, respectively, were resistant to micafungin and/or anidulafungin but did not carry any *FKS* mutation.

DISCUSSION

The increasing number of *C. glabrata* clinical isolates reported showing decreased susceptibility for echinocandins is a growing concern. Recent studies indicate that echinocandin resistance rates among *C. glabrata* clinical isolates have risen worldwide (Kiraz et al., 2010; Bourgeois et al., 2014; Guinea et al., 2014; Orasch et al., 2014; Klotz et al., 2016; Chapman et al., 2017; Hou et al., 2017). Resistance has been reported to easily develop *in vitro* (Bordallo-Cardona et al., 2017, 2018a,c; Shields et al., 2019) and in patients after echinocandin exposure (Dannaoui et al., 2012; Shields et al., 2012; Alexander et al., 2013; Bizerra et al., 2014; Sasso et al., 2017), being conferred by the presence of point mutations in hot-spot regions of *FKS1* and *FKS2* genes (Castanheira et al., 2014; Pham et al., 2014) that have been associated with higher MICs and therapeutic failure (Shields et al., 2012). Our study provides a new insight into the development of echinocandin resistance of *C. glabrata* strains both sequentially isolated from several patients and after

in vitro exposure to growing concentrations of micafungin and anidulafungin.

FKS Mutations

All isolates from five patients admitted in two Spanish hospitals that were resistant to at least one echinocandin carried mutations in *FKS2* gene (Zimbeck et al., 2010; Castanheira et al., 2014; Locke et al., 2016). The most common ones found were S663P and ΔF659, as in previous studies (Zimbeck et al., 2010; Beyda et al., 2014; Castanheira et al., 2014). Some isolates carried less frequently found substitutions L664R, D666E, and D666N (Castanheira et al., 2014; Prigent et al., 2017; Bordallo-Cardona et al., 2018b) and, although it has been found after *in vitro* exposure to the novel antifungal rezafungin (Locke et al., 2016), we believe that this is the first time that D666H mutation is reported in a clinical isolate.

From two of the studied patients, echinocandin susceptible isolates without mutations in *FKS* genes were detected before the isolation of the resistant ones, indicating that echinocandin resistance could had been acquired due to therapy, as previously reported in other studies (Cleary et al., 2008; Thompson et al., 2008; Ishikawa et al., 2010; Costa-de-Oliveira et al., 2011; Dannaoui et al., 2012; Shields et al., 2012; Alexander et al., 2013; Lewis et al., 2013; Beyda et al., 2014). All isolates with *FKS* mutations had MIC values above the clinical breakpoints established by EUCAST both for anidulafungin and for micafungin, except one isolate that was resistant to anidulafungin but not to micafungin despite harboring an *FKS* mutation (D666H). This case has been described before (Arendrup et al., 2012; Shields et al., 2012; Jensen et al., 2015; Bordallo-Cardona et al., 2017) but should be taken into account when testing only one echinocandin to detect resistance.

Glucan synthase enzyme sensitivity has been described to be affected by *FKS* mutations on different degrees (Garcia-Effron et al., 2009). In this study, S663P and ΔF659 were associated with higher echinocandin MIC values, as previously reported (Arendrup and Perlin, 2014). Isolates harboring D666H, D666N, D666E, and L664R substitutions showed lower MIC values and conferred weaker echinocandin resistance. Patient 5 simultaneously carried ΔF659 and D666N substitutions in CNM-CL10047 isolate, which had high echinocandin MIC values. A double mutation in these two amino acid positions has been previously found (F659S and D666E) (Prigent et al., 2017), but we are reporting the association of ΔF659 and D666N for the first time.

As we found sequential isolates from the same patient carrying different *FKS* mutations, we studied the possible coexistence of a mixed population of resistant isolates within the same sample, confirming its absence. Nevertheless, this experiment had some limitations, as the original samples sent from the hospital could be an already isolated colony. Serial *C. glabrata* isolates from the same patient showing different antifungal resistance profiles due to the selective pressure induced by changes in antifungal treatment have been previously reported (Cho et al., 2015; Imbert et al., 2016).

Genotyping

Strain relatedness was determined by MLST and MLP, which led to similar results evidencing that both methodologies are equally useful for genotyping purposes, as previously reported (Brisse et al., 2009). Nevertheless, in other investigations a higher number of genotypes were obtained by MLP than by MLST (Hou et al., 2018; Bordallo-Cardona et al., 2019). Isolates from the same patient seemed to have a clonal origin by using these two typing techniques, although the use of next generation sequencing in order to compare their genomes would be necessary to prove if they are isogenic. The most frequent genotype among these patients was ST3, which has been reported as one of the most prevalent STs worldwide (Dodgson et al., 2003; Lott et al., 2012; Hou et al., 2017; Biswas et al., 2018; Byun et al., 2018; Mushi et al., 2018). No association between echinocandin resistance development and genetic type was found, which was in agreement with other reports (Dodgson et al., 2003; Abbes et al., 2012). A recent study has found a link between certain STs and reduced susceptibility to fluconazole (Mushi et al., 2018), something that we did not see when performing fluconazole susceptibility to all the isolates. Genotypes were also independent of the anatomic source of the isolates (Lin et al., 2007).

MSH2

MSH2 mismatch repair gene involved in DNA repair has been described as a promoter of the acquisition of resistance to antifungals of *C. glabrata* (Healey et al., 2016b), but in this study all isolates belonging to the same patient had the same *MSH2* gene sequence, regardless their susceptibility pattern to echinocandins. Echinocandin susceptible isolates with and without *MSH2* mutations yielded echinocandin resistant isolates with *FKS* mutations. Also, *FKS* mutant isolates for three patients had a wild-type *MSH2* gene. Altogether, this supports that echinocandin resistance cannot be explained by *MSH2* mutator phenotype, as previously reported (Delliere et al., 2016; Healey et al., 2016a; Biswas et al., 2018; Byun et al., 2018; Hou et al., 2018; Singh et al., 2018; Bordallo-Cardona et al., 2019). Likewise, no clear association between *MSH2* sequence and increased fluconazole resistance or genotypes was detected on these isolates either (Biswas et al., 2018; Bordallo-Cardona et al., 2019), although a correlation with specific genetic types was previously described (Delliere et al., 2016; Byun et al., 2018; Hou et al., 2018). These results confirm that *MSH2* substitutions may be constitutive variations from the gene rather than resistance-related or genotype-related mutations (Carrete et al., 2019). Still, it cannot be dismissed that *MSH2* may just be one of a higher number of *C. glabrata* genes involved in mismatch repair mechanisms influencing on the development of antifungal resistance, as it happens for other yeasts (Legrand et al., 2007; Boyce et al., 2017).

In vitro Resistance Development

It is of interest to gain a deeper understanding of how *C. glabrata* isolates behave when *in vitro* exposed to echinocandins, and correlate these results with clinical findings or to anticipate to possible clinical cases. Susceptible isolates from Patients 1 and 2

were *in vitro* exposed to a range of growing concentrations of micafungin, obtaining echinocandin resistant and *FKS* mutant colonies after exposure to the lowest micafungin concentration considered resistant by EUCAST (0.06 mg/L) in less than 48 h of incubation. These results are in agreement with those previously reported (Bordallo-Cardona et al., 2017, 2018a). In our study, *FKS* mutations found after *in vitro* micafungin exposure were different from those isolated from the patients, something that evinces *C. glabrata*'s facility to develop resistance and to acquire different mutations under drug pressure.

It has been hypothesized that certain STs may have a better competence than others to acquire resistance through antifungal exposure at different frequencies (Lott et al., 2012; Hou et al., 2017). Therefore, we aimed to compare the potential development of *in vitro* echinocandin resistance of a set of susceptible *C. glabrata* isolates collected in 6 years from one hospital after exposure to a range of micafungin and anidulafungin concentrations, and to correlate this with their genotype. MLST and MLP revealed four different STs among these fourteen isolates. Not all strains isolated on the same year showed the same ST in all cases and no clear trend on the evolution of *C. glabrata* population in this center was found. No statistical differences were found among MPC values and spontaneous growth frequencies for both agents, something in concordance with previous results published (Bordallo-Cardona et al., 2018c). Results in our study varied between strains, being echinocandin resistant colonies harboring *FKS* mutations isolated from plates containing the first micafungin and anidulafungin concentrations considered as resistant by EUCAST. Nevertheless, a significantly higher *FKS* mutant rate was found after micafungin exposure than after anidulafungin selection.

In vitro micafungin and anidulafungin exposure allowed the selection of different *FKS* mutations grown at different concentrations and even at the same one for some strains. S663P was the most frequently found mutation following exposure to both echinocandins, although in previous *in vitro* selection studies ΔF659 was the most prevalent one (Bordallo-Cardona et al., 2018c; Shields et al., 2019),which was the second most common one in our study.

We found 53 colonies with MICs onefold or twofold above the established breakpoint for anidulafungin that did not carry any *FKS* mutations. This finding was especially detected after exposure to anidulafungin, as 50 colonies out of 258 isolated were anidulafungin resistant but *FKS* wild-type, while only three out of 296 colonies analyzed after micafungin exposure had this categorization. This similar case and also echinocandin susceptible isolates harboring *FKS* substitutions have been previously found, both in clinical isolates and after *in vitro* exposure (Castanheira et al., 2014; Pham et al., 2014; Shields et al., 2015, 2019; Locke et al., 2016). Precisely, anidulafungin resistance, according to EUCAST breakpoints, was vastly sensitive (100%; all colonies that were *FKS* mutant showed a MIC value above its clinical breakpoint for EUCAST) but showed lower specificity (80.6%; as 53 colonies out of a total of 273 that were *FKS* wild-type showed MICs above the EUCAST breakpoint) than in another study (Shields et al., 2019) for the identification of *in vitro* selected *FKS* mutant colonies. Micafungin showed higher sensitivity (99.3%; 279 out of 281 isolates that harbored *FKS* mutations were micafungin resistant according to EUCAST breakpoint) and similar specificity (99.8%; only one *FKS* wild-type colony was micafungin resistant) to those reported in that study. Taken together, this confirmed that both antifungals are suitable echinocandin resistance markers for *C. glabrata*, unlike caspofungin (Shields et al., 2013; Eschenauer et al., 2014).

We concluded that the development of echinocandin resistance in *C. glabrata* after *in vitro* exposure to micafungin and anidulafungin has no association with specific genotypes. Results obtained in all these *in vitro* studies on how echinocandin susceptible *C. glabrata* strains are able to develop resistance after exposure to low echinocandin concentrations supports the fact that *C. glabrata* is able to colonize and survive in certain reservoirs of the human body, such as the abdomen (Shields et al., 2014), the peritoneum (Grau et al., 2015), the gastrointestinal tract (Healey et al., 2017) or the mucosal surfaces (Jensen et al., 2015), due to long-term penetration of echinocandins in lower concentrations than those that prevent resistance acquisition. Since sometimes this required dose could lead to toxicity, the use of newly developed drugs that target the 1–3-β-D-glucan synthase, such as ibrexafungerp (Scynexis, Jersey City, NJ, United States), which has shown potential effectiveness against echinocandin resistant *C. glabrata* isolates (Wiederhold et al., 2018), or rezafungin (Cidara, San Diego, CA, United States), which has an extended-interval administration due to its improved pharmacodynamics (Sandison et al., 2017) could help to overcome echinocandin resistance. Nevertheless, *C. glabrata* strains have been *in vitro* exposed to both drugs, leading to similar results than those against other echinocandins (Locke et al., 2016; Jimenez-Ortigosa et al., 2017), so further research for new compounds that have a role on novel mechanisms of action is assured.

In summary, the present study analyzes the relevance of certain hypothesis raised on the increase of echinocandin resistance in *C. glabrata*, and sheds light on several important aspects related to its acquisition and development, both in genetically related serial isolates from the same patient and after *in vitro* exposure to micafungin and anidulafungin.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

AA-I and OR-M conceived and designed the experiments. OR-M, PN-R, LB-M, GM-C, and LL-P performed the experimental work. OR-M and AA-I analyzed the data. IS-R and AP-A provided clinical isolates. OZ provided materials. OR-M and AA-I wrote the manuscript. All authors read, edited, and approved the final version of the manuscript.

FUNDING

This work was supported by the Fondo de Investigación Sanitaria (Grant FI14CIII/00025 to OR-M and research projects PI13/02145 and PI16CIII/00035 to AA-I), and also supported by the Plan Nacional de I+D+i 2013–2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Ciencia, Innovación y Universidades, Spanish Network for Research in Infectious Diseases (REIPI RD16CIII/0004/0003) – co-financed by the European Development Regional Fund "A way to achieve Europe," Operative Program Intelligent Growth 2014–2020.

REFERENCES

Abbes, S., Sellami, H., Sellami, A., Hadrich, I., Amouri, I., Mahfoudh, N., et al. (2012). *Candida glabrata* strain relatedness by new microsatellite markers. *Eur. J. Clin. Microbiol. Infect. Dis.* 31, 83–91. doi: 10.1007/s10096-011-1280-4

Alexander, B. D., Johnson, M. D., Pfeiffer, C. D., Jimenez-Ortigosa, C., Catania, J., Booker, R., et al. (2013). Increasing echinocandin resistance in *Candida glabrata*: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. *Clin. Infect. Dis.* 56, 1724–1732. doi: 10.1093/cid/cit136

Arendrup, M. C., and Perlin, D. S. (2014). Echinocandin resistance: an emerging clinical problem? *Curr. Opin. Infect. Dis.* 27, 484–492. doi: 10.1097/QCO.0000000000000111

Arendrup, M. C., Perlin, D. S., Jensen, R. H., Howard, S. J., Goodwin, J., and Hope, W. (2012). Differential in vivo activities of anidulafungin, caspofungin, and micafungin against *Candida glabrata* isolates with and without FKS resistance mutations. *Antimicrob. Agents Chemother.* 56, 2435–2442. doi: 10.1128/AAC.06369-11

Asmundsdottir, L. R., Erlendsdottir, H., and Gottfredsson, M. (2013). Nationwide study of candidemia, antifungal use, and antifungal drug resistance in Iceland, 2000 to 2011. *J. Clin. Microbiol.* 51, 841–848. doi: 10.1128/JCM.02566-12

Astvad, K. M. T., Johansen, H. K., Roder, B. L., Rosenvinge, F. S., Knudsen, J. D., Lemming, L., et al. (2017). Update from 12-year nationwide fungaemia surveillance: increasing intrinsic and acquired resistance causes concern. *J. Clin. Microbiol.* 56:e1564-17. doi: 10.1128/JCM.01564-17

Beyda, N. D., John, J., Kilic, A., Alam, M. J., Lasco, T. M., and Garey, K. W. (2014). FKS mutant *Candida glabrata*: risk factors and outcomes in patients with candidemia. *Clin. Infect. Dis.* 59, 819–825. doi: 10.1093/cid/ciu407

Biswas, C., Marcelino, V. R., Van Hal, S., Halliday, C., Martinez, E., Wang, Q., et al. (2018). Whole genome sequencing of Australian *Candida glabrata* isolates reveals genetic diversity and novel sequence types. *Front. Microbiol.* 9:2946. doi: 10.3389/fmicb.2018.02946

Bizerra, F. C., Jimenez-Ortigosa, C., Queiroz-Telles, F., Perlin, D. S., and Lopes Colombo, A. (2013). "A Point Mutation in the FKS2 gene of *Candida glabrata* confers reduced sensitivity to echinocandin drugs," in *Poster P991 at the European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)*, Berlin.

Bizerra, F. C., Jimenez-Ortigosa, C., Souza, A. C., Breda, G. L., Queiroz-Telles, F., Perlin, D. S., et al. (2014). Breakthrough candidemia due to multidrug-resistant *Candida glabrata* during prophylaxis with a low dose of micafungin. *Antimicrob. Agents Chemother.* 58, 2438–2440. doi: 10.1128/AAC.02189-13

Bordallo-Cardona, M. A., Agnelli, C., Gomez-Nunez, A., Sanchez-Carrillo, C., Bouza, E., Munoz, P., et al. (2019). MSH2 gene point mutations are not antifungal resistance markers in *Candida glabrata*. *Antimicrob. Agents Chemother.* 63:e1876-18. doi: 10.1128/AAC.01876-18

Bordallo-Cardona, M. A., Escribano, P., de la Pedrosa, E. G., Marcos-Zambrano, L. J., Canton, R., Bouza, E., et al. (2017). In vitro exposure to increasing micafungin concentrations easily promotes echinocandin resistance in *Candida glabrata* isolates. *Antimicrob. Agents Chemother.* 61:e1542-16. doi: 10.1128/AAC.01542-16

Bordallo-Cardona, M. A., Escribano, P., Marcos-Zambrano, L. J., Diaz-Garcia, J., de la Pedrosa, E. G., Canton, R., et al. (2018a). Low and constant micafungin concentrations may be sufficient to lead to resistance mutations in FKS2 gene of *Candida glabrata*. *Med. Mycol.* 56, 903–906. doi: 10.1093/mmy/myx124

Bordallo-Cardona, M. A., Marcos-Zambrano, L. J., Sanchez-Carrillo, C., Bouza, E., Munoz, P., Escribano, P., et al. (2018b). Resistance to echinocandins in candida can be detected by performing the etest directly on blood culture samples. *Antimicrob. Agents Chemother.* 62:e162-18. doi: 10.1128/AAC.00162-18

Bordallo-Cardona, M. A., Marcos-Zambrano, L. J., Sanchez-Carrillo, C., de la Pedrosa, E. G. G., Canton, R., Bouza, E., et al. (2018c). Mutant prevention concentration and mutant selection window of micafungin and anidulafungin in clinical *Candida glabrata* isolates. *Antimicrob. Agents Chemother.* 62:e1982-17. doi: 10.1128/AAC.01982-17

Bourgeois, N., Laurens, C., Bertout, S., Balard, Y., Krasteva, D., Rispail, P., et al. (2014). Assessment of caspofungin susceptibility of *Candida glabrata* by the Etest(R), CLSI, and EUCAST methods, and detection of FKS1 and FKS2 mutations. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 1247–1252. doi: 10.1007/s10096-014-2069-z

Boyce, K. J., Wang, Y., Verma, S., Shakya, V. P. S., Xue, C., and Idnurm, A. (2017). Mismatch repair of DNA replication errors contributes to microevolution in the pathogenic fungus Cryptococcus neoformans. *mBio* 8:e595-17. doi: 10.1128/mBio.00595-17

Brisse, S., Pannier, C., Angoulvant, A., de Meeus, T., Diancourt, L., Faure, O., et al. (2009). Uneven distribution of mating types among genotypes of *Candida glabrata* isolates from clinical samples. *Eukaryot. Cell* 8, 287–295. doi: 10.1128/EC.00215-08

Byun, S. A., Won, E. J., Kim, M. N., Lee, W. G., Lee, K., Lee, H. S., et al. (2018). Multilocus Sequence Typing (MLST) genotypes of *Candida glabrata* bloodstream isolates in Korea: association with antifungal resistance, mutations in mismatch repair gene (MSH2), and clinical outcomes. *Front. Microbiol.* 9:1523. doi: 10.3389/fmicb.2018.01523

Carrete, L., Ksiezopolska, E., Gomez-Molero, E., Angoulvant, A., Bader, O., Fairhead, C., et al. (2019). Genome comparisons of *Candida glabrata* serial clinical isolates reveal patterns of genetic variation in infecting clonal populations. *Front. Microbiol.* 10:112. doi: 10.3389/fmicb.2019.00112

Castanheira, M., Messer, S. A., Rhomberg, P. R., and Pfaller, M. A. (2016). Antifungal susceptibility patterns of a global collection of fungal isolates: results of the SENTRY antifungal surveillance program (2013). *Diagn. Microbiol. Infect. Dis.* 85, 200–204. doi: 10.1016/j.diagmicrobio.2016.02.009

Castanheira, M., Woosley, L. N., Messer, S. A., Diekema, D. J., Jones, R. N., and Pfaller, M. A. (2014). Frequency of FKS mutations among *Candida glabrata* isolates from a 10-year global collection of bloodstream infection isolates. *Antimicrob. Agents Chemother.* 58, 577–580. doi: 10.1128/AAC.01674-13

Chapman, B., Slavin, M., Marriott, D., Halliday, C., Kidd, S., Arthur, I., et al. (2017). Changing epidemiology of candidaemia in Australia. *J. Antimicrob. Chemother.* 72, 1103–1108. doi: 10.1093/jac/dkw422

Cho, E. J., Shin, J. H., Kim, S. H., Kim, H. K., Park, J. S., Sung, H., et al. (2015). Emergence of multiple resistance profiles involving azoles, echinocandins and amphotericin B in *Candida glabrata* isolates from a neutropenia patient with prolonged fungaemia. *J. Antimicrob. Chemother.* 70, 1268–1270. doi: 10.1093/jac/dku518

Cleary, J. D., Garcia-Effron, G., Chapman, S. W., and Perlin, D. S. (2008). Reduced *Candida glabrata* susceptibility secondary to an FKS1 mutation developed during candidemia treatment. *Antimicrob. Agents Chemother.* 52, 2263–2265. doi: 10.1128/AAC.01568-07

Costa-de-Oliveira, S., Marcos Miranda, I., Silva, R. M., Pinto, E. S. A., Rocha, R., Amorim, A., et al. (2011). FKS2 mutations associated with decreased echinocandin susceptibility of *Candida glabrata* following anidulafungin therapy. *Antimicrob. Agents Chemother.* 55, 1312–1314. doi: 10.1128/AAC.00589-10

Dannaoui, E., Desnos-Ollivier, M., Garcia-Hermoso, D., Grenouillet, F., Cassaing, S., Baixench, M. T., et al. (2012). Candida spp. with acquired echinocandin resistance, France, 2004-2010. *Emerg. Infect. Dis.* 18, 86–90. doi: 10.3201/eid1801.110556

Delliere, S., Healey, K., Gits-Muselli, M., Carrara, B., Barbaro, A., Guigue, N., et al. (2016). Fluconazole and echinocandin resistance of *Candida glabrata* correlates better with antifungal drug exposure rather than with MSH2 mutator

genotype in a French cohort of patients harboring low rates of resistance. *Front. Microbiol.* 7:2038. doi: 10.3389/fmicb.2016.02038

Dodgson, A. R., Pujol, C., Denning, D. W., Soll, D. R., and Fox, A. J. (2003). Multilocus sequence typing of *Candida glabrata* reveals geographically enriched clades. *J. Clin. Microbiol.* 41, 5709–5717. doi: 10.1128/jcm.41.12.5709-5717.2003

Drlica, K. (2003). The mutant selection window and antimicrobial resistance. *J. Antimicrob. Chemother.* 52, 11–17. doi: 10.1093/jac/dkg269

Duran-Valle, M. T., Gago, S., Gomez-Lopez, A., Cuenca-Estrella, M., Jimenez Diez-Canseco, L., Gomez-Garces, J. L., et al. (2012). Recurrent episodes of candidemia due to *Candida glabrata* with a mutation in hot spot 1 of the FKS2 gene developed after prolonged therapy with caspofungin. *Antimicrob. Agents Chemother.* 56, 3417–3419. doi: 10.1128/AAC.06100-11

Eschenauer, G. A., Nguyen, M. H., Shoham, S., Vazquez, J. A., Morris, A. J., Pasculle, W. A., et al. (2014). Real-world experience with echinocandin MICs against candida species in a multicenter study of hospitals that routinely perform susceptibility testing of bloodstream isolates. *Antimicrob. Agents Chemother.* 58, 1897–1906. doi: 10.1128/AAC.02163-13

Farmakiotis, D., Tarrand, J. J., and Kontoyiannis, D. P. (2014). Drug-resistant *Candida glabrata* infection in cancer patients. *Emerg. Infect. Dis.* 20, 1833–1840. doi: 10.3201/eid2011.140685

Foulet, F., Nicolas, N., Eloy, O., Botterel, F., Gantier, J. C., Costa, J. M., et al. (2005). Microsatellite marker analysis as a typing system for *Candida glabrata*. *J. Clin. Microbiol.* 43, 4574–4579. doi: 10.1128/JCM.43.9.4574-4579.2005

Garcia-Effron, G., Lee, S., Park, S., Cleary, J. D., and Perlin, D. S. (2009). Effect of *Candida glabrata* FKS1 and FKS2 mutations on echinocandin sensitivity and kinetics of 1,3-beta-D-glucan synthase: implication for the existing susceptibility breakpoint. *Antimicrob. Agents Chemother.* 53, 3690–3699. doi: 10.1128/AAC.00443-09

Grau, S., Luque, S., Campillo, N., Samso, E., Rodriguez, U., Garcia-Bernedo, C. A., et al. (2015). Plasma and peritoneal fluid population pharmacokinetics of micafungin in post-surgical patients with severe peritonitis. *J. Antimicrob. Chemother.* 70, 2854–2861. doi: 10.1093/jac/dkv173

Guinea, J., Zaragoza, O., Escribano, P., Martin-Mazuelos, E., Peman, J., Sanchez-Reus, F., et al. (2014). Molecular identification and antifungal susceptibility of yeast isolates causing fungemia collected in a population-based study in Spain in 2010 and 2011. *Antimicrob. Agents Chemother.* 58, 1529–1537. doi: 10.1128/AAC.02155-13

Healey, K. R., Jimenez Ortigosa, C., Shor, E., and Perlin, D. S. (2016a). Genetic drivers of multidrug resistance in *Candida glabrata*. *Front. Microbiol.* 7:1995. doi: 10.3389/fmicb.2016.01995

Healey, K. R., Zhao, Y., Perez, W. B., Lockhart, S. R., Sobel, J. D., Farmakiotis, D., et al. (2016b). Prevalent mutator genotype identified in fungal pathogen *Candida glabrata* promotes multi-drug resistance. *Nat. Commun.* 7:11128. doi: 10.1038/ncomms11128

Healey, K. R., Nagasaki, Y., Zimmerman, M., Kordalewska, M., Park, S., Zhao, Y., et al. (2017). The gastrointestinal tract is a major source of echinocandin drug resistance in a murine model of *Candida glabrata* colonization and systemic dissemination. *Antimicrob. Agents Chemother.* 61, e1412–e1417. doi: 10.1128/AAC.01412-17

Hou, X., Xiao, M., Chen, S. C., Kong, F., Wang, H., Chu, Y. Z., et al. (2017). Molecular epidemiology and antifungal susceptibility of *Candida glabrata* in China (August 2009 to July 2014): a multi-center study. *Front. Microbiol.* 8:880. doi: 10.3389/fmicb.2017.00880

Hou, X., Xiao, M., Wang, H., Yu, S. Y., Zhang, G., Zhao, Y., et al. (2018). Profiling of PDR1 and MSH2 in *Candida glabrata* bloodstream isolates from a multicenter study in China. *Antimicrob. Agents Chemother.* 62:e153-18. doi: 10.1128/AAC.00153-18

Imbert, S., Castain, L., Pons, A., Jacob, S., Meyer, I., Palous, M., et al. (2016). Discontinuation of echinocandin and azole treatments led to the disappearance of an FKS alteration but not azole resistance during clonal *Candida glabrata* persistent candidaemia. *Clin. Microbiol. Infect.* 22, 891.e5–891.e8. doi: 10.1016/j.cmi.2016.07.025

Ishikawa, T., Takata, T., Akamatsu, S., Hatakenaka, K., Matsumoto, S., Maki, K., et al. (2010). Analysis of *Candida glabrata* strains with reduced sensitivity to micafungin in vitro isolated from a patient with persistent candidemia. *Jpn. J. Infect. Dis.* 63, 332–337.

Jensen, R. H., Johansen, H. K., Soes, L. M., Lemming, L. E., Rosenvinge, F. S., Nielsen, L., et al. (2015). Posttreatment antifungal resistance among colonizing candida isolates in candidemia patients: results from a systematic multicenter study. *Antimicrob. Agents Chemother.* 60, 1500–1508. doi: 10.1128/AAC.01763-15

Jimenez-Ortigosa, C., Perez, W. B., Angulo, D., Borroto-Esoda, K., and Perlin, D. S. (2017). De novo acquisition of resistance to SCY-078 in *Candida glabrata* involves FKS mutations that both overlap and are distinct from those conferring echinocandin resistance. *Antimicrob. Agents Chemother.* 61:e833-17. doi: 10.1128/AAC.00833-17

Katiyar, S. K., Alastruey-Izquierdo, A., Healey, K. R., Johnson, M. E., Perlin, D. S., and Edlind, T. D. (2012). Fks1 and Fks2 are functionally redundant but differentially regulated in *Candida glabrata*: implications for echinocandin resistance. *Antimicrob. Agents Chemother.* 56, 6304–6309. doi: 10.1128/AAC.00813-12

Kiraz, N., Dag, I., Oz, Y., Yamac, M., Kiremitci, A., and Kasifoglu, N. (2010). Correlation between broth microdilution and disk diffusion methods for antifungal susceptibility testing of caspofungin, voriconazole, amphotericin B, itraconazole and fluconazole against *Candida glabrata*. *J. Microbiol. Methods* 82, 136–140. doi: 10.1016/j.mimet.2010.05.002

Klotz, U., Schmidt, D., Willinger, B., Steinmann, E., Buer, J., Rath, P. M., et al. (2016). Echinocandin resistance and population structure of invasive *Candida glabrata* isolates from two university hospitals in Germany and Austria. *Mycoses* 59, 312–318. doi: 10.1111/myc.12472

Legrand, M., Chan, C. L., Jauert, P. A., and Kirkpatrick, D. T. (2007). Role of DNA mismatch repair and double-strand break repair in genome stability and antifungal drug resistance in *Candida albicans*. *Eukaryot. Cell* 6, 2194–2205. doi: 10.1128/EC.00299-07

Lewis, J. S. II, Wiederhold, N. P., Wickes, B. L., Patterson, T. F., and Jorgensen, J. H. (2013). Rapid emergence of echinocandin resistance in *Candida glabrata* resulting in clinical and microbiologic failure. *Antimicrob. Agents Chemother.* 57, 4559–4561. doi: 10.1128/AAC.01144-13

Lin, C. Y., Chen, Y. C., Lo, H. J., Chen, K. W., and Li, S. Y. (2007). Assessment of *Candida glabrata* strain relatedness by pulsed-field gel electrophoresis and multilocus sequence typing. *J. Clin. Microbiol.* 45, 2452–2459. doi: 10.1128/JCM.00699-07

Locke, J. B., Almaguer, A. L., Zuill, D. E., and Bartizal, K. (2016). Characterization of in vitro resistance development to the novel echinocandin CD101 in Candida species. *Antimicrob. Agents Chemother.* 60, 6100–6107. doi: 10.1128/AAC.00620-16

Lortholary, O., Renaudat, C., Sitbon, K., Madec, Y., Denoeud-Ndam, L., Wolff, M., et al. (2014). Worrisome trends in incidence and mortality of candidemia in intensive care units (Paris area, 2002-2010). *Intensive Care Med.* 40, 1303–1312. doi: 10.1007/s00134-014-3408-3

Lott, T. J., Frade, J. P., Lyon, G. M., Iqbal, N., and Lockhart, S. R. (2012). Bloodstream and non-invasive isolates of *Candida glabrata* have similar population structures and fluconazole susceptibilities. *Med. Mycol.* 50, 136–142. doi: 10.3109/13693786.2011.592153

Milazzo, L., Peri, A. M., Mazzali, C., Grande, R., Cazzani, C., Ricaboni, D., et al. (2014). Candidaemia observed at a university hospital in milan (northern Italy) and review of published studies from 2010 to 2014. *Mycopathologia* 178, 227–241. doi: 10.1007/s11046-014-9786-9

Mushi, M. F., Gross, U., Mshana, S. E., and Bader, O. (2018). High diversity of *Candida glabrata* in a tertiary hospital-Mwanza, Tanzania. *Med. Mycol.* doi: 10.1093/mmy/myy151 [Epub ahead of print].

Orasch, C., Marchetti, O., Garbino, J., Schrenzel, J., Zimmerli, S., Muhlethaler, K., et al. (2014). Candida species distribution and antifungal susceptibility testing according to european committee on antimicrobial susceptibility testing and new vs. old clinical and laboratory standards institute clinical breakpoints: a 6-year prospective candidaemia survey from the fungal infection network of switzerland. *Clin. Microbiol. Infect.* 20, 698–705. doi: 10.1111/1469-0691.12440

Pappas, P. G., Lionakis, M. S., Arendrup, M. C., Ostrosky-Zeichner, L., and Kullberg, B. J. (2018). Invasive candidiasis. *Nat. Rev. Dis. Primers* 4:18026. doi: 10.1038/nrdp.2018.26

Pfaller, M. A., Castanheira, M., Lockhart, S. R., Ahlquist, A. M., Messer, S. A., and Jones, R. N. (2012). Frequency of decreased susceptibility and resistance to echinocandins among fluconazole-resistant bloodstream isolates of *Candida glabrata*. *J. Clin. Microbiol.* 50, 1199–1203. doi: 10.1128/JCM.06112-11

Pham, C. D., Iqbal, N., Bolden, C. B., Kuykendall, R. J., Harrison, L. H., Farley, M. M., et al. (2014). Role of FKS mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance. *Antimicrob. Agents Chemother.* 58, 4690–4696. doi: 10.1128/AAC.03255-14

Prigent, G., Ait-Ammar, N., Levesque, E., Fekkar, A., Costa, J. M., El Anbassi, S., et al. (2017). Echinocandin resistance in Candida species isolates from liver transplant recipients. *Antimicrob. Agents Chemother.* 61:e1229-16. doi: 10.1128/AAC.01229-16

Sandison, T., Ong, V., Lee, J., and Thye, D. (2017). Safety and pharmacokinetics of CD101 IV, a novel echinocandin, in healthy adults. *Antimicrob. Agents Chemother.* 61:e1627-16. doi: 10.1128/AAC.01627-16

Sasso, M., Roger, C., and Lachaud, L. (2017). Rapid emergence of FKS mutations in *Candida glabrata* isolates in a peritoneal candidiasis. *Med. Mycol. Case Rep.* 16, 28–30. doi: 10.1016/j.mmcr.2017.04.004

Shields, R. K., Kline, E. G., Healey, K. R., Kordalewska, M., Perlin, D. S., Nguyen, M. H., et al. (2019). Spontaneous mutational frequency and FKS mutation rates vary by echinocandin agent against *Candida glabrata*. *Antimicrob. Agents Chemother.* 63:e1692-18. doi: 10.1128/AAC.01692-18

Shields, R. K., Nguyen, M. H., Press, E. G., and Clancy, C. J. (2014). Abdominal candidiasis is a hidden reservoir of echinocandin resistance. *Antimicrob. Agents Chemother.* 58, 7601–7605. doi: 10.1128/AAC.04134-14

Shields, R. K., Nguyen, M. H., Press, E. G., Cumbie, R., Driscoll, E., Pasculle, A. W., et al. (2015). Rate of FKS mutations among consecutive Candida isolates causing bloodstream infection. *Antimicrob. Agents Chemother.* 59, 7465–7470. doi: 10.1128/AAC.01973-15

Shields, R. K., Nguyen, M. H., Press, E. G., Kwa, A. L., Cheng, S., Du, C., et al. (2012). The presence of an FKS mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to *Candida glabrata*. *Antimicrob. Agents Chemother.* 56, 4862–4869. doi: 10.1128/AAC.00027-12

Shields, R. K., Nguyen, M. H., Press, E. G., Updike, C. L., and Clancy, C. J. (2013). Anidulafungin and micafungin MIC breakpoints are superior to that of caspofungin for identifying FKS mutant *Candida glabrata* strains and echinocandin resistance. *Antimicrob. Agents Chemother.* 57, 6361–6365. doi: 10.1128/AAC.01451-13

Singh, A., Healey, K. R., Yadav, P., Upadhyaya, G., Sachdeva, N., Sarma, S., et al. (2018). Absence of azole or echinocandin resistance in *Candida glabrata* isolates in India despite background prevalence of strains with defects in the DNA mismatch repair pathway. *Antimicrob. Agents Chemother.* 62:e195-18. doi: 10.1128/AAC.00195-18

Tang, C. M., Cohen, J., and Holden, D. W. (1992). An Aspergillus fumigatus alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. *Mol. Microbiol.* 6, 1663–1671. doi: 10.1111/j.1365-2958.1992.tb00891.x

Thompson, G. R. III, Wiederhold, N. P., Vallor, A. C., Villareal, N. C., Lewis, J. S. II, and Patterson, T. F. (2008). Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to *Candida glabrata* infection. *Antimicrob. Agents Chemother.* 52, 3783–3785. doi: 10.1128/AAC.00473-08

Vallabhaneni, S., Cleveland, A. A., Farley, M. M., Harrison, L. H., Schaffner, W., Beldavs, Z. G., et al. (2015). Epidemiology and risk factors for echinocandin nonsusceptible *Candida glabrata* bloodstream infections: data from a large multisite population-based candidemia surveillance program, 2008-2014. *Open Forum Infect. Dis.* 2:ofv163. doi: 10.1093/ofid/ofv163

White, T. J., Bruns, T., and Lee, S. B. (1990). *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics*. San Diego, CA: Academic Press.

Wiederhold, N. P., Najvar, L. K., Jaramillo, R., Olivo, M., Pizzini, J., Catano, G., et al. (2018). Oral glucan synthase inhibitor SCY-078 is effective in an experimental murine model of invasive candidiasis caused by WT and echinocandin-resistant *Candida glabrata*. *J. Antimicrob. Chemother.* 73, 448–451. doi: 10.1093/jac/dkx422

Zhao, X., and Drlica, K. (2001). Restricting the selection of antibiotic-resistant mutants: a general strategy derived from fluoroquinolone studies. *Clin. Infect. Dis.* 33(Suppl. 3), S147–S156. doi: 10.1086/321841

Zimbeck, A. J., Iqbal, N., Ahlquist, A. M., Farley, M. M., Harrison, L. H., Chiller, T., et al. (2010). FKS mutations and elevated echinocandin MIC values among *Candida glabrata* isolates from U.S. population-based surveillance. *Antimicrob. Agents Chemother.* 54, 5042–5047. doi: 10.1128/AAC.00836-10

Conflict of Interest Statement: In the past five years, AA-I has received grants from GILEAD, F2G, and Scynexis and personal fees for educational conferences from Pfizer Astellas, MSD, and GILEAD outside the submitted work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2. Capítulo 2

3.2. Capítulo 2. Estudio de la identificación molecular, la sensibilidad antifúngica y el principal mecanismo de resistencia a azoles en las especies de *Aspergillus* incluidas en un programa de vigilancia de la resistencia antifúngica establecido en España

Publicación original: **Rivero-Menendez O**, Soto-Debran JC, Medina N, Lucio J, Mellado E, Alastruey-Izquierdo A. 2019. Molecular identification, antifungal susceptibility testing and mechanisms of azole resistance in *Aspergillus* species received within a surveillance program on antifungal resistance in Spain. *Antimicrob Agents Chemother*, 63(9): e00865-19. doi: 10.1128/AAC.00865-19.

La emergencia de la resistencia antifúngica supone un problema para el manejo de las infecciones fúngicas invasivas, las cuales están asociadas a una alta mortalidad. En los últimos años, este fenómeno se ha descrito asociado tanto a especies emergentes que presentan resistencia intrínseca como a especies sensibles que son capaces de desarrollar resistencia secundaria.

La resistencia adquirida a azoles en las distintas especies de *Aspergillus* está sufriendo un progresivo aumento en todo el mundo, sobre todo en *Aspergillus fumigatus*. Esto compromete el tratamiento de las infecciones que causan estas especies, ya que los compuestos triazólicos son considerados su tratamiento de primera línea. El principal mecanismo de resistencia a azoles en *A. fumigatus* se basa en la aparición de mutaciones en el gen *cyp51A*, el cual codifica la enzima 14α-esterol-demetilasa de la ruta de biosíntesis del ergosterol. A su vez, en *Aspergillus terreus* también se han descrito mutaciones en *cyp51A* asociadas a la resistencia a estos compuestos, y en *Aspergillus flavus* en *cyp51A* y *cyp51C*.

Con el fin de monitorizar la resistencia antifúngica en las especies de *Aspergillus* más prevalentes en España, en 2014 se estableció un programa de vigilancia en el Laboratorio de Referencia e Investigación en Micología del Instituto de Salud Carlos III. Este trabajo recoge los principales resultados desde su instauración hasta finales de 2018. La determinación de la sensibilidad antifúngica de las 273 cepas incluidas en el programa permitió su categorización como sensibles o resistentes de acuerdo con los puntos de corte clínicos propuestos por EUCAST. Aquellas resistentes a al menos un antifúngico fueron identificadas molecularmente, encontrando varias especies crípticas de *Aspergillus* entre ellas.

Las mutaciones en Cyp51 fueron caracterizadas en las cepas de *A. fumigatus*, *A. terreus* y *A. flavus sensu stricto* (*s.s.*) que fueron clasificadas como resistentes. Se detectó el mecanismo de resistencia TR_{34}/L98H en tres cepas de *A. fumigatus s.s.*, la mutación puntual G54R en dos y el mecanismo TR_{46}/Y121F/T289A en otra. Diecisiete cepas resistentes de esta especie no mostraron mutaciones en *cyp51A*, de las cuales diez presentaron resistencia solo a isavuconazol. Se encontró la sustitución D344N en *cyp51A* en tres cepas de *A. terreus s.s.*, en una de ellas en combinación con M217I, y en otra una mutación nueva, A249G. En las cepas resistentes de *A. flavus s.s.* se hallaron las alteraciones P220L y H349R en *cyp51A* y *cyp51C*, respectivamente, las cuales necesitan ser investigadas para conocer si están implicadas en la resistencia a los azoles.

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

SUSCEPTIBILITY

Molecular Identification, Antifungal Susceptibility Testing, and Mechanisms of Azole Resistance in *Aspergillus* Species Received within a Surveillance Program on Antifungal Resistance in Spain

Olga Rivero-Menendez,[a] Juan Carlos Soto-Debran,[a] Narda Medina,[a] Jose Lucio,[a] Emilia Mellado,[a,b] Ana Alastruey-Izquierdo[a,b]

[a]Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain
[b]Spanish Network for the Research in Infectious Diseases (REIPI RD16/CIII/0004/0003), Instituto de Salud Carlos III, Madrid, Spain

ABSTRACT Antifungal resistance is one of the major causes of the increasing mortality rates for fungal infections, especially for those caused by *Aspergillus* spp. A surveillance program was established in 2014 in the Spanish National Center for Microbiology for tracking resistance in the most prevalent *Aspergillus* species. A total of 273 samples were included in the study and were initially classified as susceptible or resistant according to EUCAST breakpoints. Several *Aspergillus* cryptic species were found within the molecularly identified isolates. Cyp51 mutations were characterized for *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus flavus sensu stricto* strains that were classified as resistant. Three *A. fumigatus sensu stricto* strains carried the TR_{34}/L98H resistance mechanism, while two harbored G54R substitution and one harbored the TR_{46}/Y121F/T289A mechanism. Seventeen strains had no mutations in *cyp51A*, with ten of them resistant only to isavuconazole. Three *A. terreus sensu stricto* strains harbored D344N substitution in *cyp51A*, one of them combined with M217I, and another carried an A249G novel mutation. Itraconazole-resistant *A. flavus sensu stricto* strains harbored P220L and H349R alterations in *cyp51A* and *cyp51C*, respectively, that need further investigation on their implication in azole resistance.

KEYWORDS *Aspergillus*, Cyp51, EUCAST, antifungal resistance, azoles, isavuconazole, itraconazole, posaconazole, surveillance program, voriconazole

Invasive fungal infections (IFIs) have suffered a significant increase over the last decades, due mainly to the rise in patients subjected to strong immunosuppression (1, 2). These infections present high associated mortality and morbidity rates (1, 3, 4), and a broad range of molds have been reported to cause them (such as *Aspergillus* spp., *Scedosporium* spp., *Fusarium* spp., or Mucorales), with a shift toward new species that are emerging.

One of the reasons for the increased mortality caused by IFIs is the development of antifungal resistance. New emerging species present decreased susceptibility to most antifungal drugs, while secondary resistance is increasingly reported in molds, especially in *Aspergillus* spp. (5). Azoles, which are first-line antifungal therapy for *Aspergillus*, generally have a good activity against these species, although an increase in *Aspergillus fumigatus* clinical isolates displaying MIC values above the clinical breakpoints for azole drugs established by CLSI and EUCAST has been described in recent years in several countries (6–8), and reports on single cases of *A. fumigatus* strains showing triazole resistance have been made throughout the world (9). Most *A. fumigatus* triazole resistance cases are explained by point mutations in the *cyp51A* gene, which encodes the 14α-sterol-demethylase of the ergosterol biosynthesis pathway (10). Point muta-

Citation Rivero-Menendez O, Soto-Debran JC, Medina N, Lucio J, Mellado E, Alastruey-Izquierdo A. 2019. Molecular identification, antifungal susceptibility testing, and mechanisms of azole resistance in *Aspergillus* species received within a surveillance program on antifungal resistance in Spain. Antimicrob Agents Chemother 63:e00865-19. https://doi.org/10.1128/AAC.00865-19.

Address correspondence to Ana Alastruey-Izquierdo, anaalastruey@isciii.es.

Received 26 April 2019
Returned for modification 13 June 2019
Accepted 30 June 2019
Accepted manuscript posted online 8 July 2019
Published 23 August 2019

tions associated with azole resistance have also been reported in *cyp51A* in *Aspergillus terreus* and in *cyp51C* in *Aspergillus flavus* (11–15). Resistance in *Aspergillus* has also been described in cryptic or sibling species, which can only be identified to the species level using molecular tools and are classified in "complex" of closely related species when convenient identification techniques are not performed (16). These species usually present decreased susceptibility to several antifungal drugs (17, 18).

The first multicenter epidemiological study carried out in Spain in molds (FILPOP) reported triazole resistance rates ranging from 10% to 12.7%, based on different species and antifungals tested, but no secondary resistance in *A. fumigatus* was found (19). As a consequence, a surveillance program on antifungal resistance was implemented in 2014 by the Mycology Reference Laboratory of the Spanish National Center for Microbiology in order to monitor antifungal resistance in the most frequently found *Aspergillus* species in our country. In this study, we aim to describe the antifungal susceptibility and mechanisms of azole resistance in the isolates included in the program through the end of 2018.

RESULTS

Strains in this study. In total, 306 samples were received from 45 hospitals from the implementation of the surveillance program on antifungal resistance in July 2014 through the end of 2018. Three samples were identified as *Candida albicans* and were transferred to the surveillance program on antifungal resistance for yeasts for analysis; 27 samples were identified as molds that did not belong to the genus *Aspergillus* and, therefore, were not further analyzed; and three samples were contaminated and were excluded from the study; thus, 273 samples from 33 hospitals were analyzed.

Morphological identification. Macroscopic and microscopic studies allowed the identification of *Aspergillus* strains to the complex level. Among them, 57.9% (158/273) of the strains were identified as part of the *A. fumigatus* complex, 21.6% (59/273) belonged to the *Aspergillus terreus* complex, 12.8% (35/273) were part of the *Aspergillus flavus* complex, and 5.5% (15/273) and 1.5% (4/273) belonged to the *Aspergillus nidulans* complex and the *Aspergillus niger* complex, respectively. Two *Aspergillus* strains were classified as *Aspergillus* species.

Antifungal susceptibility testing. Antifungal susceptibility testing results are summarized in Table 1, showing geometric means (GMs), MIC/minimal effective concentration (MEC) causing inhibition of 50% of the isolates tested (MIC_{50}/MEC_{50}), MIC/MEC causing inhibition of 90% of the isolates tested (MIC_{90}/MEC_{90}), and MIC/MEC ranges of all antifungals tested against strains belonging to an *Aspergillus* complex with more than 10 isolates. *Aspergillus* species showed a wide range of MIC values for all antifungals tested. Azoles were active against *A. fumigatus* complex, *A. terreus* complex, *A. flavus* complex, and *A. nidulans* complex strains, showing lower MIC values for itraconazole and posaconazole than for voriconazole and isavuconazole. Terbinafine showed a discrete activity against all strains tested. Amphotericin B was more active against *A. fumigatus* complex than against *A. terreus* and *A. flavus* complexes and showed moderate activity against *A. nidulans* complex. Echinocandins displayed good activity against all strains.

The number of *Aspergillus* strains analyzed that had MIC values above the clinical breakpoints established by EUCAST is included in Table 2.

Molecular identification. Among the *A. fumigatus* complex isolates, 46 strains with resistance to at least one antifungal were molecularly identified in order to identify cryptic species. The most prevalent species identified was *A. fumigatus sensu stricto* (23 strains), followed by several cryptic species of the complex, as follows: *Aspergillus lentulus* (13 strains), *Aspergillus udagawae* (four strains), *Aspergillus fumigatiaffinis* (three strains), and *Aspergillus felis* and *Aspergillus parafelis* (one strain each). One strain that was resistant to isavuconazole was only identified to the complex level because of absence of amplification of target genes. Thirteen *A. terreus* complex strains resistant to itraconazole, posaconazole, and/or isavuconazole were molecularly identified; 12 were *A. terreus sensu stricto*, and one belonged to the cryptic species *Aspergillus citrinoterreus*.

TABLE 1 GM and MIC/MEC values and ranges for tested antifungals[a] and *Aspergillus* complex strains ($n \geq 10$), as determined by the EUCAST broth microdilution method

Aspergillus complex (no. of strains tested)	MIC parameter	Value (mg/liter) for:								
		AMB	ITC	VRC	POS	ISA	TRB	CAS	MFG	AFG
A. fumigatus complex (158)	GM	0.633	0.663	0.726	0.139	1.124	3.431	0.312	0.016	0.024
	MIC_{50}/MEC_{50}	0.5	0.5	0.5	0.12	1	4	0.25	0.015	0.03
	MIC_{90}/MEC_{90}	8	4	4	0.5	4	8	0.5	0.03	0.06
	Range	0.12–32	0.015–16	0.06–16	0.015–4	0.25–16	0.25–32	0.03–4	0.004–4	0.007–8
A. terreus complex (59)	GM	1.638	0.282	1.036	0.098	1.084	0.315	0.358	0.017	0.025
	MIC_{50}/MEC_{50}	2	0.25	1	0.12	1	0.25	0.5	0.015	0.03
	MIC_{90}/MEC_{90}	8	0.5	2	0.25	2	0.5	1	0.06	0.06
	Range	0.25–16	0.03–16	0.25–16	0.015–1	0.25–8	0.06–1	0.03–2	0.004–4	0.007–8
A. flavus complex (35)	GM	1.400	0.541	0.837	0.193	1.224	0.157	0.517	0.068	0.065
	MIC_{50}/MEC_{50}	1	0.5	1	0.25	1	0.12	0.25	0.03	0.03
	MIC_{90}/MEC_{90}	4	1	1	0.5	2	2	32	4	8
	Range	0.5–32	0.12–16	0.25–16	0.03–2	0.5–16	0.03–4	0.06–32	0.015–4	0.007–8
A. nidulans complex (15)	GM	2.639	0.522	0.396	0.154	0.500	0.412	0.497	0.043	0.057
	MIC_{50}/MEC_{50}	2	0.5	0.25	0.12	0.25	0.25	0.25	0.06	0.06
	MIC_{90}/MEC_{90}	16	1	1	0.5	1	2	4	0.5	0.5
	Range	0.5–16	0.12–4	0.12–1	0.03–1	0.25–8	0.06–2	0.03–8	0.004–2	0.007–4
All (273)	GM	0.916	0.534	0.773	0.138	1.082	1.139	0.349	0.021	0.029
	MIC_{50}/MEC_{50}	0.5	0.5	0.5	0.12	1	2	0.25	0.015	0.03
	MIC_{90}/MEC_{90}	8	2	2	0.5	4	8	1	0.06	0.06
	Range	0.06–32	0.015–16	0.06–16	0.015–4	0.25–16	0.03–32	0.015–32	0.004–4	0.007–8

[a]MIC values for amphotericin B (AMB), itraconazole (ITC), voriconazole (VRC), posaconazole (POS), isavuconazole (ISA), and terbinafine (TRB), and MEC values for caspofungin (CAS), micafungin (MFG), and anidulafungin (AFG).

Only two strains from the *A. flavus* complex were resistant to itraconazole, and these were identified as *A. flavus sensu stricto*. Out of six *A. nidulans* complex strains showing resistance to itraconazole and/or isavuconazole, four were *A. nidulans sensu stricto*, and four belonged to the cryptic species *Aspergillus spinulosporus*.

Characterization of molecular mechanisms of azole resistance in *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus flavus sensu stricto*. Further characterization was performed of *A. fumigatus*, *A. flavus*, and *A. terreus sensu stricto* strains that were resistant to at least one azole drug according to EUCAST breakpoints. Table 3 shows *cyp51A* mutations found in azole-resistant *A. fumigatus* and *A. terreus* strains and *cyp51A*, *cyp51B*, and *cyp51C* mutations found in azole-resistant *A. flavus* strains. Twenty-three *A. fumigatus* strains were azole resistant. Three of them harbored the TR_{34}/L98H mutation and were panazole resistant, while two strains carried a G54R substitution showing resistance to itraconazole and posaconazole, and another one harbored the

TABLE 2 Number of *Aspergillus* strains with MIC values above the established EUCAST breakpoint to amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole

Aspergillus complex or species	No. (%)[a] of strains with MIC values above their established EUCAST breakpoint				
	AMB	ITC	VRC	POS	ISA[b]
A. fumigatus complex (158)	19 (12%)	17 (10.8%)	17 (10.8%)	21 (13.3%)	28 (27.7%)
A. fumigatus sensu stricto	0 (0%)	11 (64.7%)	7 (41.2%)	13 (61.9%)	17 (60.7%)
A. terreus complex (59)	NA[c]	2 (3.4%)	NA	3 (5.1%)	12 (44.4%)
A. terreus sensu stricto		2 (100%)		2 (66.7%)	11 (91.7%)
A. flavus complex (35)	NA	2 (5.7%)	NA	NA	NA
A. flavus sensu stricto		2 (100%)			
A. nidulans complex (15)	NA	1 (6.7%)	NA	NA	6 (46.2%)
A. nidulans sensu stricto		1 (100%)			4 (66.7%)

[a]Percentages for *sensu stricto* isolates correspond to % of resistant *sensu stricto* isolates among the total resistant isolates of the complex.
[b]Percentages for isavuconazole were calculated according to the number of strains where isavuconazole was tested (101 *for A. fumigatus*, 27 for *A. terreus*, and 13 for *A. nidulans*).
[c]NA, not applicable due to the lack of EUCAST breakpoint.

TABLE 3 Characterization of molecular mechanisms of azole resistance by studying *cyp51* gene alterations from azole-resistant *A. fumigatus*, *A. terreus*, and *A. flavus sensu stricto* isolates

Species	Strain[a]	MIC (mg/liter)[b]				Mutation(s) found (gene)
		ITC	VRC	POS	ISA	
A. fumigatus	CNM-CM7582	**16**	**4**	**0.5**	ND	TR_{34}/L98H (*cyp51A*)
	CNM-CM7609	**16**	**16**	**1**	ND	TR_{34}/L98H (*cyp51A*)
	CNM-CM9399	**16**	**4**	**0.5**	**8**	TR_{34}/L98H (*cyp51A*)
	CNM-CM9114	**16**	0.5	**2**	0.5	G54R (*cyp51A*)
	CNM-CM9501	**16**	0.5	**2**	0.5	G54R (*cyp51A*)
	CNM-CM8057	**16**	**16**	**1**	**16**	TR_{46}/Y121F/T289A (*cyp51A*)
	CNM-CM7510	**16**	2	**0.5**	ND	None (*cyp51A*)
	CNM-CM7552	**16**	1	**0.5**	ND	None (*cyp51A*)
	CNM-CM8822	**4**	**8**	**0.5**	**4**	None (*cyp51A*)
	CNM-CM8900	1	**4**	**0.5**	**2**	None (*cyp51A*)
	CNM-CM8914	0.5	0.25	0.12	**2**	None (*cyp51A*)
	CNM-CM8915	0.5	0.5	0.12	**2**	None (*cyp51A*)
	CNM-CM8916	0.5	0.25	0.12	**2**	None (*cyp51A*)
	CNM-CM8917	0.5	0.5	0.06	**2**	None (*cyp51A*)
	CNM-CM8922	1	0.5	0.06	**2**	None (*cyp51A*)
	CNM-CM8925	1	0.5	0.25	**2**	None (*cyp51A*)
	CNM-CM9120	1	0.25	0.25	**2**	None (*cyp51A*)
	CNM-CM9307	0.5	1	0.12	**2**	None (*cyp51A*)
	CNM-CM9327	1	2	**0.5**	**2**	None (*cyp51A*)
	CNM-CM9361	1	2	0.25	**2**	None (*cyp51A*)
	CNM-CM9471	**16**	**8**	**0.5**	**8**	None (*cyp51A*)
	CNM-CM9491	1	0.5	0.12	**2**	None (*cyp51A*)
	CNM-CM9494	**8**	2	**0.5**	**4**	None (*cyp51A*)
	Range	**0.5–16**	0.25–16	0.12–2	0.5–16	
A. flavus	CNM-CM7668	**16**	0.25	1	ND	P220L (*cyp51A*)
	CNM-CM9326	**16**	16	2	16	H349R (*cyp51C*)
	Range	16–16	0.25–16	1–2	**16–16**	
A. terreus	CNM-CM9079	0.25	2	0.06	**2**	D344N (*cyp51A*)
	CNM-CM9280	0.5	2	0.12	**2**	D344N (*cyp51A*)
	CNM-CM9490	**4**	8	**0.5**	**8**	D344N (*cyp51A*)
	CNM-CM7846	**16**	16	**1**	ND	M217I; D344N (*cyp51A*)
	CNM-CM9284	0.5	1	0.12	**2**	A249G (*cyp51A*)
	CNM-CM8056	0.5	2	0.06	**2**	None (*cyp51A*)
	CNM-CM8671	0.5	2	0.25	**2**	None (*cyp51A*)
	CNM-CM8852	2	2	0.25	**2**	None (*cyp51A*)
	CNM-CM8952	0.5	8	0.25	**8**	None (*cyp51A*)
	CNM-CM8981	0.25	1	0.06	**2**	None (*cyp51A*)
	CNM-CM9108	0.25	2	0.06	**2**	None (*cyp51A*)
	CNM-CM9285	0.25	1	0.12	**2**	None (*cyp51A*)
	Range	0.25–16	1–16	0.06–1	**2–8**	

[a]CNM-CM, Mold Collection of the Spanish National Center for Microbiology.
[b]Boldface numbers indicate MIC values that are above the EUCAST clinical breakpoints.

TR_{46}/Y121F/T289A mechanism and was resistant to all azoles. Seventeen out of 23 azole-resistant *A. fumigatus* strains did not have mutations in the *cyp51A* gene; ten of these were only resistant to isavuconazole. The seven remaining strains were resistant to at least two azoles.

Twelve *A. terreus* strains were azole resistant according to EUCAST breakpoints, of which two were resistant to itraconazole, posaconazole, and isavuconazole and had high MICs to voriconazole. Both had a D344N substitution in *cyp51A*, in one case combined with another mutation, M217I, in the same gene. The remaining ten strains were only resistant to isavuconazole (MIC = 2 mg/liter), and while seven carried wild-type *cyp51A*, two carried the D344N alteration, and one harbored an A249G novel mutation that was not present in azole-susceptible strains.

Two *A. flavus* strains were itraconazole resistant. While both of them carried several *cyp51C* substitutions (M54T and S240A in both strains, and D246G, E421D, and N423D in one strain) that were also present in azole-susceptible *A. flavus* strains, one strain

harbored a P220L mutation in the *cyp51A* gene, and the other carried an H349R substitution in *cyp51C*. These alterations were not carried by azole-susceptible isolates and have not been described before.

DISCUSSION

IFIs are an increasing health concern worldwide, as more than one million deaths are attributed to them every year (20). Variable rates of antifungal resistance were found in a population-based survey performed in Spain on molds (19), with no secondary resistance in *A. fumigatus*. An antifungal resistance surveillance program for *Aspergillus* spp. was established in our reference laboratory. Identification and antifungal susceptibility testing (AFST) results of clinical strains received within this program from its implementation in July 2014 through the end of 2018 are reviewed in this report. Molecular mechanisms of azole resistance were further characterized for those *A. fumigatus sensu stricto*, *A. terreus sensu stricto*, and *A. flavus sensu stricto* isolates that presented azole MICs above the breakpoints established by EUCAST.

Out of a total of 273 *Aspergillus* sp. isolates received within the antifungal resistance surveillance program, *A. fumigatus* complex strains represented more than half of the strains identified (57.9%), followed by *A. terreus* complex (21.6%), *A. flavus* complex (12.8%), and *A. nidulans* (5.5%) complex strains. As samples received within the program constitute a biased subset of isolates under the suspicion of being antifungal resistant, *Aspergillus* complex species prevalence in Spanish centers are not fully representative based on these data. In the FILPOP and FILPOP2 prospective surveillance studies *A. fumigatus sensu stricto* was the most prevalent species isolated, followed by *A. flavus sensu stricto*, *A. terreus sensu stricto*, and *A. tubingensis* in FILPOP (19) and *A. niger sensu stricto*, *A. flavus sensu stricto*, and *A. terreus sensu stricto* in FILPOP2 (21).

Overall, voriconazole and isavuconazole, which have been described to display similar *in vitro* activities (22, 23), showed higher MICs *in vitro* than those of itraconazole and posaconazole in the isolates tested. These differences were particularly remarkable against *A. terreus* complex strains. Similar MIC ranges for voriconazole (EUCAST epidemiological cutoff [ECOFF], >2 mg/liter) and isavuconazole were observed in previous studies against *A. terreus* isolates (22). *A. terreus* and *A. flavus* strains have been reported to display high MIC values to amphotericin B (24–26), although neither clinical breakpoints nor ECOFF values have yet been set for them by EUCAST. Accordingly, strains from this work showed MIC_{90} values of 8 mg/liter for those within the *A. terreus* complex and 4 mg/liter for those belonging to the *A. flavus* complex.

In the FILPOP and FILPOP2 studies, 12% and 11.5% of the identified strains belonged to cryptic or sibling *Aspergillus* species, respectively (19, 21). Although some cryptic *Aspergillus* species present among the isolates received within the program may have been missed due to the fact that molecular identification was only performed for strains that showed MICs above EUCAST clinical breakpoints to at least one antifungal, several sibling species were identified in the current study; the multidrug-resistant species *A. lentulus* was the most common one (13 identified strains), in agreement with results from a multicenter international surveillance study (27). Strains identified as part of this species from the *A. fumigatus* complex presented low susceptibility to amphotericin B and to azoles, especially to voriconazole (data not shown), as reported by previous studies (17, 18). Four *Aspergillus udagawae* and three *A. fumigatiaffinis* strains were also identified. One isolate was identified as *A. citrinoterreus*, from the *A. terreus* complex, which has been reported as the most prevalent cryptic species from this complex in Spain (15).

Despite previous studies in Spain showing low rates of *A. fumigatus* azole resistance (19, 21, 28), resistance to at least one azole was found in 23 strains molecularly identified as *A. fumigatus sensu stricto* Three strains had the most frequent mechanism of azole resistance described worldwide (TR_{34}/L98H) (29–31) and one TR_{46}/Y121F/T289A mechanism (32). These two resistance-related changes have been previously described in Spanish isolates (19, 33, 34), TR_{34}/L98H is associated with a panazole resistance profile, and TR_{46}/Y121F/T289A is related to voriconazole and

isavuconazole resistance and variable MICs for itraconazole and posaconazole (35, 36), although the isolate carrying it in this study was resistant to all azoles. Two other *A. fumigatus* strains harbored a substitution of glycine for arginine at position 54 of *cyp51A*, which has been linked to cross-resistance to itraconazole and posaconazole (37, 38), in agreement with the MICs obtained in this study. The remaining seventeen azole-resistant strains had no mutations in *cyp51A*; two of them were multiazole resistant, while the rest had different azole resistance profiles. Azole-resistant *A. fumigatus* isolates lacking *cyp51A* mutations have been previously reported (39, 40), evidencing the need to investigate further *cyp51A*-independent mechanisms of azole resistance that can be present, such as the overexpression of efflux pumps or *cyp51B* (9). Interestingly, ten of these 17 isolates with no mutations in *cyp51A* were resistant only to isavuconazole, with MIC values of 2 mg/liter. Even though ECOFF value for isavuconazole for *A. fumigatus* is 2 mg/liter, its clinical breakpoint was set by EUCAST as 1 mg/liter (41) based on the use of standard dosing against a mouse model of disseminated aspergillosis (42). On the basis of this established breakpoint, *cyp51A* wild-type isolates classified as isavuconazole resistant have been reported (23, 36, 43). Nevertheless, a recent study on isavuconazole dose escalation proved its effectiveness for treating patients infected with an *A. fumigatus* with an isavuconazole MIC of 2 mg/liter (36), which could result in an increase of one 2-fold dilution step of the EUCAST clinical breakpoint or in the categorization of isolates with an isavuconazole MIC of 2 mg/liter as intermediate, as previously suggested (23). For the time being, EUCAST recommends to repeatedly perform AFST, including additional markers for azole resistance (itraconazole and voriconazole), and to sequence *cyp51A* when isolates have a MIC of 2 mg/liter.

Only two isolates of *A. terreus sensu stricto* had MIC values above the established breakpoints for itraconazole and posaconazole. One of them harbored M217I mutation in *cyp51A*, which has been related to itraconazole resistance and high MIC values of voriconazole and posaconazole (14), as shown by this isolate that also carried the D344N substitution. This alteration has been reported together with M217V substitution in an isolate only resistant to posaconazole (15). The other itraconazole- and posaconazole-resistant isolate, which was also isavuconazole resistant, carried the D344N mutation alone and showed high MICs to voriconazole as well. Mutations in M217 have been suggested to correlate with M220 alterations in the *A. fumigatus cyp51A* gene, which are linked to itraconazole and posaconazole resistance and variable voriconazole and isavuconazole MIC values (44, 45). Another amino acid change in this position, M217T, has also been reported in *A. terreus* isolates resistant only to posaconazole (15). Nevertheless, further research is needed in order to confirm the role of these several mutations in M217 and D344N alteration in the development of *A. terreus* azole resistance. Similarly to *A. fumigatus sensu stricto* strains, ten *A. terreus sensu stricto* strains were only resistant to isavuconazole, with a MIC of 2 mg/liter, a 1-fold dilution above the breakpoint. Two of them carried D344N substitution, while another harbored a novel mutation (A249G) that was not present in susceptible strains and which, therefore, needs to be further studied. The remaining seven strains did not carry any *cyp51A* alterations. In a study where *in vitro* activity of isavuconazole and voriconazole were compared, a high number of *A. terreus* isolates was found to be isavuconazole resistant but voriconazole susceptible, even though MIC distributions for both azoles were symmetric. If an isavuconazole ECOFF value of 2 mg/liter for *A. terreus* strains was applied to those isolates, susceptibility categorization would be the same for both voriconazole and isavuconazole (22).

In previous studies, *A. flavus* strains with MIC values higher than the epidemiological cutoff value established for voriconazole by CLSI, 1 mg/liter, were reported to sometimes harbor *cyp51A*, *cyp51B*, and *cyp51C* mutations, as *A. flavus* has three *cyp51* genes. These strains presented different susceptibility patterns, as some of them had reduced susceptibility to all azoles (46) while others showed intermediate MIC values for itraconazole and posaconazole (11, 13). Two *A. flavus sensu stricto* strains (6%) within the program were classified as itraconazole resistant, based on the EUCAST clinical

breakpoint, and had high MICs against voriconazole, posaconazole, and isavuconazole. *cyp51C* substitutions M54T, S250A, D246G, E421D, and N423D, found in these isolates, seem to have no effect on azole resistance, as they were also present in azole-susceptible strains. Nevertheless, both azole-resistant isolates carried two novel substitutions that were not found in azole-susceptible isolates; one of them carried a P220L mutation on the *cyp51A* gene, and the other harbored an H349R alteration in *cyp51C*. Further investigation is warranted in order to study the implications of their role in azole resistance.

Even though no azole resistance was found among *A. nidulans* strains in the FILPOP and FILPOP2 studies, in this study, one strain was itraconazole resistant and six isolates were isavuconazole resistant according to EUCAST breakpoints. Azole resistance mechanisms were not further studied, as it is not clear how this species develops it. Nevertheless, two *cyp51* genes homologous to those of *A. fumigatus* have been described in *A. nidulans* (47).

Limitations of this study include the fact that resistance rates are not representative, as they are biased due to the fact that only isolates suspected to be antifungal resistant were received by the program. Nevertheless, this study highlights the interest in establishing an antifungal resistance surveillance program in order to get a deeper insight into antifungal resistance mechanisms, which can help in the future to implement specific control measures and to design adapted strategies to diagnose and manage azole resistance in *Aspergillus* species. An important number of azole-resistant *A. fumigatus* strains were found with different mechanisms of resistance, and *A. flavus* and *A. terreus* azole resistance mechanisms were studied for the first time in clinical isolates from Spain.

MATERIALS AND METHODS

Strains. From July 2014 to December 2018, 306 samples belonging to patients from 45 Spanish hospitals were received that were suspected of being resistant (as tested by any antifungal susceptibility method or due to the lack of clinical response) to at least one antifungal agent.

Morphological identification. Strains were morphologically identified to species complex level using malt extract agar (2% malt extract; Oxoid SA, Madrid, Spain), potato dextrose agar (Oxoid SA), dermasel agar base (Oxoid SA), and Czapek-Dox agar (Difco, Soria Melguizo SA, Madrid, Spain) for subculturing the strains to determine their macroscopic and microscopic morphology. Cultures were incubated at 30°C. Fungal morphological features were examined macroscopically and microscopically by conventional methods (48).

Antifungal susceptibility testing. Antifungal susceptibility testing (AFST) was performed following the European Committee on Antifungal Susceptibility Testing (EUCAST) reference method 9.3.1 (49). Antifungals used were amphotericin B (Sigma-Aldrich Química, Madrid, Spain), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer SA, Madrid, Spain), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland (tested from January 2017), terbinafine (Novartis, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, NJ), micafungin (Astellas Pharma, Inc., Tokyo, Japan), and anidulafungin (Pfizer SA). The final concentrations tested ranged from 0.03 to 16 mg/liter for amphotericin B, terbinafine, and caspofungin; from 0.015 to 8 mg/liter for itraconazole, voriconazole, posaconazole, and isavuconazole; from 0.007 to 4 mg/liter for anidulafungin; and from 0.004 to 2 mg/liter for micafungin. *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains in all tests performed. MICs for amphotericin B, itraconazole, voriconazole, posaconazole, isavuconazole, and terbinafine, and minimal effective concentrations (MECs) for anidulafungin, caspofungin and micafungin were visually read after 24 and 48 h of incubation at 35°C in a humid atmosphere. Clinical breakpoints for interpreting AFST results have been established by EUCAST for some A*spergillus* species for amphotericin B, itraconazole, voriconazole, posaconazole and isavuconazole (50). These breakpoints were used for classifying as susceptible or resistant to each antifungal all species complexes as identified by morphological methods in the first stage and the molecularly identified isolates as available. Breakpoints of echinocandins and terbinafine have not yet been set. Geometric mean (GM), MIC_{50}/MEC_{50} (MIC/MEC causing inhibition of 50% of the isolates tested), and MIC_{90}/MEC_{90} (MIC/MEC causing inhibition of 90% of the isolates tested) were determined. For calculation purposes, the MIC/MEC values that exceeded the maximum concentration tested were transformed to the next dilution (i.e., if MIC/MEC was >8 mg/liter, it was expressed as 16 mg/liter), and values that were less than or equal to the minimum concentration tested were transformed to equal (i.e., if MIC/MEC was ≤0.03 mg/liter, it was expressed as 0.03 mg/liter).

Molecular identification. *Aspergillus* strains that were classified as resistant to at least one antifungal according to breakpoints were subjected to molecular identification as follows. Isolates were subcultured in glucose-yeast extract-peptone (GYEP) liquid medium (0.3% yeast extract and 1% peptone; Difco, Soria Melguizo) with 2% glucose (Sigma-Aldrich, Spain) for 24 h to 48 h at 30°C. After mechanical disruption of the mycelium by vortex mixing with glass beads, genomic DNA of isolates was extracted using the

phenol-chloroform method (51). Molecular identification was performed by PCR amplifying and sequencing internal transcribed spacer 1 (ITS1)-5.8S-ITS2 regions (52) and a portion of the β-tubulin gene (53). PCR conditions were set as previously described (19). PCR products were purified using Illustra ExoProStar 1-step technology (GE Healthcare Life Sciences, UK), and subsequently sequenced by Sanger method with an ABI3730XL sequencer (Applied Biosystems, Foster City, CA). DNA sequences were analyzed with DNAStar Lasergene 12 software (DNAStar Inc., USA) and compared with reference sequences from the GenBank (https://www.ncbi.nlm.nih.gov/GenBank/) and Mycobank (http://www.mycobank.org/) databases with InfoQuest FP software version 4.50 (Bio-Rad Laboratories, Madrid, Spain), as well as with the in-house database belonging to the Mycology Reference Laboratory of the Spanish National Center for Microbiology (restricted access).

Characterization of molecular mechanisms of azole resistance in *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus terreus*. Molecular mechanisms of azole resistance were studied by sequencing the main azole target genes for *A. fumigatus sensu stricto* (*cyp51A*, including its promoter region), *A. flavus sensu stricto* (*cyp51A*, *cyp51B*, and *cyp51C*) and *A. terreus sensu stricto* (*cyp51A*) strains that were resistant to at least one azole. All *cyp51* genes were amplified and sequenced as previously described (12, 14, 29). DNA sequences were compared with the *cyp51A* sequence of reference strain A1163 of *A. fumigatus* (NCBI accession number DS499598.1), *cy51A* sequence of reference strain NIH2624 of *A. terreus* (NCBI accession number XM_001215095.1), *cyp51A* (NCBI accession number XM_002375082.1), *cyp51B* (NCBI accession number XM_002379089.1), and *cyp51C* (NCBI accession number XM_002383890.1) sequences of reference strain NRRL3357 of *A. flavus* in order to detect point mutations related to azole resistance.

ACKNOWLEDGMENTS

O.R.-M. holds a predoctoral fellowship from the Fondo de Investigación Sanitaria (FI14CIII/00025). J.C.S.-D. holds a predoctoral fellowship from the Fondo de Investigación Sanitaria (F17CIII/00027). J.L. holds a predoctoral fellowship from the Fondo de Investigación Sanitaria (F17CIII/00037). A.A.-I. is supported by a research project from the Fondo de Investigación Sanitaria (PI13/02145 and PI16CIII/00035); she has received research grants or honoraria as a speaker or advisor from Gilead Sciences, MSD, Astellas, Pfizer, F2G, Amplix, and Scynexis.

REFERENCES

1. Chen SC, Playford EG, Sorrell TC. 2010. Antifungal therapy in invasive fungal infections. Curr Opin Pharmacol 10:522–530. https://doi.org/10.1016/j.coph.2010.06.002.
2. Kriengkauykiat J, Ito JI, Dadwal SS. 2011. Epidemiology and treatment approaches in management of invasive fungal infections. Clin Epidemiol 3:175–191. https://doi.org/10.2147/CLEP.S12502.
3. Pagano L, Akova M, Dimopoulos G, Herbrecht R, Drgona L, Blijlevens N. 2011. Risk assessment and prognostic factors for mould-related diseases in immunocompromised patients. J Antimicrob Chemother 66 Suppl 1:i5–14. https://doi.org/10.1093/jac/dkq437.
4. Sun KS, Tsai CF, Chen SC, Huang WC. 2017. Clinical outcome and prognostic factors associated with invasive pulmonary aspergillosis: an 11-year follow-up report from Taiwan. PLoS One 12:e0186422. https://doi.org/10.1371/journal.pone.0186422.
5. Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. 2017. The global problem of antifungal resistance: prevalence, mechanisms, and management. Lancet Infect Dis 17:e383–e392. https://doi.org/10.1016/S1473-3099(17)30316-X.
6. Howard SJ, Pasqualotto AC, Denning DW. 2010. Azole resistance in allergic bronchopulmonary aspergillosis and *Aspergillus* bronchitis. Clin Microbiol Infect 16:683–688. https://doi.org/10.1111/j.1469-0691.2009.02911.x.
7. van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, Kuijper EJ, Van Tiel FH, Melchers WJ, Verweij PE. 2011. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007–2009. Emerg Infect Dis 17:1846–1854. https://doi.org/10.3201/eid1710.110226.
8. Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, Denning DW. 2010. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. J Antimicrob Chemother 65:2116–2118. https://doi.org/10.1093/jac/dkq279.
9. Rivero-Menendez O, Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M. 2016. Triazole resistance in *Aspergillus* spp.: a worldwide problem? J Fungi (Basel) 2:E21. https://doi.org/10.3390/jof2030021.
10. 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. https://doi.org/10.1128/AAC.01599-09.
11. Liu W, Sun Y, Chen W, Liu W, Wan Z, Bu D, Li R. 2012. The T788G mutation in the *cyp51C* gene confers voriconazole resistance in *Aspergillus flavus* causing aspergillosis. Antimicrob Agents Chemother 56:2598–2603. https://doi.org/10.1128/AAC.05477-11.
12. Paul RA, Rudramurthy SM, Meis JF, Mouton JW, Chakrabarti A. 2015. A novel Y319H substitution in *cyp51C* associated with azole resistance in *Aspergillus flavus*. Antimicrob Agents Chemother 59:6615–6619. https://doi.org/10.1128/AAC.00637-15.
13. Choi MJ, Won EJ, Joo MY, Park YJ, Kim SH, Shin MG, Shin JH. 2019. Microsatellite typing and resistance mechanism analysis of voriconazole-resistant *Aspergillus flavus* isolates in South Korean hospitals. Antimicrob Agents Chemother 63:e01610-18. https://doi.org/10.1128/AAC.01610-18.
14. Arendrup MC, Jensen RH, Grif K, Skov M, Pressler T, Johansen HK, Lass-Flörl C. 2012. *In vivo* emergence of *Aspergillus terreus* with reduced azole susceptibility and a *cyp51A* M217I alteration. J Infect Dis 206:981–985. https://doi.org/10.1093/infdis/jis442.
15. Zoran T, Sartori B, Sappl L, Aigner M, Sánchez-Reus F, Rezusta A, Chowdhary A, Taj-Aldeen SJ, Arendrup MC, Oliveri S, Kontoyiannis DP, Alastruey-Izquierdo A, Lagrou K, Cascio GL, Meis JF, Buzina W, Farina C, Drogari-Apiranthitou M, Grancini A, Tortorano AM, Willinger B, Hamprecht A, Johnson E, Klingspor L, Arsic-Arsenijevic V, Cornely OA, Meletiadis J, Prammer W, Tullio V, Vehreschild J-J, Trovato L, Lewis RE, Segal E, Rath P-M, Hamal P, Rodriguez-Iglesias M, Roilides E, Arikan-Akdagli S, Chakrabarti A, Colombo AL, Fernández MS, Martin-Gomez MT, Badali H, Petrikkos G, Klimko N, Heimann SM, Uzun O, Roudbary M, de la Fuente S, Houbraken J, Risslegger B, Lass-Flörl C, Lackner M. 2018. Azole-resistance in *Aspergillus terreus* and related species: an emerging problem or a rare phenomenon? Front Microbiol 9:516. https://doi.org/10.3389/fmicb.2018.00516.
16. Balajee SA, Houbraken J, Verweij PE, Hong SB, Yaghuchi T, Varga J, Samson RA. 2007. *Aspergillus* species identification in the clinical setting. Stud Mycol 59:39–46. https://doi.org/10.3114/sim.2007.59.05.
17. Alastruey-Izquierdo A, Alcazar-Fuoli L, Cuenca-Estrella M. 2014. Antifun-

gal susceptibility profile of cryptic species of *Aspergillus*. Mycopathologia 178:427–433. https://doi.org/10.1007/s11046-014-9775-z.
18. Nedel WL, Pasqualotto AC. 2014. Treatment of infections by cryptic *Aspergillus* species. Mycopathologia 178:441–445. https://doi.org/10.1007/s11046-014-9811-z.
19. Alastruey-Izquierdo A, Mellado E, Peláez T, Pemán J, Zapico S, Alvarez M, Rodríguez-Tudela JL, Cuenca-Estrella M, FILPOP Study Group. 2013. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). Antimicrob Agents Chemother 57:3380–3387. https://doi.org/10.1128/AAC.00383-13.
20. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. Sci Transl Med 4:165rv13. https://doi.org/10.1126/scitranslmed.3004404.
21. Alastruey-Izquierdo A, Alcazar-Fuoli L, Rivero-Menéndez O, Ayats J, Castro C, García-Rodríguez J, Goterris-Bonet L, Ibáñez-Martínez E, Linares-Sicilia MJ, Martin-Gomez MT, Martín-Mazuelos E, Pelaez T, Peman J, Rezusta A, Rojo S, Tejero R, Anza DV, Viñuelas J, Zapico MS, Cuenca-Estrella M, the FILPOP2 Project from GEMICOMED (SEIMC) and REIPI. 2018. Molecular identification and susceptibility testing of molds isolated in a prospective surveillance of triazole resistance in Spain (FILPOP2 Study). Antimicrob Agents Chemother 62. https://doi.org/10.1128/AAC.00358-18.
22. Astvad KMT, Hare RK, Arendrup MC. 2017. Evaluation of the *in vitro* activity of isavuconazole and comparator voriconazole against 2635 contemporary clinical *Candida* and *Aspergillus* isolates. Clin Microbiol Infect 23:882–887. https://doi.org/10.1016/j.cmi.2017.03.023.
23. Jorgensen KM, Astvad KMT, Hare RK, Arendrup MC. 2019. EUCAST susceptibility testing of isavuconazole: MIC data for contemporary clinical mould and yeast isolates. Antimicrob Agents Chemother. https://doi.org/10.1128/AAC.00073-19.
24. Blum G, Perkhofer S, Haas H, Schrettl M, Würzner R, Dierich MP, Lass-Flörl C. 2008. Potential basis for amphotericin B resistance in *Aspergillus terreus*. Antimicrob Agents Chemother 52:1553–1555. https://doi.org/10.1128/AAC.01280-07.
25. Hadrich I, Makni F, Neji S, Cheikhrouhou F, Bellaaj H, Elloumi M, Ayadi A, Ranque S. 2012. Amphotericin B *in vitro* resistance is associated with fatal *Aspergillus flavus* infection. Med Mycol 50:829–834. https://doi.org/10.3109/13693786.2012.684154.
26. Reichert-Lima F, Lyra L, Pontes L, Moretti ML, Pham CD, Lockhart SR, Schreiber AZ. 2018. Surveillance for azoles resistance in *Aspergillus* spp. highlights a high number of amphotericin B-resistant isolates. Mycoses 61:360–365. https://doi.org/10.1111/myc.12759.
27. van der Linden JW, Arendrup MC, Warris A, Lagrou K, Pelloux H, Hauser PM, Chryssanthou E, Mellado E, Kidd SE, Tortorano AM, Dannaoui E, Gaustad P, Baddley JW, Uekotter A, Lass-Florl C, Klimko N, Moore CB, Denning DW, Pasqualotto AC, Kibbler C, Arikan-Akdagli S, Andes D, Meletiadis J, Naumiuk L, Nucci M, Melchers WJ, Verweij PE. 2015. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. Emerg Infect Dis 21:1041–1044. https://doi.org/10.3201/eid2106.140717.
28. Escribano P, Pelaez T, Munoz P, Bouza E, Guinea J. 2013. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? Antimicrob Agents Chemother 57:2815–2820. https://doi.org/10.1128/AAC.02487-12.
29. Mellado E, Garcia-Effron G, Alcázar-Fuoli L, Melchers WJG, Verweij PE, Cuenca-Estrella M, Rodríguez-Tudela JL. 2007. A new *Aspergillus fumigatus* resistance mechanism conferring *in vitro* cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. Antimicrob Agents Chemother 51:1897–1904. https://doi.org/10.1128/AAC.01092-06.
30. Chowdhary A, Kathuria S, Xu J, Meis JF. 2013. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. PLoS Pathog 9:e1003633. https://doi.org/10.1371/journal.ppat.1003633.
31. Morio F, Aubin GG, Danner-Boucher I, Haloun A, Sacchetto E, Garcia-Hermoso D, Bretagne S, Miegeville M, Le Pape P. 2012. High prevalence of triazole resistance in *Aspergillus fumigatus*, especially mediated by TR34/L98H, in a French cohort of patients with cystic fibrosis. J Antimicrob Chemother 67:1870–1873. https://doi.org/10.1093/jac/dks160.
32. van der Linden JW, Camps SM, Kampinga GA, Arends JP, Debets-Ossenkopp YJ, Haas PJ, Rijnders BJ, Kuijper EJ, van Tiel FH, Varga J, Karawajczyk A, Zoll J, Melchers WJ, Verweij PE. 2013. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. Clin Infect Dis 57:513–520. https://doi.org/10.1093/cid/cit320.
33. Rodriguez-Tudela JL, Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A, Monzon A, Cuenca-Estrella M. 2008. Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. Antimicrob Agents Chemother 52:2468–2472. https://doi.org/10.1128/AAC.00156-08.
34. Pelaez T, Monteiro MC, Garcia-Rubio R, Bouza E, Gomez-Lopez A, Mellado E. 2015. First detection of *Aspergillus fumigatus* azole-resistant strain due to *cyp51A* TR46/Y121F/T289A in an azole-naive patient in Spain. New Microbes New Infect 6:33–34. https://doi.org/10.1016/j.nmni.2015.04.005.
35. van Ingen J, van der Lee HA, Rijs TA, Zoll J, Leenstra T, Melchers WJ, Verweij PE. 2015. Azole, polyene and echinocandin MIC distributions for wild-type, TR34/L98H and TR46/Y121F/T289A *Aspergillus fumigatus* isolates in the Netherlands. J Antimicrob Chemother 70:178–181. https://doi.org/10.1093/jac/dku364.
36. Buil JB, Bruggemann RJM, Wasmann RE, Zoll J, Meis JF, Melchers WJG, Mouton JW, Verweij PE. 2018. Isavuconazole susceptibility of clinical *Aspergillus fumigatus* isolates and feasibility of isavuconazole dose escalation to treat isolates with elevated MICs. J Antimicrob Chemother 73:134–142. https://doi.org/10.1093/jac/dkx354.
37. Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL. 2003. A point mutation in the 14α-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 47:1120–1124. https://doi.org/10.1128/aac.47.3.1120-1124.2003.
38. Nascimento AM, Goldman GH, Park S, Marras SA, Delmas G, Oza U, Lolans K, Dudley MN, Mann PA, Perlin DS. 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother 47:1719–1726. https://doi.org/10.1128/aac.47.5.1719-1726.2003.
39. Arendrup MC, Mavridou E, Mortensen KL, Snelders E, Frimodt-Moller N, Khan H, Melchers WJ, Verweij PE. 2010. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. PLoS One 5:e10080. https://doi.org/10.1371/journal.pone.0010080.
40. Denning DW, Park S, Lass-Florl C, Fraczek MG, Kirwan M, Gore R, Smith J, Bueid A, Moore CB, Bowyer P, Perlin DS. 2011. High-frequency triazole resistance found in nonculturable *Aspergillus fumigatus* from lungs of patients with chronic fungal disease. Clin Infect Dis 52:1123–1129. https://doi.org/10.1093/cid/cir179.
41. Arendrup MC, Meletiadis J, Mouton JW, Guinea J, Cuenca-Estrella M, Lagrou K, Howard SJ, Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). 2016. EUCAST technical note on isavuconazole breakpoints for *Aspergillus*, itraconazole breakpoints for *Candida* and updates for the antifungal susceptibility testing method documents. Clin Microbiol Infect 22:571.e1-4. https://doi.org/10.1016/j.cmi.2016.01.017.
42. Seyedmousavi S, Bruggemann RJ, Meis JF, Melchers WJ, Verweij PE, Mouton JW. 2015. Pharmacodynamics of isavuconazole in an *Aspergillus fumigatus* mouse infection model. Antimicrob Agents Chemother 59:2855–2866. https://doi.org/10.1128/AAC.04907-14.
43. Howard SJ, Lass-Florl C, Cuenca-Estrella M, Gomez-Lopez A, Arendrup MC. 2013. Determination of isavuconazole susceptibility of *Aspergillus* and *Candida* species by the EUCAST method. Antimicrob Agents Chemother 57:5426–5431. https://doi.org/10.1128/AAC.01111-13.
44. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 2004. Substitutions at methionine 220 in the 14α-sterol demethylase (*cyp51A*) of *Aspergillus fumigatus* are responsible for resistance *in vitro* to azole antifungal drugs. Antimicrob Agents Chemother 48:2747–2750. https://doi.org/10.1128/AAC.48.7.2747-2750.2004.
45. Wiederhold NP, Gil VG, Gutierrez F, Lindner JR, Albataineh MT, McCarthy DI, Sanders C, Fan H, Fothergill AW, Sutton DA. 2016. First detection of TR34/L98H and TR46/Y121F/T289A *cyp51* mutations in *Aspergillus fumigatus* isolates in the United States. J Clin Microbiol 54:168–171. https://doi.org/10.1128/JCM.02478-15.
46. Sharma C, Kumar R, Kumar N, Masih A, Gupta D, Chowdhary A. 2018. Investigation of multiple resistance mechanisms in voriconazole-resistant *Aspergillus flavus* clinical isolates from a chest hospital surveillance in Delhi, India. Antimicrob Agents Chemother 62:e01928-17. https://doi.org/10.1128/AAC.01928-17.
47. Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. 2001. Identification of two different 14-α sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. J Clin Microbiol 39:2431–2438. https://doi.org/10.1128/JCM.39.7.2431-2438.2001.
48. de Hoog G, Guarro J, Tran CS, Wintermans RGF, Gene J. 1995. Hypho-

mycetes, p 380–1007. *In* de Hoog GS, Guarro J. (ed), Atlas of clinical fungi. John Wiley & Sons, Chichester, England.
49. Arendrup MC, Meletiadis J, Mouton JW, Lagrou K, Howard SJ, Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing. 2017. EUCAST DEFINITIVE DOCUMENT E.DEF 9.3.1: Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9_3_1_Mould_testing__definitive.pdf. Accessed 8 April 2019. EUCAST, Växjö, Sweden.
50. EUCAST. 2018. Antifungal breakpoint tables for interpretation of MICs v9.0. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/Antifungal_breakpoints_v_9.0_180212.pdf. Accessed 8 April 2019. EUCAST, Växjö, Sweden.
51. Tang CM, Cohen J, Holden DW. 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol Microbiol 6:1663–1671. https://doi.org/10.1111/j.1365-2958.1992.tb00891.x.
52. White TBT, Lee SB. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press, San Diego, CA.
53. Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA. 2005. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot Cell 4:625–632. https://doi.org/10.1128/EC.4.3.625-632.2005.

3.3. Capítulo 3

3.3. Capítulo 3. Desarrollo, estandarización y validación de una metodología basada en el análisis de curvas de fusión de alta resolución para detectar la resistencia a azoles en *Aspergillus fumigatus*

Publicación original:

Bernal-Martinez L, Gil H, **Rivero-Menendez O**, Gago S, Cuenca-Estrella M, Mellado E, Alastruey Izquierdo A. 2017. Development and validation of a high resolution melting assay to detect azole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother*, 61(12): e01083-17. doi: 0.1128/AAC.01083-17.

Aspergillus fumigatus es la causa más frecuente de aspergilosis invasiva en individuos inmunocomprometidos, siendo los triazoles la terapia de primera línea para su tratamiento. Sin embargo, en la última década se ha detectado un progresivo aumento de aislados clínicos resistentes a esta clase de antifúngicos en todo el mundo, evidenciando la importancia que tiene la rápida detección de este fenómeno para la elección de una terapia apropiada. El mecanismo de resistencia principal a los azoles se basa en la aparición de alteraciones en el gen *cyp51A*, el cual codifica la diana de estos fármacos, la 14 α-esterol demetilasa. Se han descrito varias mutaciones en este gen directamente relacionadas con la aparición de resistencia, las cuales están asociadas a distintitos perfiles de sensibilidad a esta familia de compuestos.

Este trabajo consistió en el desarrollo de una técnica que, mediante la amplificación simultánea bajo las mismas condiciones por PCR cuantitativa y la posterior diferenciación utilizando análisis de curvas de fusión de alta resolución, permite detectar y distinguir en un solo ensayo las mutaciones puntuales en Cyp51A en las posiciones G54, Y121, M220 y G448 y las principales repeticiones en tándem en el promotor del gen (TR_{34}, TR_{46} y TR_{53}) en cepas clínicas y ambientales de *A. fumigatus*. La estandarización del método se realizó utilizando 30 cepas resistentes de esta especie caracterizadas fenotípica y molecularmente y permitió la detección de todas las alteraciones por la agrupación en clústeres de aislados con el mismo mecanismo de resistencia. Su validación se llevó a cabo con un panel ciego de 80 cepas de *A. fumigatus* sensibles y resistentes a los azoles con distintas sustituciones en *cyp51A*, las cuales fueron clasificadas adecuadamente con esta técnica.

La principal ventaja de esta metodología reside en la reducción del tiempo necesario para obtener resultados acerca del perfil de sensibilidad a azoles y el mecanismo de resistencia asociado con respecto a otras técnicas.

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

Development and Validation of a High-Resolution Melting Assay To Detect Azole Resistance in *Aspergillus fumigatus*

L. Bernal-Martínez,[a,b] H. Gil,[a,c] O. Rivero-Menéndez,[a,b] S. Gago,[a] M. Cuenca-Estrella,[a,b] E. Mellado,[a,b] A. Alastruey-Izquierdo[a,b]

Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain[a]; Spanish Network for Research in Infectious Diseases, Instituto de Salud Carlos III, Madrid, Spain[b]; European Program for Public Health Microbiology Training, European Centre for Disease Prevention and Control, Stockholm, Sweden[c]

ABSTRACT The global emergence of azole-resistant *Aspergillus fumigatus* strains is a growing public health concern. Different patterns of azole resistance are linked to mutations in *cyp51A*. Therefore, accurate characterization of the mechanisms underlying azole resistance is critical to guide selection of the most appropriate antifungal agent for patients with aspergillosis. This study describes a new sequencing-free molecular screening tool for early detection of the most frequent mutations known to be associated with azole resistance in *A. fumigatus*. PCRs targeting *cyp51A* mutations at positions G54, Y121, G448, and M220 and targeting different tandem repeats (TRs) in the promoter region were designed. All PCRs were performed simultaneously, using the same cycling conditions. Amplicons were then distinguished using a high-resolution melting assay. For standardization, 30 well-characterized azole-resistant *A. fumigatus* strains were used, yielding melting curve clusters for different resistance mechanisms for each target and allowing detection of the most frequent azole resistance mutations, i.e., G54E, G54V, G54R, G54W, Y121F, M220V, M220I, M220T, M220K, and G448S, and the tandem repeats TR_{34}, TR_{46}, and TR_{53}. Validation of the method was performed using a blind panel of 80 *A. fumigatus* azole-susceptible or azole-resistant strains. All strains included in the blind panel were properly classified as susceptible or resistant with the developed method. The implementation of this screening method can reduce the time needed for the detection of azole-resistant *A. fumigatus* isolates and therefore facilitate selection of the best antifungal therapy in patients with aspergillosis.

KEYWORDS antifungal resistance, azoles, *cyp51A*, aspergillosis, *Aspergillus fumigatus*, TR_{34}/L98H, TR_{46}/Y121F/T289A, high-resolution melting

Received 25 May 2017 **Returned for modification** 10 July 2017 **Accepted** 3 September 2017

Accepted manuscript posted online 11 September 2017

Citation Bernal-Martínez L, Gil H, Rivero-Menéndez O, Gago S, Cuenca-Estrella M, Mellado E, Alastruey-Izquierdo A. 2017. Development and validation of a high-resolution melting assay to detect azole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 61:e01083-17. https://doi.org/10.1128/AAC.01083-17.

Address correspondence to A. Alastruey-Izquierdo, anaalastruey@isciii.es.

L.B.-M. and H.G. contributed equally to this work.

In recent years, azole resistance in *Aspergillus fumigatus* has been increasingly reported in clinical settings, representing a growing public health concern (1). In the Netherlands, azole resistance in *A. fumigatus* has been increasing since 1999, with resistance rates ranging from 4.3% to 19.2% in 2013 and from 3.8% to 13.3% in 2014 (2, 3). Similarly, an increase from 5% in 2004 to 20% in 2009 was observed in the United Kingdom (4, 5). Now the presence of azole-resistant strains has been reported in many countries from all continents, with strains being isolated from both environmental and clinical samples (6).

Azoles target 14α-sterol demethylase, a key enzyme of the ergosterol biosynthesis pathway, which is encoded in *Aspergillus* by *cyp51A*. Alteration of this gene is the major mechanism leading to azole resistance (7). Several point mutations in *cyp51A* have been described, although only some of them have been confirmed as being responsible for phenotypic resistance; those include mutations at positions glycine 54, methionine 220, and glycine 448 (8–10). However, the most frequent mechanism of resistance in *A. fumigatus* determined to date is a combination of a point mutation at position leucine

98 with a tandem repeat (TR) insertion of 34 bp in the promoter region of *cyp51A* (11). Recent studies indicate that approximately 50% of the increase in azole-resistant strains is due to the TR_{34}/L98H alteration (12). Other emerging mechanisms of resistance have also been described, such as two point mutations (Y121F and T289A) in combination with a 46-bp TR in the promoter (13), the point mutation Y121F alone (14), or a 53-bp TR with no associated point mutations (15, 16). These mutations confer different susceptibility profiles; G54 has been associated with resistance to itraconazole and posaconazole (8, 17), M220 with resistance to itraconazole, high MICs for voriconazole, and variable MICs for posaconazole (10), and G448 with voriconazole resistance and some reduction in itraconazole and posaconazole susceptibility (18). TR_{34}/L98H is described as being responsible for pan-azole resistance (11), TR_{46}/Y121F/T289A confers resistance to posaconazole and voriconazole, with variable susceptibilities to itraconazole (19, 20), and TR_{53} confers resistance to voriconazole and itraconazole and lower susceptibility to posaconazole (16).

The analysis of high-resolution melting (HRM) curves using fluorescent DNA-binding dyes with improved saturation properties allows precise assessment of sequences and can be used to identify single point mutations (21), reducing the time for characterization without the need for sequencing. Fast effective diagnosis using HRM assays has been performed for microbiological applications using various platforms (22–25). Since fast identification of azole resistance is critical for the selection of the proper antifungal drug for patients with invasive fungal disease, we have developed a screening tool based on this technology, targeting the most common *cyp51A* mutations associated with azole resistance in *A. fumigatus*.

(This work was presented in part at the 7th Advances Against Aspergillosis conference, Manchester, United Kingdom, 3 to 5 March 2016.)

RESULTS

Azole-resistant mutation discrimination by HRM analysis. The real-time PCR (RT-PCR)-amplified fragments ranged from 70 to 144 bp, with cycle thresholds of the amplification below 30 for all samples. After HRM analysis and standardization, normalized melting curve clusters for different resistance mechanisms were obtained for each target (Fig. 1).

The average melting temperature (T_m) values obtained for strains with mutations are summarized in Table 1. The specific amino acid changes at position G54 could not be distinguished, but any change from glycine was clearly differentiated from the wild-type (WT) sequence (Fig. 1A). For the Y121 target, two melting curve clusters allowed differentiation of tyrosine from phenylalanine (Fig. 1B); this also happened in the case of the G448S mutation (Fig. 1E). The three TRs were distinguished from the WT sequence by the melting curve clusters (Fig. 1C and D). Three of the four described mutations (i.e., M220I, M220T, and M220V) at position M220 were specifically differentiated, and all of them could be distinguished by the melting curves clusters (Fig. 1F). The mutation M220K presented a T_m of 82.9°C (Table 1) and was located in the same cluster as the WT strains, which complicated its proper detection. However, the PCR designed for M220K amplified only the strains that harbored this mutation, distinguishing them specifically from the others (Fig. 1G).

Validation of the method. A total of 80 strains (34 azole-resistant strains and 46 azole-susceptible strains) were included in the panel. All 46 azole-susceptible strains tested were classified as WT (see Table S1 in the supplemental material). Nine strains harbored mutations at position G54, i.e., G54E ($n = 3$), G54W ($n = 2$), G54V ($n = 3$), and G54R ($n = 1$). These mutations could not be distinguished, although they were clearly differentiated from the WT sequence and the strains were thus characterized as resistant. The two strains with Y121F were properly identified. For the M220 target, this methodology was able to specifically differentiate the strains in the blind panel that were harboring M220V ($n = 2$), M220I ($n = 1$), M220T ($n = 1$), or M220K ($n = 2$). The only strain in the panel with the G448S mutation was also properly identified. Strains in the panel that were carrying the TR (TR_{34} [$n = 14$], TR_{46} [$n = 2$], or TR_{53} [$n = 2$]) were specifically identified.

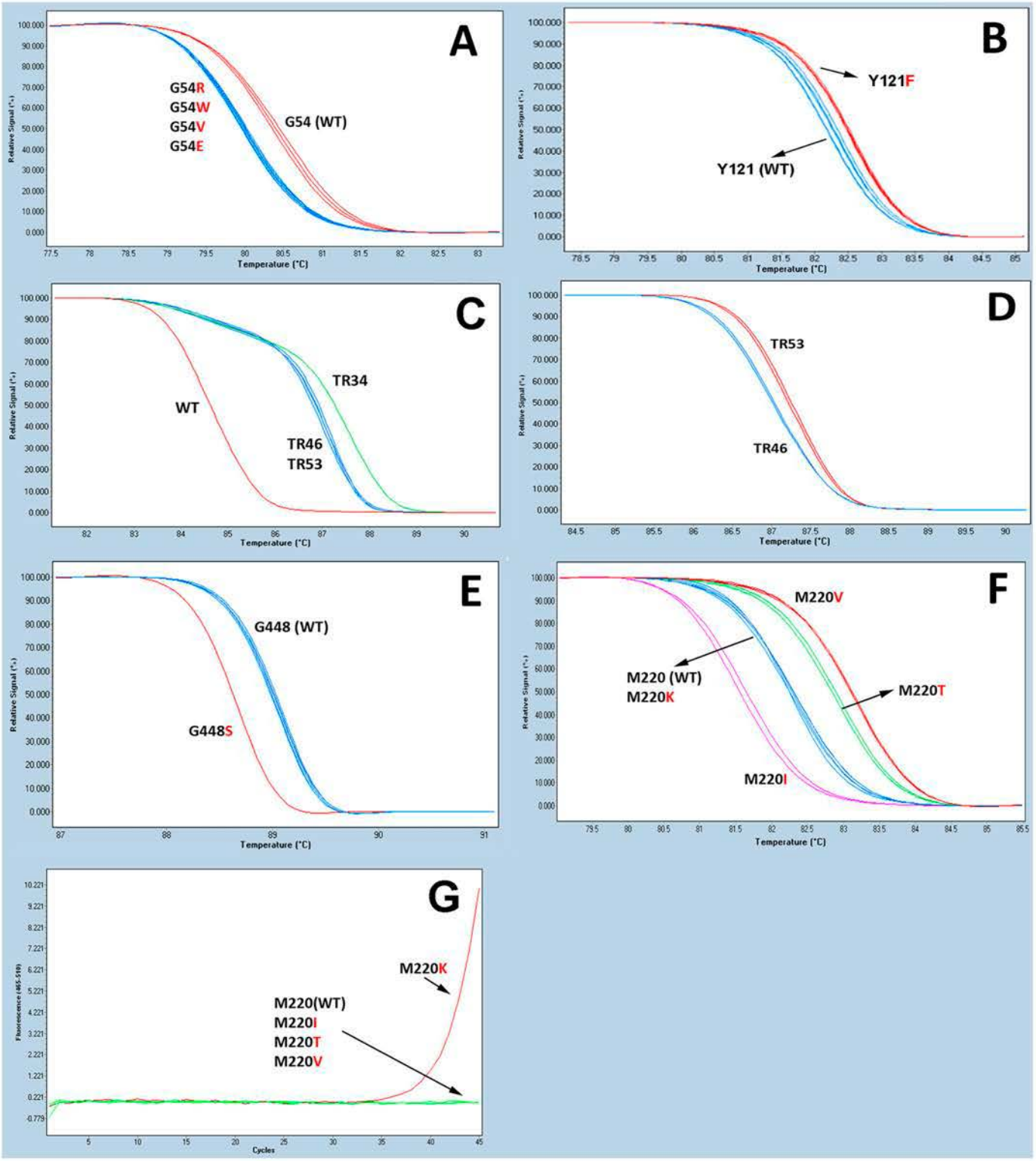


FIG 1 Representative HRM assays for the different targets used for the method, with the normalized profiles obtained. (A) G54 target. (B) Y121 target. (C) TR target. (D) TR target for differentiating TR46 and TR53. (E) G448 target. (F) M220 target. (G) Specific amplification of an *A. fumigatus* strain harboring the M220K mutation.

DISCUSSION

Azoles are currently the preferred agents for the treatment and prevention of invasive aspergillosis (IA). Voriconazole is the first-line therapy for invasive aspergillosis and posaconazole is recommended for primary prophylaxis, with voriconazole and

TABLE 1 Average T_m values for the targets and mutations included in this study

HRM target and mutation	T_m (°C)[a]
G54	
Wild type	80.6
G54R	80.1
G54V	80.2
G54E	80.3
G54W	79.9
Y121	
Wild type	82.2
Y121F	82.5
M220	
Wild type	82.9
M220K	82.9
M220I	82.1
M220T	83.4
M220V	82.6
G448	
Wild type	89.2
G448S	88.6
TR	
Wild type	84.6
TR_{34}	88.2
TR_{46}	87.1
TR_{53}	87.2

[a]T_m, melting temperature; TR, tandem repeat.

itraconazole as alternative agents (26, 27). Recently, isavuconazole was also approved for the treatment of IA, showing MICs similar to those obtained for voriconazole (28, 29, 30). The use of liposomal amphotericin B is indicated as an alternative treatment, although it can be associated with high toxicity (31). Combined therapies with azoles and echinocandins, mainly voriconazole and anidulafungin, have been tested and seem to be beneficial for IA treatment (32–34). As alternative rescue therapy, posaconazole or itraconazole is prescribed (27).

Azole-resistant *A. fumigatus* isolates have been increasingly reported in recent years (35–37). Although the number of patients affected is still limited (38), treatment options are clearly reduced and lead to poor outcomes (5, 39), as mutations or alterations in the *cyp51A* gene lead to different azole susceptibility profiles (8, 16, 17, 20). Thus, early diagnosis and a proper antifungal prescription have direct impacts on patient survival (40). In addition, susceptibility testing (especially for molds) is not routinely performed in all clinical laboratories and, when available, testing takes 48 to 72 h to be completed. This delays the detection of resistance and, therefore, the administration of an appropriate antifungal agent if different from the standard. Close surveillance of clinical azole-resistant strains and exhaustive screening of environmental isolates are vital for the management of antifungal resistance in human-pathogenic fungi (41).

To address all of these challenges, we have developed a single HRM assay to detect each of the most frequent mutations related to azole resistance (changes at G54, Y121, M220, or G448 and promoter insertions of variable sizes [TR_{34}, TR_{46}, or TR_{53}]). We were able to clearly differentiate the azole-resistant isolates from the WT isolates by HRM analysis, showing the reliability of this diagnostic tool for the rapid identification of known azole resistance mechanisms in both clinical and environmental *A. fumigatus* strains. This assay could be used as a screening method, reducing the time needed to obtain information on the azole resistance profile to a single day. In addition, the method can also be useful to detect the molecular mechanism of phenotypic resistance when the susceptibility profile has already

been determined, with the option of testing only the mutations that can explain the resistance pattern obtained.

Several molecular methods for the detection of mutations responsible for azole resistance were developed previously. Tuohy et al. developed a HRM assay to characterize the G54 codon in *cyp51A*, but other mutations associated with resistance were not included (42). Nested PCR assays for detecting mutations at position M220 (without discriminating among different base changes), L98H, and TR_{34} have also been described (43, 44). In contrast to nested PCR assays, our assay involves a closed-tube technique that reduces the possibility of contamination and the time to obtain results, since there is no need for sequencing. Other methods based on RT-PCR have been described but are able to detect fewer mutations, require the use of expensive fluorescent probes, and/or are performed in several steps (45–49).

Another advantage of the method developed is the potential detection of additional mutations in the amplified regions that have not been previously described, without the need to expand the assay, as well as the possibility of including new amplification regions if new resistance mechanisms arise. Moreover, the technique is very flexible and can be adapted to particular situations were only some of the mechanisms need to be detected, optimizing assay costs.

Although our HRM assay is a useful method for the identification of azole-resistant isolates, reducing the time for characterization without the need for sequencing, it also has some limitations. Mutations that include changes between adenine and thymine are harder to detect with this methodology. Indeed, we could not properly differentiate the strains harboring the M220K mutation from the WT strains, due to this change. Modification of the $MgCl_2$ concentration or the use of additives such as betaine or dimethyl sulfoxide, as suggested previously (50), did not improve the differentiation. To address this limitation, we have included an extra PCR that specifically detects this mutation and runs simultaneously with the rest of the assay.

Our method has not targeted L98 and T289 because these point mutations have never been reported alone. Moreover, laboratory-generated strains containing these single point mutations are not azole resistant (11, 51). In our study, the use of the PCR targeting TR was enough to identify all of the included TR_{34}/L98H and TR_{46}/Y121F/T289A isolates.

In addition, for some mutations, such as G448, M220T, M220I, and G54R, and $TR_{53,}$ a limited number of strains were available for testing. Even though the results were robust, more strains should be tested to confirm them. Another limitation is that this technique still requires the isolation of the strains. The identification of resistance mutations directly from clinical samples, such as blood, serum, or bronchoalveolar lavage fluid samples, could clearly reduce the response time (45, 48). However, the small amounts of *Aspergillus* in clinical samples represent a challenge. A multicopy target approach is recommended but is not possible for azole resistance characterization, since it is based on a single-copy target (52). Three studies detecting *cyp51A* mutations directly from respiratory samples have been published recently (44–46). However, those methods also have limitations; one of the studies detected only TR_{34}, L98H, Y121F, and T289A mutations via melting curve analysis (45, 46) and the other used nested PCR assays combined with sequencing (44).

In conclusion, the current study is the first to detect the most frequent azole-resistant strains simultaneously, based on HRM technology. This method is simple to perform, enabling rapid accurate detection of *A. fumigatus*-resistant strains. It is highly sensitive and specific, and it is suitable for routine screening clinical diagnostics, as the equipment needed is usually available in hospitals. The fast detection of resistant *A. fumigatus* strains with this technology will allow selection of proper antifungal treatments, improving the management of *A. fumigatus*-infected patients. Moreover, in the future, this approach could represent a good alternative to replace phenotypic methods such as antifungal susceptibility testing in clinical laboratories, allowing quick, precise, and reliable detection of the most frequent mutations.

TABLE 2 Phenotypic and genotypic characteristics of the strains used to standardize the method

Modification	No. of strains (n = 30)	Triazole phenotype[a]			Substitution or sequence
		ICZ	VCZ	PCZ	
Wild type	4	S	S	S	None
G54R	1	R	S	R	GGG to **A**GG
G54W	2	R	S	R	GGG to **T**GG
G54V	3	R	S	R	GGG to G**T**G
G54E	3	R	S	R	GGG to G**A**G
Y121F	2[b]	S[c]	R[c]	S[c]	TAT to T**T**T
M220K	2	R	S[d]	R	ATG to A**A**G
M220T	1	R	S[d]	S[d]	ATG to A**C**G
M220I	1	R	S[d]	R	ATG to AT**A**
M220V	2	R	S[d]	S[d]	ATG to **G**TG
G448S	1	S	R	R	GGT to **A**GT
TR_{34}	6	R	R	R	GAATCACGCGGTCCGATGTGTGCTGAGCCGAAT[e]
TR_{46}	2[b]	R[f]	R	R	GAATCACGCGGTCCGATGTGTGCTGAGCCGAATGAAAGTTGTCTA[e]
TR_{53}	2	R	R	S[d]	GAATCACGCGGTCCGATGTGTGCTGAGCCGAATGAAAGTTGTCTAATGTCTA[e]

[a]ICZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole; R, resistant; S, susceptible.
[b]Same strains containing the mutations TR_{46} and Y121F.
[c]The triazole phenotype is based on the results published for the only strain identified with this single point mutation (14).
[d]Elevated MIC.
[e]Sequence of the tandem repeat in the promoter region.
[f]Resistant phenotype variable.

MATERIALS AND METHODS

Strains analyzed in the study. Thirty well-characterized *A. fumigatus* clinical strains (26 azole-resistant strains and 4 azole-susceptible strains) from the mold collection of the Spanish National Centre for Microbiology were selected for establishment of the method (Table 2). Azole susceptibility was assessed using the EUCAST broth microdilution method (53). Strains were classified as susceptible or resistant according to EUCAST breakpoints for *A. fumigatus* and azoles (resistant to itraconazole and voriconazole with MICs of >2 mg/liter and resistant to posaconazole with MICs of >0.25 mg/liter) (54) (Table 2). The *cyp51A* gene, including its promoter region, was amplified and sequenced following the procedure described previously (11) for the detection of specific mutations associated with azole resistance (Table 2). In a second step, a blind panel of 80 strains of *A. fumigatus*, including 46 susceptible strains and 34 azole-resistant strains, was used for validation of the method.

DNA extraction from *A. fumigatus* strains. Strains were subcultured for 24 to 48 h at 30°C in glucose-yeast extract-peptone medium (0.3% yeast extract and 1% peptone; Difco, Soria Melguizo) with 2% glucose (Sigma-Aldrich Química, Madrid, Spain). After mechanical disruption of the fungus by

TABLE 3 Primers used in this study

Target and primers	Amplicon length (bp)	Sequence (5′ to 3′)	Source
G54	70		
G54F		TCATTGGGTCCCATTTCTG	49
G54R		GCACGCAAAGAAGAACTTG	49
Y121	72		
Y121F1		CATTGACGACCCCCGTTT	This study
Y121R1		TTTTCTGCTCCATCAGCTTG	This study
M220	77		
M220F		TCATGACCTGGACAAGGGC	49
M220R		TCGCTTCTTGTTATGCGGC	This study
M220K	144		
M220Kmod4F		TTTACTCCCATCAATTTTAA	This study
M220KR		TGATTTCTGAGAGTCCTTGTCAC	This study
G448	131		
G448F		TACTAAGGAGCAGGAGAA	This study
G448R		TGACATAAGCGAATTTCT	This study
TR	117		
TRF		GCACCACTTCAGAGTTG	This study
TRR1		ACCGTAGTATGAGTTAGG	This study

TABLE 4 Parameters established for HRM analysis

HRM target	Premelting temperature (°C)	Postmelting temperature (°C)	Threshold	Sensitivity
G54	77.4–78.4	83.7–84.7	0	0.30
Y121	78.2–79.2	84.2–85.2	0	0.20
M220	80.2–81.2	85.7–86.7	0	0.20
G448	86.9–87.9	90.1–91.1	0	0.20
TR_{34}	81.3–82.3	90.0–91.0	0	0.30
TR_{46}, TR_{53}	84.3–85.3	89.3–90.3	1	0.30

vortex-mixing of the mycelium with glass beads, genomic DNA was extracted using a phenol-chloroform method, following the protocol described previously (55). The extracted DNA was quantified with a Nanodrop 8000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and stored at −20°C until processing.

Amplification of targets. Five PCRs to amplify fragments containing the targeted mutations (G54, Y121, M220, and G448) in *cyp51A* and its promoter region, in which the three tandem repeats (TR_{34}, TR_{46}, and TR_{53}) are located, were developed. Because the M220 PCR failed to identify M220K, an extra PCR to specifically detect that mutation was also included in the method. Primers (Table 3) were designed using Beacon Designer 7.0 software (Premier Biosoft, Palo Alto, CA, USA) and were synthesized by Sigma-Aldrich. Reactions were performed in a 20-μl final reaction volume containing 1 μl of LightCycler 480 ResoLight dye (Roche Diagnostics, Mannheim, Germany), 10 μl of SensiMix DNA (Quantace; Ecogen, Madrid, Spain), 1 μM each primer, water (PCR-grade; Roche, Madrid, Spain), and 25 ng of DNA. All reactions were performed with the same cycling conditions, i.e., an initial denaturation step of 5 min at 95°C followed by 45 cycles of denaturation (10 s at 95°C), annealing (10 s at 60°C), and extension (10 s at 72°C). All samples were performed in duplicate; each experiment included positive controls, which contained the described mutations, and negative controls, in which water was added as the template.

High-resolution melting analysis. After amplification, melting curves were generated by ramping from 65°C to 95°C at 0.02°C/s, with 25 acquisitions per degree and a final cooling step of 30s at 40°C. HRM analysis was performed using LightCycler 480 Gene Scanning software (Roche) in the LightCycler 480 instrument II (Roche). The melting curve data were manually adjusted and the fluorescence values were normalized, fixing the premelting and postmelting slider settings to range from 77.4 to 87.9°C and from 83.7 to 91.1°C, respectively, with a threshold of 0 or 1 for the specific amplification and a sensitivity range of 0.20 to 0.30. In the case of the TR target, we performed a second analysis to discriminate between TR_{46} and TR_{53}, adjusting the premelting and postmelting slider settings to 84.3 to 85.3°C and 89.3 to 90.3°C, respectively. The optimized conditions for each HRM assay are shown in Table 4.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01083-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

This study was financed by a research project from the Fondo de Investigación Sanitaria (FIS) (project PI13/02145). It was supported by Plan Nacional de I+D+i and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, and the Spanish Network for Research in Infectious Diseases (REIPI grant RD12/0015), cofinanced by the European Development Regional Fund. L.B.-M. has a research contract from the Spanish Network for Research in Infectious Diseases (REIPI grant RD12/0015). O.R.-M. holds a predoctoral fellowship from the Fondo de Investigaciones Sanitarias (grant FI14CIII/00025). S.G. was supported by a research fellowship from the Fondo de Investigaciones Sanitarias of the Spanish Ministry of Science and Innovation (grant FI10/00464). E.M. was supported by a project from the Spanish Fondo de Investigación Sanitaria (FIS grant PI15CIII/00019).

In the past 5 years, M.C.-E. has received grant support from Astellas Pharma, bioMérieux, Gilead Sciences, Merck Sharp & Dohme, Pfizer, Schering-Plough, Soria Melguizo SA, Ferrer International, the European Union, the ALBAN Program, the Spanish Agency for International Cooperation, the Spanish Ministry of Culture and Education, the Spanish Health Research Fund, the Instituto de Salud Carlos III, the Ramon Areces Foundation, and the Mutua Madrileña Foundation. He has been an advisor/consultant to the Panamerican Health Organization, Astellas Pharma, Gilead Sciences,

and Merck Sharp & Dohme. A.A.-I. has received grant or travel support from Gilead Sciences and Pfizer. The other authors declare no conflicts of interest.

REFERENCES

1. European Centre for Disease Prevention and Control. 2013. Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species. European Centre for Disease Prevention and Control, Stockholm, Sweden. https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/risk-assessment-impact-environmental-usage-of-triazoles-on-Aspergillus-spp-resistance-to-medical-triazoles.pdf.
2. Snelders E, van der Lee HA, Kuijpers J, Rijs AJ, Varga J, Samson RA, Mellado E, Donders AR, Melchers WJ, Verweij PE. 2008. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. PloS Med 5:e219. https://doi.org/10.1371/journal.pmed.0050219.
3. van der Linden JW, Arendrup MC, Melchers WJ, Verweij PE. 2016. Azole resistance of *Aspergillus fumigatus* in immunocompromised patients with invasive aspergillosis. Emerg Infect Dis 22:158–159. https://doi.org/10.3201/eid2201.151308.
4. Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, Denning DW. 2010. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. J Antimicrob Chemother 65:2116–2118. https://doi.org/10.1093/jac/dkq279.
5. Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, Laverdiere M, Arendrup MC, Perlin DS, Denning DW. 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerg Infect Dis 15:1068–1076. https://doi.org/10.3201/eid1507.090043.
6. Rivero-Menendez O, Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M. 2016. Triazole resistance in *Aspergillus* spp.: a worldwide problem? J Fungi (Basel) 2:1–21. https://doi.org/10.3390/jof2030021.
7. Denning DW, Perlin DS. 2011. Azole resistance in *Aspergillus*: a growing public health menace. Future Microbiol 6:1229–1232. https://doi.org/10.2217/fmb.11.118.
8. Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL. 2003. A point mutation in the 14α-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 47:1120–1124. https://doi.org/10.1128/AAC.47.3.1120-1124.2003.
9. Manavathu EK, Alangaden GJ, Chandrasekar PH. 2003. Differential activity of triazoles in two-drug combinations with the echinocandin caspofungin against *Aspergillus fumigatus*. J Antimicrob Chemother 51:1423–1425. https://doi.org/10.1093/jac/dkg242.
10. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 2004. Substitutions at methionine 220 in the 14α-sterol demethylase (*cyp51A*) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. Antimicrob Agents Chemother 48:2747–2750. https://doi.org/10.1128/AAC.48.7.2747-2750.2004.
11. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Melchers WJ, Verweij PE, Cuenca-Estrella M, Rodriguez-Tudela JL. 2007. A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. Antimicrob Agents Chemother 51:1897–1904. https://doi.org/10.1128/AAC.01092-06.
12. Jensen RH, Hagen F, Astvad KM, Tyron A, Meis JF, Arendrup MC. 2016. Azole-resistant *Aspergillus fumigatus* in Denmark: a laboratory-based study on resistance mechanisms and genotypes. Clin Microbiol Infect 22:570–579. https://doi.org/10.1016/j.cmi.2016.04.001.
13. van der Linden JW, Camps SM, Kampinga GA, Arends JP, Debets-Ossenkopp YJ, Haas PJ, Rijnders BJ, Kuijper EJ, Van Tiel FH, Varga J, Karawajczyk A, Zoll J, Melchers WJ, Verweij PE. 2013. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. Clin Infect Dis 57:513–520. https://doi.org/10.1093/cid/cit320.
14. Lescar J, Meyer I, Akshita K, Srinivasaraghavan K, Verma C, Palous M, Mazier D, Datry A, Fekkar A. 2014. *Aspergillus fumigatus* harbouring the sole Y121F mutation shows decreased susceptibility to voriconazole but maintained susceptibility to itraconazole and posaconazole. J Antimicrob Chemother 69:3244–3247. https://doi.org/10.1093/jac/dku316.
15. Mellado E, Alcazar-Fuoli L, Pajkrt D, Verweij PE, Melchers WJ, Cuenca-Estrella M, Rodriguez-Tudela JL. 2007. Alteration of the *cyp51A* gene promoter contribute to *Aspergillus fumigatus* multiple triazole resistance, abstr M-521. Abstr 47th Intersci Conf Antimicrob Agents Chemother.
16. Hodiamont CJ, Dolman KM, Ten Berge IJ, Melchers WJ, Verweij PE, Pajkrt D. 2009. Multiple-azole-resistant *Aspergillus fumigatus* osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. Med Mycol 47:217–220. https://doi.org/10.1080/13693780802545600.
17. Mann PA, Parmegiani RM, Wei SQ, Mendrick CA, Li X, Loebenberg D, DiDomenico B, Hare RS, Walker SS, McNicholas PM. 2003. Mutations in *Aspergillus fumigatus* resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14α-demethylase. Antimicrob Agents Chemother 47:577–581. https://doi.org/10.1128/AAC.47.2.577-581.2003.
18. Pelaez T, Gijon P, Bunsow E, Bouza E, Sanchez-Yebra W, Valerio M, Gama B, Cuenca-Estrella M, Mellado E. 2012. Resistance to voriconazole due to a G448S substitution in *Aspergillus fumigatus* in a patient with cerebral aspergillosis. J Clin Microbiol 50:2531–2534. https://doi.org/10.1128/JCM.00329-12.
19. Pelaez T, Monteiro MC, Garcia-Rubio R, Bouza E, Gomez-Lopez A, Mellado E. 2015. First detection of *Aspergillus fumigatus* azole-resistant strain due to Cyp51A TR46/Y121F/T289A in an azole-naive patient in Spain. New Microbes New Infect 6:33–34. https://doi.org/10.1016/j.nmni.2015.04.005.
20. van Ingen J, van der Lee HA, Rijs TA, Zoll J, Leenstra T, Melchers WJ, Verweij PE. 2015. Azole, polyene and echinocandin MIC distributions for wild-type, TR34/L98H and TR46/Y121F/T289A *Aspergillus fumigatus* isolates in the Netherlands. J Antimicrob Chemother 70:178–181. https://doi.org/10.1093/jac/dku364.
21. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. 2003. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 49:853–860. https://doi.org/10.1373/49.6.853.
22. Erali M, Voelkerding KV, Wittwer CT. 2008. High resolution melting applications for clinical laboratory medicine. Exp Mol Pathol 85:50–58. https://doi.org/10.1016/j.yexmp.2008.03.012.
23. Hoek KG, Gey van Pittius NC, Moolman-Smook H, Carelse-Tofa K, Jordaan A, van der Spuy GD, Streicher E, Victor TC, van Helden PD, Warren RM. 2008. Fluorometric assay for testing rifampin susceptibility of *Mycobacterium tuberculosis* complex. J Clin Microbiol 46:1369–1373. https://doi.org/10.1128/JCM.02343-07.
24. Lin JH, Tseng CP, Chen YJ, Lin CY, Chang SS, Wu HS, Cheng JC. 2008. Rapid differentiation of influenza A virus subtypes and genetic screening for virus variants by high-resolution melting analysis. J Clin Microbiol 46:1090–1097. https://doi.org/10.1128/JCM.02015-07.
25. Slinger R, Bellfoy D, Desjardins M, Chan F. 2007. High-resolution melting assay for the detection of *gyrA* mutations causing quinolone resistance in *Salmonella enterica* serovars Typhi and Paratyphi. Diagn Microbiol Infect Dis 57:455–458. https://doi.org/10.1016/j.diagmicrobio.2006.09.011.
26. Leroux S, Ullmann AJ. 2013. Management and diagnostic guidelines for fungal diseases in infectious diseases and clinical microbiology: critical appraisal. Clin Microbiol Infect 19:1115–1121. https://doi.org/10.1111/1469-0691.12426.
27. Patterson TF, Thompson GR, III, Denning DW, Fishman JA, Hadley S, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Nguyen MH, Segal BH, Steinbach WJ, Stevens DA, Walsh TJ, Wingard JR, Young JA, Bennett JE. 2016. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis 63:e1–e60. https://doi.org/10.1093/cid/ciw326.
28. Gregson L, Goodwin J, Johnson A, McEntee L, Moore CB, Richardson M, Hope WW, Howard SJ. 2013. *In vitro* susceptibility of *Aspergillus fumigatus* to isavuconazole: correlation with itraconazole, voriconazole, and posaconazole. Antimicrob Agents Chemother 57:5778–5780. https://doi.org/10.1128/AAC.01141-13.
29. Howard SJ, Lass-Florl C, Cuenca-Estrella M, Gomez-Lopez A, Arendrup MC. 2013. Determination of isavuconazole susceptibility of *Aspergillus* and *Candida* species by the EUCAST method. Antimicrob Agents Chemother 57:5426–5431. https://doi.org/10.1128/AAC.01111-13.

30. Maertens JA, Raad II, Marr KA, Patterson TF, Kontoyiannis DP, Cornely OA, Bow EJ, Rahav G, Neofytos D, Aoun M, Baddley JW, Giladi M, Heinz WJ, Herbrecht R, Hope W, Karthaus M, Lee DG, Lortholary O, Morrison VA, Oren I, Selleslag D, Shoham S, Thompson GR, III, Lee M, Maher RM, Schmitt-Hoffmann AH, Zeiher B, Ullmann AJ. 2016. Isavuconazole versus voriconazole for primary treatment of invasive mould disease caused by *Aspergillus* and other filamentous fungi (SECURE): a phase 3, randomised-controlled, non-inferiority trial. Lancet 387:760–769. https://doi.org/10.1016/S0140-6736(15)01159-9.
31. Sabra R, Branch RA. 1990. Amphotericin B nephrotoxicity. Drug Saf 5:94–108. https://doi.org/10.2165/00002018-199005020-00003.
32. Ledoux MP, Toussaint E, Denis J, Herbrecht R. 2017. New pharmacological opportunities for the treatment of invasive mould diseases. J Antimicrob Chemother 72:i48–i58. https://doi.org/10.1093/jac/dkx033.
33. Seyedmousavi S, Bruggemann RJ, Melchers WJ, Rijs AJ, Verweij PE, Mouton JW. 2013. Efficacy and pharmacodynamics of voriconazole combined with anidulafungin in azole-resistant invasive aspergillosis. J Antimicrob Chemother 68:385–393. https://doi.org/10.1093/jac/dks402.
34. Seyedmousavi S, Mouton JW, Melchers WJ, Bruggemann RJ, Verweij PE. 2014. The role of azoles in the management of azole-resistant aspergillosis: from the bench to the bedside. Drug Resist Updat 17:37–50. https://doi.org/10.1016/j.drup.2014.06.001.
35. Jeanvoine A, Rocchi S, Reboux G, Grenouillet F, Benassarou M, Chirouze C, Millon L. 2016. Sinus aspergillosis due to an azole-resistant *Aspergillus fumigatus* strain carrying the TR34/L98H mutation in immunocompetent host. Infect Dis 48:765–766. https://doi.org/10.1080/23744235.2016.1193791.
36. Mellado E, De La Camara R, Buendia B, Rodriguez-Tudela JL, Cuenca-Estrella M. 2013. Breakthrough pulmonary *Aspergillus fumigatus* infection with multiple triazole resistance in a Spanish patient with chronic myeloid leukemia. Rev Iberoam Micol 30:64–68. https://doi.org/10.1016/j.riam.2012.09.002.
37. Prigitano A, Esposto MC, Biffi A, De Lorenzis G, Favuzzi V, Koncan R, Lo Cascio G, Barao Ocampo M, Colombo C, Pizzamiglio G, Romano L, Tortorano AM. 2017. Triazole resistance in *Aspergillus fumigatus* isolates from patients with cystic fibrosis in Italy. J Cyst Fibros 16:64–69. https://doi.org/10.1016/j.jcf.2016.06.006.
38. Alanio A, Sitterle E, Liance M, Farrugia C, Foulet F, Botterel F, Hicheri Y, Cordonnier C, Costa JM, Bretagne S. 2011. Low prevalence of resistance to azoles in *Aspergillus fumigatus* in a French cohort of patients treated for haematological malignancies. J Antimicrob Chemother 66:371–374. https://doi.org/10.1093/jac/dkq450.
39. van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, Kuijper EJ, Van Tiel FH, Melchers WJ, Verweij PE. 2011. Clinical implications of azole resistance in *Aspergillus fumigatus*, the Netherlands, 2007–2009. Emerg Infect Dis 17:1846–1854. https://doi.org/10.3201/eid1710.110226.
40. Bernal-Martinez L, Alastruey-Izquierdo A, Cuenca-Estrella M. 2016. Diagnostics and susceptibility testing in *Aspergillus*. Future Microbiol 11:315–328. https://doi.org/10.2217/fmb.15.140.
41. van der Linden JW, Arendrup MC, Warris A, Lagrou K, Pelloux H, Hauser PM, Chryssanthou E, Mellado E, Kidd SE, Tortorano AM, Dannaoui E, Gaustad P, Baddley JW, Uekotter A, Lass-Florl C, Klimko N, Moore CB, Denning DW, Pasqualotto AC, Kibbler C, Arikan-Akdagli S, Andes D, Meletiadis J, Naumiuk L, Nucci M, Melchers WJ, Verweij PE. 2015. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. Emerg Infect Dis 21:1041–1044. https://doi.org/10.3201/eid2106.140717.
42. Tuohy MJ, Reja V, Park S, Perlin DS, Wnek M, Procop GW, Yen-Lieberman B. 2010. Use of a high-resolution melt assay to characterize codon 54 of the *cyp51A* gene of *Aspergillus fumigatus* on a Rotor-Gene 6000 instrument. Antimicrob Agents Chemother 54:2248–2251. https://doi.org/10.1128/AAC.01712-09.
43. Spiess B, Seifarth W, Merker N, Howard SJ, Reinwald M, Dietz A, Hofmann WK, Buchheidt D. 2012. Development of novel PCR assays to detect azole resistance-mediating mutations of the *Aspergillus fumigatus cyp51A* gene in primary clinical samples from neutropenic patients. Antimicrob Agents Chemother 56:3905–3910. https://doi.org/10.1128/AAC.05902-11.
44. Zhao Y, Garnaud C, Brenier-Pinchart MP, Thiebaut-Bertrand A, Saint-Raymond C, Camara B, Hamidfar R, Cognet O, Maubon D, Cornet M, Perlin DS. 2016. Direct molecular diagnosis of aspergillosis and CYP51A profiling from respiratory samples of French patients. Front Microbiol 7:1164. https://doi.org/10.3389/fmicb.2016.01164.
45. Chong GL, van de Sande WW, Dingemans GJ, Gaajetaan GR, Vonk AG, Hayette MP, van Tegelen DW, Simons GF, Rijnders BJ. 2015. Validation of a new *Aspergillus* real-time PCR assay for direct detection of *Aspergillus* and azole resistance of *Aspergillus fumigatus* on bronchoalveolar lavage fluid. J Clin Microbiol 53:868–874. https://doi.org/10.1128/JCM.03216-14.
46. Chong GM, van der Beek MT, von dem Borne PA, Boelens J, Steel E, Kampinga GA, Span LF, Lagrou K, Maertens JA, Dingemans GJ, Gaajetaan GR, van Tegelen DW, Cornelissen JJ, Vonk AG, Rijnders BJ. 2016. PCR-based detection of *Aspergillus fumigatus* Cyp51A mutations on bronchoalveolar lavage: a multicentre validation of the AsperGenius assay in 201 patients with haematological disease suspected for invasive aspergillosis. J Antimicrob Chemother 71:3528–3535. https://doi.org/10.1093/jac/dkw323.
47. Klaassen CH, de Valk HA, Curfs-Breuker IM, Meis JF. 2010. Novel mixed-format real-time PCR assay to detect mutations conferring resistance to triazoles in *Aspergillus fumigatus* and prevalence of multi-triazole resistance among clinical isolates in the Netherlands. J Antimicrob Chemother 65:901–905. https://doi.org/10.1093/jac/dkq041.
48. White PL, Posso RB, Barnes RA. 2015. Analytical and clinical evaluation of the PathoNostics AsperGenius assay for detection of invasive aspergillosis and resistance to azole antifungal drugs during testing of serum samples. J Clin Microbiol 53:2115–2121. https://doi.org/10.1128/JCM.00667-15.
49. Garcia-Effron G, Dilger A, Alcazar-Fuoli L, Park S, Mellado E, Perlin DS. 2008. Rapid detection of triazole antifungal resistance in *Aspergillus fumigatus*. J Clin Microbiol 46:1200–1206. https://doi.org/10.1128/JCM.02330-07.
50. Song C, Castellanos-Rizaldos E, Bejar R, Ebert BL, Makrigiorgos GM. 2015. DMSO increases mutation scanning detection sensitivity of high-resolution melting in clinical samples. Clin Chem 61:1354–1362. https://doi.org/10.1373/clinchem.2015.245357.
51. Snelders E, Camps SM, Karawajczyk A, Rijs AJ, Zoll J, Verweij PE, Melchers WJ. 2015. Genotype-phenotype complexity of the TR_{46}/Y121F/T289A *cyp51A* azole resistance mechanism in *Aspergillus fumigatus*. Fungal Genet Biol 82:129–135. https://doi.org/10.1016/j.fgb.2015.06.001.
52. Alanio A, Bretagne S. 2017. Performance evaluation of multiplex PCR including *Aspergillus*: not so simple! Med Mycol 55:56–62. https://doi.org/10.1093/mmy/myw080.
53. Arendrup MC, Guinea J, Cuenca-Estrella M, Meletiadis J, Mouton JW, Lagrou K, Howard SJ. 2015. EUCAST definitive document E.DEF 9.3: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. EUCAST, Basel, Switzerland. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9_3_Mould_testing_definitive.pdf.
54. EUCAST. 2016. Antifungal breakpoint tables for interpretation of MICs v 8.0. EUCAST, Basel, Switzerland. http://www.eucast.org./fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/Antifungal_breakpoints_v_8.0_November_2015.pdf.
55. Tang CM, Cohen J, Holden DW. 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol Microbiol 6:1663–1671. https://doi.org/10.1111/j.1365-2958.1992.tb00891.x.

3.3.1. Material suplementario

Tabla S1. Panel de las 80 cepas sensibles y resistentes a los triazoles utilizadas para validar la técnica.

Strain N	G54	Y121	M220	M220K	G448	TR	DNA sequencing result	Triazole phenotype
1	WT	WT	WT	WT	WT	WT	WT	S
2	WT	WT	WT	WT	WT	WT	WT	S
3	G54 (R/W/V/E)	WT	WT	WT	WT	WT	G54E	R
4	WT	WT	M220V	WT	WT	WT	M220V	R
5	G54 (R/W/V/E)	WT	WT	WT	WT	WT	G54E	R
6	G54 (R/W/V/E)	WT	WT	WT	WT	WT	G54W	R
7	G54 (R/W/V/E)	WT	WT	WT	WT	WT	G54R	R
8	WT	WT	M220I	WT	WT	WT	M220I	R
9	WT	WT	M220T	WT	WT	WT	M220T	R
10	G54 (R/W/V/E)	WT	WT	WT	WT	WT	G54E	R
11	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
12	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
13	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
14	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
15	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
16	WT	WT	WT	WT	WT	WT	WT	S
17	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
18	WT	WT	WT	WT	WT	WT	WT	S
19	WT	WT	WT	WT	WT	WT	WT	S
20	G54 (R/W/V/E)	WT	WT	WT	WT	WT	G54V	R
21	WT	Y121F	WT	WT	WT	TR_{46}	TR_{46}/Y121F	R
22	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
23	WT	WT	WT	WT	WT	WT	WT	S
24	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
25	WT	WT	WT	M220K	WT	WT	M220K	R
26	WT	WT	WT	WT	WT	WT	WT	S
27	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
28	WT	WT	WT	WT	WT	WT	WT	S
29	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
30	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
31	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
32	WT	WT	WT	WT	WT	WT	WT	S
33	WT	Y121F	WT	WT	WT	TR_{46}	TR_{46}/Y121F	R
34	WT	WT	WT	WT	WT	TR_{53}	TR_{53}	R
35	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
36	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
37	G54 (R/W/V/E)	WT	WT	WT	WT	WT	G54W	R
38	WT	WT	WT	WT	WT	WT	WT	S
39	WT	WT	WT	WT	WT	WT	WT	S
40	WT	WT	WT	WT	WT	WT	WT	S
41	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
42	G54 (R/W/V/E)	WT	WT	WT	WT	WT	G54V	R

43	WT	WT	WT	WT	WT	TR_{53}	TR_{53}	R
44	WT	WT	WT	WT	WT	WT	WT	S
45	G54 (R/W/V/E)	WT	WT	WT	WT	WT	G54V	R
46	WT	WT	WT	WT	WT	WT	WT	S
47	WT	WT	WT	WT	WT	WT	WT	S
48	WT	WT	WT	WT	WT	WT	WT	S
49	WT	WT	WT	WT	G448S	WT	G448S	R
50	WT	WT	WT	WT	WT	WT	WT	S
51	WT	WT	WT	WT	WT	WT	WT	S
52	WT	WT	WT	WT	WT	WT	WT	S
53	WT	WT	WT	WT	WT	WT	WT	S
54	WT	WT	WT	WT	WT	WT	WT	S
55	WT	WT	WT	WT	WT	WT	WT	S
56	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
57	WT	WT	WT	WT	WT	WT	WT	S
58	WT	WT	WT	WT	WT	WT	WT	S
59	WT	WT	WT	WT	WT	WT	WT	S
60	WT	WT	WT	WT	WT	WT	WT	S
61	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
62	WT	WT	WT	WT	WT	TR_{34}	TR_{34}	R
63	WT	WT	WT	WT	WT	WT	WT	S
64	WT	WT	WT	WT	WT	WT	WT	S
65	WT	WT	WT	WT	WT	WT	WT	S
66	WT	WT	WT	WT	WT	WT	WT	S
67	WT	WT	WT	WT	WT	WT	WT	S
68	WT	WT	WT	WT	WT	WT	WT	S
69	WT	WT	WT	WT	WT	WT	WT	S
70	WT	WT	WT	WT	WT	WT	WT	S
71	WT	WT	WT	WT	WT	WT	WT	S
72	WT	WT	WT	WT	WT	WT	WT	S
73	WT	WT	WT	WT	WT	WT	WT	S
74	WT	WT	WT	WT	WT	WT	WT	S
75	WT	WT	WT	WT	WT	WT	WT	S
76	WT	WT	WT	WT	WT	WT	WT	S
77	WT	WT	WT	M220K	WT	WT	M220K	R
78	WT	WT	M220V	WT	WT	WT	M220V	R
79	WT	WT	WT	WT	WT	WT	WT	S
80	WT	WT	WT	WT	WT	WT	WT	S

3.4. Capítulo 4

3.4.1. Artículo 1 del capítulo 4. Estudio de la actividad *in vitro* del manogepix, comparada con la de otros antifúngicos en uso, frente a un conjunto de especies fúngicas emergentes mediante el uso de las metodologías EUCAST y CLSI

Publicación original:

Rivero-Menendez O, Cuenca-Estrella M, Alastruey-Izquierdo A. 2019. *In vitro* activity of APX001A against rare moulds using EUCAST and CLSI methodologies. *J Antimicrob Chemother*, 74: 1295-1299. doi: 10.1093/jac/dkz022.

El manogepix, anteriormente denominado APX001A o E1210, es un nuevo compuesto con capacidad antifúngica de amplio espectro que inhibe la proteína Gwt1, la cual juega un papel indispensable en la integridad de la pared celular de los hongos. Se ha demostrado que esta droga tiene actividad *in vitro* frente a muchas especies de *Candida* y *Aspergillus*, así como es eficaz en formulación oral, intravenosa o intraperitoneal para tratar modelos murinos con infecciones causadas por los principales patógenos fúngicos de estos géneros. A su vez, *Cryptococcus neoformans*, *Coccidioides immitis* y *Coccidioides posadasii* también han sido probadas con éxito frente a este compuesto. Sin embargo, los estudios que ensayan el manogepix frente a *Fusarium*, *Scedosporium* o Mucorales, cuyas especies se caracterizan por presentar resistencia intrínseca a los antifúngicos en uso, son escasos. Asimismo, algunas de las especies crípticas de *Aspergillus* exhiben sensibilidad reducida a los tratamientos disponibles, y la efectividad de este compuesto no ha sido comprobada frente a ellas.

Por ello, en este trabajo se investigó la actividad *in vitro* del manogepix frente a una colección de 200 cepas pertenecientes a diversas especies de *Scedosporium*, *Lomentospora*, *Fusarium*, *Alternaria* y Mucorales, así como a las especies crípticas más frecuentes de *Aspergillus*. Su eficacia fue comparada con la de la anfotericina B, el posaconazol y la micafungina mediante la determinación de la sensibilidad antifúngica de todas las cepas por EUCAST y CLSI.

A pesar de que el manogepix, por lo general, no mostró actividad frente a las especies de Mucorales y solo fue moderadamente eficaz frente a *Fusarium solani* y *Alternaria alternata*, fue activo frente a las especies de *Scedosporium* y *Lomentospora*, además de frente a las crípticas de *Aspergillus*. Estos resultados son acordes a los obtenidos en otros estudios en los que se probó este compuesto frente a alguna de las especies de estos géneros. Por tanto, el manogepix se postula como un buen candidato para el tratamiento de algunos de los hongos emergentes que causan infecciones que son difíciles de curar con las terapias antifúngicas actualmente disponibles.

J Antimicrob Chemother 2019; **74**: 1295–1299
doi:10.1093/jac/dkz022 Advance Access publication 6 February 2019

Journal of Antimicrobial Chemotherapy

In vitro activity of APX001A against rare moulds using EUCAST and CLSI methodologies

Olga Rivero-Menendez, Manuel Cuenca-Estrella and Ana Alastruey-Izquierdo*

Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain; Spanish Network for the Research in Infectious Diseases (RD16/CIII/0004/0003), Instituto de Salud Carlos III, Madrid, Spain

*Corresponding author. Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain. Tel: +34-91-822-3784; Fax: +34-91-509-7966; E-mail: anaalastruey@isciii.es

Received 2 November 2018; returned 30 November 2018; revised 19 December 2018; accepted 4 January 2019

Background: APX001A (E1210) is a novel broad-spectrum antifungal agent that inhibits Gwt1p, a protein that plays an important role in fungal cell wall integrity. Previous studies have shown that APX001A has broad activity against most species of *Candida*, *Aspergillus*, *Scedosporium*, *Fusarium* and Mucorales.

Objectives: To investigate the *in vitro* activity of APX001A against 200 isolates belonging to 20 different species of *Fusarium*, *Scedosporium*, *Lomentospora*, *Alternaria*, cryptic species of *Aspergillus* and Mucorales.

Methods: APX001A and comparators were tested using EUCAST and CLSI methodologies for broth microdilution susceptibility testing of antifungal agents.

Results: APX001A was generally inactive against Mucorales, but active against all cryptic species of *Aspergillus* and *Scedosporium*/*Lomentospora* species.

Conclusions: APX001A shows encouraging *in vitro* activity against some emerging fungi that are hard to treat with currently available antifungals.

Introduction

In recent decades there has been an increasing number of invasive fungal infections (IFIs) caused primarily by yeasts (*Candida* spp. and *Cryptococcus* spp.) and filamentous fungi (*Aspergillus* spp., *Fusarium* spp. and *Scedosporium* spp.).[1] The spectrum of yeasts and moulds causing these infections is broadening in diversity, as other species are emerging.

Despite antifungal therapy, the morbidity and mortality rates of IFIs remain high (30%–80%), and at present only three antifungal classes are used for treatment: polyenes, azoles and echinocandins. Several limitations related to their modest clinical efficacy, development of resistance and significant toxicity have been described for all of them,[1] making clear the need to expand the search for new antifungal formulations with novel mechanisms of action.

APX001A (E1210) is a novel broad-spectrum antifungal agent that inhibits Gwt1p, a protein that plays an important role in fungal cell wall integrity. During fungal glycophosphatidylinositol biosynthesis, APX001A is able to inhibit the inositol acylation step, giving rise to defects in various steps in cell wall biosynthesis.[2] It also alters cell wall growth, hyphal elongation[3] and attachment of fungal cells to biological substrates. Previous studies have shown that APX001A has broad activity against most species of *Candida*,[4–8] *Aspergillus*,[9,10] *Cryptococcus*[11] and multiresistant fungi such as *Scedosporium* and *Fusarium*,[9,12] as well as other moulds, including the order Mucorales.

The aim of this study was to evaluate the *in vitro* activity of APX001A and other antifungal agents against rare moulds by comparing the results obtained by EUCAST and CLSI reference standard methodologies for broth microdilution susceptibility testing of antifungal agents.

Materials and methods

A total of 10 isolates from 20 different species of Mucorales, *Scedosporium*/*Lomentospora*, *Fusarium* and cryptic *Aspergillus* isolated between 2000 and 2016 were tested. All isolates were obtained from clinical samples (respiratory, cutaneous, ocular, ear, biopsies, abscess, blood cultures and wounds) and identified to species level by standard microscopic morphology and by sequencing the internal transcribed spacer region of the rDNA, part of the β-tubulin gene for *Scedosporium* spp. and *Aspergillus* spp. and elongation factor α for *Fusarium* spp., following methods previously reported.[13]

Antifungal susceptibility testing was performed following EUCAST reference method 9.3.1[14] and CLSI M38A.[15]

Antifungals used were APX001A (range = 0.015–8 mg/L; Amplyx Pharmaceuticals Inc., San Diego, CA, USA), amphotericin B (range = 0.03–16 mg/L; Sigma-Aldrich Quimica, Madrid, Spain), posaconazole (range = 0.015–8 mg/L; Sigma-Aldrich Quimica) and micafungin (range = 0.004–2 mg/L; Astellas Pharma Inc., Tokyo, Japan).

3.4.2. Artículo 2 del capítulo 4. Estudio de la actividad *in vitro* de la olorofima, comparada con la de otros antifúngicos en uso, frente a aislados clínicos de especies crípticas de *Aspergillus* mediante el uso las metodologías EUCAT y CLSI

Publicación original:

Rivero-Menendez O, Cuenca-Estrella M, Alastruey-Izquierdo A. 2019. *In vitro a*ctivity of olorofim (F901318) against clinical isolates of cryptic species of *Aspergillus* by EUCAST and CLSI methodologies. *J Antimicrob Chemother*, 74(6): 1586-1590. doi: 10.1093/jac/dkz078.

La olorofima (F901318) es el principal representante de las orotomidas, una nueva clase de compuestos con capacidad antifúngica. Su mecanismo de acción compromete la producción de pirimidina, la cual es esencial para las células fúngicas, mediante la inhibición de la dihidroorotato deshidrogenasa, enzima encargada de catalizar la conversión del dihidroorotato en orotato en su ruta de biosíntesis.

A pesar de que no es efectivo frente a *Candida* y Mucorales, su actividad *in vitro* ha sido confirmada frente a especies de *Penicillium*, *Aspergillus, Fusarium*, *Scedosporium* y dermatofitos, y también frente a *Histoplasma capsulatum*, *Coccidioides posadasii* y *Coccidioides immitis*. Además, se ha administrado con éxito en formulación oral, intraperitoneal e intravenosa a modelos murinos de infección con varias especies de *Aspergillus* y con *Coccidioides*. Sin embargo, no se conoce su actividad frente a las principales especies crípticas de *Aspergillus,* muchas de las cuales presentan sensibilidad disminuida a los antifúngicos en uso.

Por consiguiente, el objetivo de este trabajo fue el de estudiar la eficacia *in vitro* de la olorofima frente a 150 aislados clínicos de especies crípticas de *Aspergillus* pertenecientes a seis complejos de especies. Para ello, se determinó la sensibilidad antifúngica de todas las cepas frente a este nuevo compuesto por EUCAST y CLSI y se comparó con la exhibida por las mismas frente a la anfotericina B, el voriconazol, el posaconazol y la micafungina.

La olorofima fue el único compuesto con actividad *in vitro* frente a todas las especies crípticas de *Aspergillus* probadas, incluyendo aquellas que son resistentes a más de una familia de antifúngicos en uso, como *Aspergillus lentulus*, *Aspergillus fumigatiaffinis* o *Aspergillus calidoustus*. Estos resultados son prometedores, y alientan al desarrollo de nuevos estudios que permitan comprobar su eficacia *in vivo* frente a estas especies causantes de infecciones difíciles de tratar.

J Antimicrob Chemother 2019; **74**: 1586–1590
doi:10.1093/jac/dkz078 Advance Access publication 19 March 2019

Journal of Antimicrobial Chemotherapy

In vitro activity of olorofim (F901318) against clinical isolates of cryptic species of *Aspergillus* by EUCAST and CLSI methodologies

Olga Rivero-Menendez, Manuel Cuenca-Estrella and Ana Alastruey-Izquierdo *

Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain; Spanish Network for the Research in Infectious Diseases (REIPI RD16/CIII/0004/0003), Instituto de Salud Carlos III, Madrid, Spain

*Corresponding author. Mycology Reference Laboratory, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera de Majadahonda a Pozuelo Km 2, 28220, Majadahonda, Madrid, Spain. Tel: +34 91 822 3784; Fax: +34 91 509 7966; E-mail: anaalastruey@isciii.es
orcid.org/0000-0001-8651-4405

Received 24 August 2018; returned 28 September 2018; revised 25 January 2019; accepted 31 January 2019

Objectives: To investigate the *in vitro* activity of olorofim (F901318), a novel broad-spectrum antifungal agent, against 150 strains belonging to 16 different cryptic species of *Aspergillus* by EUCAST and CLSI methodologies.

Methods: Olorofim, amphotericin B, micafungin, posaconazole and voriconazole were tested against cryptic species belonging to *Aspergillus fumigatus* complex (n=57), *Aspergillus ustus* complex (n=25), *Aspergillus niger* complex (n=20), *Aspergillus flavus* complex (n=20), *Aspergillus circumdati* complex (n=15) and *Aspergillus terreus* complex (n=13) using EUCAST and CLSI methodologies for broth microdilution susceptibility testing of antifungal agents.

Results: Olorofim was the only drug with activity against all cryptic species of *Aspergillus* tested, including the multiresistant species *Aspergillus lentulus*, *Aspergillus fumigatiaffinis* and *Aspergillus calidoustus*. Geometric means of MICs for olorofim were lower (0.017, 0.015 and 0.098 mg/L, respectively, for EUCAST; and 0.015, 0.015 and 0.048 mg/L, respectively, for CLSI) than for amphotericin B (4.438, 12.699 and 0.554 mg/L, respectively, for EUCAST; and 0.758, 1.320 and 0.447 mg/L, respectively, for CLSI), voriconazole (2.549, 2.297 and 5.856 mg/L, respectively, for EUCAST; and 2.071, 1.741 and 5.657 mg/L, respectively, for CLSI) and posaconazole (0.307, 0.308 and 12.996 mg/L, respectively, for EUCAST; and 0.391, 0.215 and 9.514 mg/L, respectively, for CLSI).

Conclusions: Olorofim shows encouraging *in vitro* activity against cryptic species of *Aspergillus* that can be hard to treat with current antifungal therapies. Further studies are warranted in order to assess its efficacy.

Introduction

Aspergillus spp. are some of the most common opportunistic human pathogens worldwide, especially in immunocompromised patients. They are the most frequent moulds isolated in clinical samples[1] and can produce a wide range of infections, such as invasive aspergillosis, chronic pulmonary aspergillosis or allergic bronchopulmonary aspergillosis. The leading cause of these life-threatening infections is *Aspergillus fumigatus* (85%), followed by *Aspergillus flavus* (5%–10%), *Aspergillus terreus* (2%–10%) and *Aspergillus niger* (2%–3%).[2]

However, the advances of molecular tools for identification during the last decade have led to the description of new species within the genus *Aspergillus* that are considered cryptic or sibling because they are difficult to differentiate by classical methods. Their prevalence in the clinical setting has been reported to be between 11% and 19% in three studies.[3–5] It has been recommended that *Aspergillus* isolates should be classified to the 'species complex' level,[6] as a way of gathering all these closely related cryptic species if appropriate speciation techniques are not performed. These species are important in the clinical setting because of their susceptibility profile, as azoles and amphotericin B frequently show poor activity against them, and some are considered to be MDR.[3,7] Therefore, new antifungal drugs that act via novel mechanisms are needed to overcome this ever-growing problem of resistance to current therapies.

The orotomides are a new chemical class of drugs whose most representative antifungal is olorofim (F901318). This synthetic small molecule inhibits dihydroorotate dehydrogenase (DHOH), which catalyses the conversion of dihydroorotate to orotate in the pyrimidine biosynthesis pathway.[8] Although it shows no activity against yeasts and Mucorales, olorofim has potent *in vitro* efficacy against a broad spectrum of pathogenic moulds such as *Coccidioides immitis*, *Coccidioides posadasii*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Penicillium* spp., *Scedosporium* spp., *Fusarium* spp. and *Aspergillus* spp., including azole-resistant *A. fumigatus*.[8–13] Murine models of invasive aspergillosis have

4. Discusión

4. DISCUSIÓN

Las mejoras terapéuticas desarrolladas en las últimas décadas han aumentado la supervivencia de los pacientes inmunodeprimidos en estado crítico, pero también han propiciado la progresión de otras enfermedades como las infecciones causadas por bacterias, virus, parásitos y hongos. En los últimos tiempos, la eficiencia de la prevención y el tratamiento de estas enfermedades infecciosas se ha visto comprometida por el desarrollo de la resistencia a los antimicrobianos, lo que supone una amenaza para la salud pública global. La Organización Mundial de la Salud (OMS) ha catalogado el abordaje de este problema como de alta prioridad, además de haber desarrollado en 2015 un plan de acción global al respecto. Entre los principales objetivos que este plan incluye destacan la mejora de la vigilancia epidemiológica de los microorganismos resistentes y la optimización del uso de los compuestos antimicrobianos, además de fomentar la creación de planes de acción nacionales que permitan afrontar la cuestión de forma específica en cada país (OMS, 2015).

En este contexto, el tratamiento de las infecciones fúngicas supone un reto para la microbiología clínica. A pesar de los avances en su diagnóstico e identificación, aún resulta complicado diagnosticarlas de forma temprana, lo que conlleva un mayor uso de la terapia antifúngica empírica y profiláctica con el fin de disminuir las altas tasas de mortalidad a las que se asocia el retraso en el inicio del tratamiento de estas infecciones (Patel *et al.*, 2009; Patterson, 2009). El abundante empleo de los antifúngicos en la práctica clínica ha propiciado la aparición de cepas resistentes, facilitando tanto el desarrollo de la resistencia secundaria por parte de los hongos patógenos más habituales como la emergencia de especies que presentan resistencia intrínseca. Además, otros de los condicionantes que se relacionan habitualmente con la aparición de resistencias ya existen en nuestro medio, como las escasas alternativas terapéuticas o un posible origen ambiental del fenómeno. Las cifras de resistencia antifúngica no son elevadas actualmente, aunque pueden aumentar en los próximos años, por lo que deben desarrollarse e implementarse programas de vigilancia.

4.1. Caracterización molecular del desarrollo clínico y en condiciones de laboratorio de la resistencia a equinocandinas en *Candida glabrata*

En los últimos años se ha observado un cambio epidemiológico en las especies que originan las infecciones invasoras. Esta evolución ha sido especialmente notoria en aquellas del género *Candida*, aumentando la prevalencia de otras especies en detrimento de *C. albicans*. En España, el estudio CANDIPOP analizó prospectivamente la epidemiología y sensibilidad de las cepas aisladas de pacientes con fungemia ingresados de 2010 a 2011 en 29 hospitales, identificando a *C. albicans* (44,6%), *C. parapsilosis* (24,5%) y *C. glabrata* (13,2%) como las especies de este género con mayor frecuencia en nuestro país (Guinea *et al.*, 2014). Con este estudio se evidenció que la prevalencia de *C. glabrata* como agente etiológico de candidemia se había incrementado en un 5,2% con respecto a un estudio epidemiológico similar realizado diez años antes en los

principales centros hospitalarios de Barcelona (Almirante *et al.*, 2005). Esta especie presenta de forma habitual valores de CMI elevados a los triazoles, sobre todo a fluconazol, hecho que se ha relacionado con su aumento en pacientes en tratamiento o profilaxis con compuestos azólicos. Las equinocandinas son, por tanto, la terapia de primera línea para el tratamiento de las candidiasis invasoras producidas por C*. glabrata*, y solo se recomienda el uso del fluconazol en casos en los que se pueda administrar en altas dosis y la CMI de la cepa que la causa sea inferior a 32 mg/L (Pappas *et al.*, 2018).

En algunas zonas geográficas se ha advertido, de forma paralela al incremento de la resistencia a los azoles, un progresivo aumento del número de cepas de esta especie resistentes a equinocandinas. Varios estudios poblacionales realizados en Estados Unidos evidenciaron un aumento en la proporción de cepas resistentes a equinocandinas de *C. glabrata* causantes de candidemia, siendo este incremento del 4,9% al 12,3% entre 2001 y 2010 en un hospital de Carolina del Norte (Alexander *et al.*, 2013) y del 4,2% en 2008 al 7,8% en 2014 en ochenta hospitales de cuatro áreas metropolitanas del país (Vallabhaneni *et al.*, 2015). En Europa Central, un estudio suizo recoge un 9,4% de cepas resistentes a caspofungina de esta especie aisladas de hemocultivos entre 2004 y 2009 (Orasch *et al.*, 2014), mientras que en Dinamarca, tras no hallar ninguna cepa resistente de *C. glabrata* entre las causantes de candidemia en el periodo de 2004 a 2007, en 2008-2012 se encontró un 1,4% de ellas, aumentando este porcentaje hasta 2,7% del 2012 al 2015 (Astvad *et al.*, 2018).

A diferencia de lo ocurrido en Estados Unidos y algunos países del centro y norte de Europa, los estudios poblacionales realizados en España hasta la fecha no han detectado un aumento de la resistencia a equinocandinas en *C. glabrata* (Peman *et al.*, 2012; Guinea *et al.*, 2014). Sin embargo, al analizar retrospectivamente el perfil de sensibilidad a esta familia de compuestos en los aislados clínicos de *C. glabrata* recibidos en el Laboratorio de Referencia e Investigación en Micología del Centro Nacional de Microbiología del Instituto de Salud Carlos III de 2007 a 2017 se observó que, pese a que hasta el año 2013 el porcentaje de resistencia no superó el 3%, a partir de 2014 se produjo un aumento considerable, llegando a porcentajes del 44% (en 2016) (**Figura 3**). Es importante señalar que este incremento coincide con el inicio del programa de vigilancia de resistencia a los antifúngicos, por lo que es probable que se hayan recibido mayoritariamente cepas resistentes: la mitad de las cepas recibidas en 2016 a través del programa de vigilancia (n=26) fueron resistentes a al menos una equinocandina, siendo diez de ellas resistentes tanto a anidulafungina como a micafungina, mientras que solo seis de las 17 recibidas ese mismo año fuera de este programa superaron los puntos de corte establecidos por EUCAST para estos antifúngicos. No obstante, esto evidencia que la resistencia a las equinocandinas en nuestro país está presente y puede suponer un problema en el futuro. En total, se recibieron 450 cepas de *C. glabrata* en los diez años analizados, de las cuales 37 (8,2%) fueron resistentes a equinocandinas. A pesar de que este porcentaje de resistencia no es representativo del país, puesto que se basa en un estudio de vigilancia pasiva, resulta relevante debido a la ya comentada sensibilidad disminuida de esta especie a los azoles.

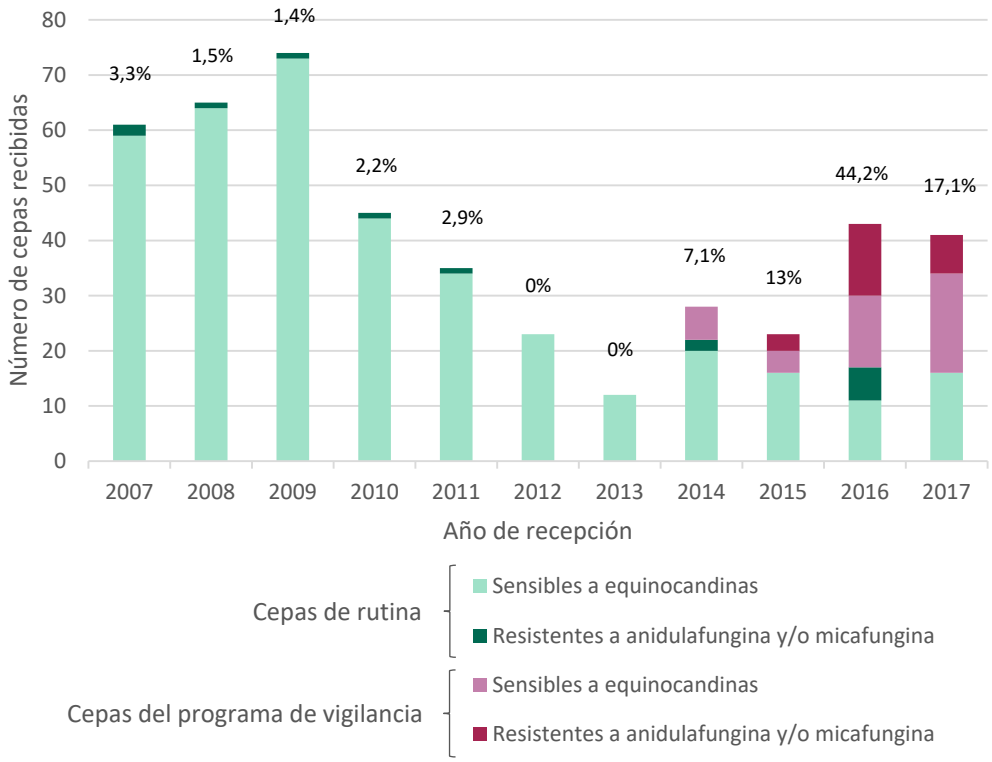


Figura 3. Número de cepas de *C. glabrata* sensibles y resistentes a equinocandinas recibidas en el Laboratorio de Referencia e Investigación en Micología del Instituto de Salud Carlos III de 2007 a 2017. Los porcentajes representan las cepas resistentes con respecto al total de las recibidas cada año.

Se ha descrito que las cepas de *C. glabrata* son más propensas a adquirir resistencia a los antifúngicos que las de otras especies de *Candida*. Concretamente, la resistencia a equinocandinas en esta especie se desarrolla con facilidad tanto en los pacientes (Shields *et al.*, 2012; Alexander *et al.*, 2013; Bizerra *et al.*, 2014; Sasso *et al.*, 2017) como *in vitro* (Bordallo-Cardona *et al.*, 2017; Bordallo-Cardona *et al.*, 2018a; Bordallo-Cardona *et al.*, 2018b; Shields *et al.*, 2019) tras la exposición a estos compuestos. Dicha resistencia está asociada con la aparición de mutaciones puntuales en los *hot-spots* de los genes *FKS1* y *FKS2* (Castanheira *et al.*, 2014; Pham *et al.*, 2014a) y a altos valores de CMIs. Se ha observado que la presión ejercida por las equinocandinas es capaz de generar resistencia adquirida tanto tras su uso como profilaxis (Bizerra *et al.*, 2014), como tras un tratamiento prolongado (Thomson *et al.*, 2008; Costa-de-Oliveira *et al.*, 2011; Dannaoui *et al.*, 2012) e, incluso, a los pocos días del inicio de la terapia (Dannaoui *et al.*, 2012; Lewis *et al.*, 2013; Prigent *et al.*, 2017; Sasso *et al.*, 2017), lo que evidencia la rapidez con la que las cepas de *C. glabrata* causantes de infecciones son capaces de desarrollar estas alteraciones en sus genes *FKS*, complicando el pronóstico de los enfermos.

Para conocer en profundidad los principales aspectos relacionados con el desarrollo de la resistencia a equinocandinas en esta especie en el ámbito clínico, se estudiaron una serie de aislados secuenciales de cinco pacientes ingresados en dos hospitales de la Comunidad de Madrid con distintos perfiles de resistencia a estos fármacos que fueron recibidos en el

Laboratorio de Referencia e Investigación en Micología. Además, de dos de ellos también se dispuso de aislados sensibles previos a aquellos resistentes (**Tabla 5**), y su comportamiento fue analizado tras ser expuestos *in vitro* de forma directa y de forma progresiva a concentraciones crecientes de micafungina. Algunos trabajos han señalado la posible asociación entre el genotipo de *C. glabrata* y el desarrollo de resistencia a los antifúngicos (Lott *et al.*, 2012; Hou *et al.*, 2017), por lo que también se estudió dicha correlación en un conjunto de cepas clínicas aisladas entre 2012 y 2017 en un hospital de la Comunidad de Madrid tras ser expuestas en condiciones de laboratorio a anidulafungina y micafungina.

Tabla 5. Mutaciones en Fks2, sensibilidad *in vitro* por la metodología EUCAST a anidulafungina, micafungina y fluconazol y genotipado por MLP y MLST de los aislados secuenciales de *C. glabrata* de cinco pacientes ingresados en dos hospitales de la Comunidad de Madrid. En negrita se muestran los valores de CMI por encima del punto de corte establecido para *C. glabrata* para esos antifúngicos.

Pte	Cepa	Fecha aislamiento	Mutación Fks2	CMI EUCAST (mg/L)			Genotipado	
				AND	MCF	FLC	MLP* (pb)	MLST (ST)
1	CNM-CL9829	14/03/16	--	0,007	0,007	4	205-243-134-267-262-325	ST3
	CNM-CL9835	17/03/16	--	0,015	0,007	4		
	CNM-CL9975	17/04/16	D666H	**0,125**	0,03	4		
	CNM-CL9877	17/06/16	L664R	**0,125**	**0,06**	2		
	CNM-CL9889	07/07/16	L664R	**0,125**	**0,125**	2		
2	CNM-CL9857	21/05/16	--	0,03	0,007	2	205-243-134-267-262-325	ST3
	CNM-CL9883	23/06/16	D666E	**0,25**	**0,06**	4		
	CNM-CL9897	17/07/16	S663P	**2**	**>2**	32		
3	CNM-CL9931	21/10/16	ΔF659	**2**	**2**	2	187-251-122-270-265-296	ST2
	CNM-CL9939	14/11/16	ΔF659	**2**	**2**	**64**		
	CNM-CL9991	23/11/16	ΔF659	**2**	**2**	**>64**		
4	CNM-CL9932	21/10/16	S663P	**2**	**2**	32	205-243-134-267-262-325	ST3
	CNM-CL9981	12/11/16	S663P	**2**	**>2**	32		
	CNM-CL9992	24/11/16	S663P	**2**	**>2**	**64**		
5	CNM-CL9646	25/02/15	D666N	**0,125**	**0,06**	**64**	237-236-128-270-262-290	ST149
	CNM-CL9775	11/11/15	D666N	**0,125**	**0,06**	2		
	CNM-CL9988	16/11/16	D666N	**0,25**	**0,06**	2		
	CNM-CL10047	16/01/17	ΔF659 + D666N	**>4**	**2**	**64**		

Pte: paciente. * MLP: *ERG3-MTI-RPM2-GLM4-GLM5-GLM6*.

Los aislados estudiados se caracterizaron genotípicamente mediante MLST (Dodgson *et al.*, 2003) y MLP (Foulet *et al.*, 2005; Abbes *et al.*, 2012) con el fin de estudiar si el desarrollo de la resistencia a equinocandinas en *C. glabrata* se distribuye de forma monoclonal o controlada o

si, por el contrario, distintas cepas de esta especie son capaces de adquirir resistencia. Estas dos técnicas dieron lugar a los mismos resultados en este trabajo, aunque en otros estudios similares se obtuvieron un mayor número de genotipos por MLP que por MLST (Hou *et al.*, 2018; Bordallo-Cardona *et al.*, 2019), posiblemente debido al uso de otros marcadores como microsatélites. En MLST, la asignación de números de alelo para cada marcador permitió definir el perfil alélico (ST) de cada aislado con la ayuda de una base de datos informatizada, la cual posibilitó la comparación de los genotipos obtenidos con los de cepas incluidas en otros estudios. El ST3, uno de los más prevalentes en todo el mundo (Dodgson *et al.*, 2003; Lott *et al.*, 2012; Hou *et al.*, 2017; Biswas *et al.*, 2018b, Byun *et al.*, 2018; Mushi *et al.*, 2018), fue el más frecuentemente detectado entre las cepas analizadas. También destacó el ST149, identificado en los aislados del paciente estudiado del Hospital 12 de Octubre y en dos de las cepas sensibles del Hospital Puerta de Hierro, el cual ha sido recientemente descrito en aislados clínicos de otro hospital de Madrid (Bordallo-Cardona *et al.*, 2019). Esto revela que el desarrollo de la resistencia a equinocandinas en *C. glabrata* sigue una distribución policlonal, lo cual convierte a este fenómeno en un problema de índole universal. No se observó correlación entre el genotipo y el sitio anatómico del que fueron obtenidos los aislados de cada paciente, como se había propuesto anteriormente (Lin *et al.*, 2007), ni con las CMIs de los aislados secuenciales de los pacientes o el desarrollo de la resistencia a equinocandinas (Dodgson *et al.*, 2003; Abbes *et al.*, 2012), ni siquiera en el caso de aquellas con ST3, el cual ha sido asociado a un peor pronóstico clínico (Byun *et al.*, 2018; Bordallo-Cardona *et al.*, 2019). Tampoco se apreció asociación entre las variantes genéticas y la sensibilidad a fluconazol, pese a haber sido descrita previamente (Mushi *et al.*, 2018). Todos los aislados pertenecientes al mismo paciente parecieron tener un origen clonal por MLST y MLP, lo que evidenció la capacidad de estas cepas para desarrollar resistencia tras el inicio de la terapia antifúngica (Dannaoui *et al.*, 2012; Lewis *et al.*, 2013).

Todas aquellas cepas con valores de CMI por encima de los puntos de corte clínicos establecidos por EUCAST para anidulafungina y/o micafungina presentaron mutaciones en el *hot-spot* 1 del gen *FKS2* previamente asociadas a la adquisición de resistencia a esta familia de compuestos (Zimbeck *et al.*, 2010; Castanheira *et al.*, 2014; Locke *et al.*, 2016), siendo S663P y ΔF659 los cambios aminoacídicos más comúnmente detectados en esta proteína, al igual que en anteriores trabajos (Zimbeck *et al.*, 2010; Beyda *et al.*, 2014; Castanheira *et al.*, 2014). Otras alteraciones menos frecuentes, como L664R, D666N o D666E también fueron encontradas en distintos aislados (Castanheira *et al.*, 2014; Prigent *et al.*, 2017; Bordallo-Cardona *et al.*, 2018c). Además, se describió por primera vez la mutación D666H en una cepa clínica, la cual fue resistente a anidulafungina pero no a micafungina, mientras que el resto de aislados con mutaciones en los genes *FKS* presentaron resistencia a ambos antifúngicos.

En este estudio, S663P y ΔF659 produjeron valores más elevados de CMIs que L664R, D666H, D666N o D666E, las cuales se relacionaron con un perfil de resistencia más discreto (Arendrup y Perlin, 2014). Esto corrobora el hecho de que la sensibilidad de la 1-3-β-D-glucano sintasa se ve afectada en distinto grado en función de la mutación presente en las proteínas Fks (Garcia-Effron

et al., 2009b). Una de las cepas presentó dos mutaciones ΔF659 y D666N y CMIs altas para equinocandinas. En un artículo anteriormente publicado se describió una triple mutación en una cepa con sustituciones en estas dos posiciones, F659S y D666E, además de en S663A (Prigent *et al.*, 2017), pero la asociación de ΔF659 y D666N ha sido encontrada en este trabajo por primera vez.

La facilidad con la que *C. glabrata* adquiere mutaciones en los genes *FKS* en respuesta a la presión antifúngica fue constatada tanto en el ámbito clínico, al encontrar distintas mutaciones en *FKS* en aislados secuenciales con el mismo genotipo en dos de los pacientes estudiados, como en el laboratorio, al exponer los aislados sensibles disponibles de estos dos pacientes a concentraciones crecientes de micafungina y hallar en las colonias crecidas mutaciones en *FKS* distintas a las detectadas en los aislados clínicos resistentes. Las alteraciones en estos genes aparecieron en las colonias que crecieron a partir de la concentración más baja considerada como resistente por EUCAST (0,06 mg/L) en 48 horas de exposición, al igual que ocurrió al exponer el resto de cepas a anidulafungina, encontrando colonias con mutaciones en estos genes a partir de una concentración de 0,12 mg/L en el mismo tiempo. Esta capacidad de *C. glabrata* de desarrollar resistencia a concentraciones bajas de equinocandina (Bordallo-Cardona *et al.*, 2018a) puede suponer un problema, ya que esta especie es capaz de colonizar y sobrevivir en determinadas zonas del cuerpo humano, como el abdomen, el peritoneo, el tracto gastrointestinal o la mucosa oral (Shields *et al.*, 2014; Grau *et al.*, 2015; Jensen *et al.*, 2015; Healey *et al.*, 2017), donde el antifúngico no es capaz de penetrar en dosis terapéuticas. Con el fin de profundizar en esta problemática, se estudió la concentración mínima de antifúngico capaz de inhibir por completo el crecimiento fúngico (MPC; *mutant prevention concentration*) y la ventana de selección de mutantes (MSW; *mutant selection window*), que define el rango de CMIs en donde se selecciona la población de mutantes (Zhao y Drlica, 2001; Drlica, 2003; Canton y Morosini, 2011). No se encontraron diferencias estadísticas significativas entre la media geométrica de las MPCs obtenidas para ambos compuestos, y se confirmaron los resultados publicados por Bordallo-Cardona y colaboradores (Bordallo-Cardona *et al.*, 2018b), en los que se determinó que se precisa de concentraciones superiores a 2 mg/L para prevenir la aparición *in vitro* de colonias resistentes con alteraciones en los genes *FKS* de *C. glabrata*. El uso de nuevos compuestos con mayor vida media y mejor perfusión a los tejidos, como el ibrexafungerp y la rezafungina, podría ayudar a prevenir el desarrollo de mutantes resistentes (Lee *et al.*, 2019; Squires *et al.*, 2019), aunque también se ha demostrado la aparición de mutaciones en los genes *FKS* de cepas sensibles tras la exposición *in vitro* a estos compuestos (Locke *et al.*, 2016; Jimenez-Ortigosa *et al.*, 2017).

El número de aislados con mutaciones en *FKS* entre el total de colonias analizadas fue significativamente superior tras la exposición a micafungina (58%) que a anidulafungina (42%). A pesar de que en otros estudios *in vitro* se utiliza un abordaje diferente al empleado en nuestro trabajo; en general, el número de colonias con mutaciones en sus genes *FKS* detectadas entre

todas las estudiadas es más elevado tras exponer las cepas a micafungina que a anidulafungina (Bordallo-Cardona *et al.*, 2018b; Shields *et al.*, 2019).

Se desarrollaron distintas mutaciones en las proteínas Fks, siendo S663P y ΔF659 las más frecuentemente detectadas, al igual que ocurre en los aislados clínicos resistentes a equinocandinas (Shields *et al.*, 2012; Alexander *et al.*, 2013; Beyda *et al.*, 2014). Todas las cepas con mutaciones en los genes *FKS* fueron clasificadas como resistentes a anidulafungina, mientras que dos cepas con sustituciones en *FKS* se categorizaron como sensibles a micafungina. Arendrup y colaboradores demostraron en un modelo murino de candidiasis por *C. glabrata* que cepas clasificadas como resistentes a anidulafungina y sensibles a micafungina pero con mutaciones en los genes *FKS* respondían al tratamiento con dosis terapéuticas de micafungina de la misma forma que aquellos ratones infectados por cepas sensibles con genes *FKS* salvajes (Arendrup *et al.*, 2012b). No se halló ninguna colonia resistente a micafungina y sensible a anidulafungina. Por otro lado, 53 colonias (19,4%) que no presentaron mutaciones en los *FKS* fueron categorizadas como resistentes a anidulafungina, mientras que solo una cepa con *FKS* salvajes fue clasificada como resistente a micafungina. Aunque se ha sugerido que las mutaciones en los genes *FKS* pueden ser mejor predictor de fallo terapéutico que la determinación de las CMIs (Shields *et al.*, 2012), las discrepancias encontradas entre los aislados de nuestro estudio ponen de manifiesto la necesidad de realizar un mayor número de experimentos para poder hacer esta afirmación. A la luz de los resultados obtenidos, se recomienda estudiar el perfil de sensibilidad de la micafungina y la anidulafungina en paralelo o repetir las pruebas de sensibilidad antifúngica a aquellas cepas con valores de CMI cercanos a los puntos de corte, además de amplificar y secuenciar las regiones *hot-spot* de los genes *FKS* para clarificar el papel de las distintas mutaciones en el desarrollo de resistencia.

Una de las hipótesis planteadas para explicar la rápida adquisición de resistencia a los antifúngicos en *C. glabrata* se basa en las alteraciones en el gen *MSH2*, las cuales se han relacionado con el desarrollo de la resistencia y con la aparición de mutaciones en los genes *FKS* en el caso de la resistencia a equinocandinas (Healey *et al.*, 2016b). Al estudiar este gen implicado en los mecanismos de reparación del ADN en los aislados de los pacientes, se observó que todos los aislados del mismo paciente poseían la misma secuencia de *MSH2*, independientemente de su perfil de sensibilidad a equinocandinas, y que tanto los aislados salvajes en *MSH2* como aquellos con mutaciones dieron lugar a aislados resistentes a equinocandinas con mutaciones en los genes *FKS*. Por tanto, las mutaciones en *MSH2* no justifican el desarrollo de resistencia a equinocandinas, tal y como ha sido defendido en otros trabajos (Delliere *et al.*, 2016; Healey *et al.*, 2016a; Biswas *et al.*, 2018b; Byun *et al.*, 2018; Hou *et al.*, 2018; Singh *et al.*, 2018). Del mismo modo, no se encontró relación entre la secuencia de este gen y la resistencia a fluconazol en estos aislados. La presencia de alteraciones en el *MSH2* de *C. glabrata* también ha sido asociada con determinados genotipos (Delliere *et al.*, 2016; Byun *et al.*, 2018; Hou *et al.*, 2018), pero tampoco se advirtió un vínculo entre ambos en este trabajo. Esta falta de correlación tanto con la propensión a adquirir resistencia antifúngica como con los

genotipos apoya el hecho de que estas mutaciones en el gen *MSH2* puedan tratarse de variaciones constitutivas del mismo, tal y como sostiene un trabajo basado en el estudio del genoma completo de cepas de *C. glabrata* (Carrete *et al.*, 2019). Además de este gen, se están estudiando otros involucrados en los mecanismos de reparación del desapareamiento de bases que también podrían jugar un papel en el desarrollo de la resistencia antifúngica, como *PMS1* y *MSH6* (Healey y Perlin, 2018), lo que ha llevado a considerar que los fallos en los mecanismos de reparación del ADN podrían formar parte de una estrategia de adaptación evolutiva que *C. glabrata* ha desarrollado para diversificar genéticamente sus poblaciones y, así, sobrevivir con mayor facilidad en el huésped al colonizarlo, siendo capaz de diseminarse y de adaptarse a la presencia de antifúngico (Healey y Perlin, 2018).

Las conclusiones recogidas en este estudio arrojan luz sobre los principales aspectos relacionados con la adquisición y el desarrollo de la resistencia a equinocandinas en *C. glabrata* a través del análisis de varias de las hipótesis planteadas a raíz de su incremento en algunas áreas geográficas, y podrían ser de gran utilidad para abordar este fenómeno si se confirma su emergencia en nuestro entorno.

4.2. Estudio de la identificación molecular, la sensibilidad antifúngica y el principal mecanismo de resistencia a azoles en las especies de *Aspergillus* incluidas en un programa de vigilancia de la resistencia antifúngica

Del mismo modo que en el CANDIPOP se estudió la epidemiología y la resistencia antifúngica de las levaduras causantes de infecciones (Guinea *et al.*, 2014), el estudio FILPOP analizó la epidemiología en hongos filamentosos en España entre 2010 y 2011 (Alastruey-Izquierdo *et al.*, 2013). En este estudio se detectó un 10,8% de cepas resistentes a anfotericina B y un rango de resistencia a azoles del 10% al 12,7%, en función del compuesto de esta familia estudiado. Esta resistencia se detectó principalmente en especies que de forma general son inherentemente resistentes a varios o a todos los antifúngicos, como aquellas pertenecientes a los géneros *Scedosporium*, *Fusarium* o al orden Mucorales. También se encontró un alto porcentaje de resistencia en las especies crípticas del género *Aspergillus*, las cuales han sido descritas en los últimos años como consecuencia de la mejora en las herramientas de identificación. Estos resultados evidenciaron la emergencia de especies de hongos filamentosos que presentan resistencia antifúngica intrínseca que se está produciendo en España, algo en consonancia con lo que está ocurriendo en todo el mundo (Roden *et al.*, 2005; Al-Hatmi *et al.*, 2016b; Gautier *et al.*, 2016; Ramirez-Garcia *et al.*, 2018; Skiada *et al.*, 2018).

Sin embargo, a pesar de los cada vez más numerosos artículos en los que se informa del aumento en las tasas de resistencia secundaria a azoles en *Aspergillus* (Rivero-Menendez *et al.*, 2016; Zoran *et al.*, 2018; Choi *et al.*, 2019), en el estudio FILPOP no se detectaron cepas resistentes de las especies de *Aspergillus* más frecuentes, como *A. fumigatus*, *A. terreus*, *A. flavus* o *A. niger* (Alastruey-Izquierdo *et al.*, 2013). Para estudiar la aparición de resistencia en

nuestro país, en julio de 2014 se implementó un programa de vigilancia de la resistencia a los antifúngicos y se realizó un nuevo estudio epidemiológico prospectivo, denominado FILPOP2, en el que se utilizaron placas de agar suplementado con itraconazol con el propósito de aumentar la sensibilidad de la detección de cepas resistentes (Alastruey-Izquierdo *et al.*, 2018). En este trabajo, realizado entre 2015 y 2016, un 1,2% de los *A. fumigatus* identificados fueron resistentes a al menos un azol al aplicar los puntos de corte clínicos establecidos por EUCAST (EUCAST, 2018), mientras que en *A. niger* y *A. terreus* se encontraron porcentajes de resistencia del 15,4% y 13%, respectivamente. Por otra parte, en el estudio FILPOP2 se constató de nuevo la presencia de especies fúngicas emergentes con CMIs elevadas a varios antifúngicos, detectando cepas de *Fusarium*, *Scedosporium* o Mucorales, así como de las especies crípticas de *Aspergillus*. Este trabajo se incluye en el apartado de anexos de esta tesis.

El segundo capítulo de esta tesis se centra en la caracterización fenotípica y molecular de las cepas de *Aspergillus* spp. recibidas en el programa de vigilancia de la resistencia antifúngica del Laboratorio de Referencia e Investigación en Micología desde su instauración hasta el final de 2018. En este periodo se recibieron 306 aislados de diferentes localizaciones anatómicas sospechosos de ser resistentes a al menos un antifúngico, los cuales fueron identificados a nivel de género o de complejo de especies mediante el estudio microscópico y macroscópico de la morfología del hongo. Tras descartar aquellos aislados contaminados o identificados como levaduras o como pertenecientes a géneros de hongos filamentosos distintos a *Aspergillus*, se analizaron 273 cepas provenientes de 33 hospitales españoles. Una vez determinado el perfil de sensibilidad antifúngica de estos aislados de *Aspergillus* spp. utilizando la metodología EUCAST, se realizó la identificación a nivel de especie mediante el uso de herramientas moleculares de aquellos en los que se confirmó su baja sensibilidad a al menos un antifúngico tras aplicar los puntos de corte clínicos establecidos por EUCAST para la especie principal del complejo de especies de *Aspergillus* al que pertenecían.

De estos 273 aislados de *Aspergillus* spp., más de la mitad se identificaron como parte del complejo de *A. fumigatus* (57,9%), seguido de aquellos pertenecientes a los complejos de *A. terreus* (21,6%), *A. flavus* (12,8%) y *A. nidulans* (5,5%).

Se observaron valores elevados de CMI a anfotericina B entre las cepas de los complejos de *A. terreus* y *A. flavus*, lo cual concuerda con lo recogido en otros estudios prospectivos de la resistencia antifúngica en *Aspergillus* spp. llevados a cabo en diferentes países (Diekema *et al.*, 2003; Baddley *et al.*, 2009; Escribano *et al.*, 2012; Reichert-Lima *et al.*, 2018). Esto supone una complicación añadida para aquellas cepas de estas especies que también muestran sensibilidad reducida a los azoles, ya que la anfotericina B en su forma liposomal se considera una opción para la terapia de la aspergilosis invasora (Garcia-Vidal y Alastruey-Izquierdo *et al.*, 2018).

Las cepas clasificadas como resistentes según los puntos de corte de EUCAST fueron identificadas por métodos moleculares. Esto permitió detectar algunas especies crípticas de *Aspergillus*, algo relevante en el caso de tener que administrar tratamiento debido a la

resistencia intrínseca a una o más clases de antifúngicos que muchas de estas especies presentan. La especie críptica más frecuentemente identificada entre las cepas resistentes fue *A. lentulus*, perteneciente al complejo de *A. fumigatus*, al igual que en un estudio de vigilancia multicéntrico realizado en 19 países durante dos años para la detección de resistencia a azoles en aislados clínicos de este complejo (Van der Linden *et al.*, 2015). *A. lentulus* tiene CMIs elevadas a los azoles y los polienos, lo que empeora el pronóstico de las infecciones que causa en individuos inmunodeprimidos. Otras dos de las especies crípticas de este complejo, *A. fumigatiaffinis* y *A. udagawae*, fueron también identificadas entre las resistentes, confirmando la multirresistencia ya descrita para la primera (Alastruey-Izquierdo *et al.*, 2014) y los elevados valores de CMI a anfotericina B y variables a azoles de la segunda (Balajee *et al.*, 2006; Vinh *et al.*, 2009). Por otra parte, una cepa se identificó como *Aspergillus citrinoterreus*, especie perteneciente al complejo de *A. terreus* de sensibilidad variable a azoles y a anfotericina B descrita recientemente en España (Guinea *et al.*, 2015), la cual se considera la especie críptica de este complejo más frecuente (Risslegger *et al.*, 2017; Zoran *et al.*, 2018).

El programa de vigilancia permitió detectar la resistencia a al menos un azol de 23 cepas de *A. fumigatus s.s.*, 12 *A. terreus s.s.* y dos *A. flavus s.s.* A continuación, se realizó la caracterización molecular de las modificaciones en los genes *cyp51* de estas cepas, encontrando mutaciones puntuales previamente descritas en seis aislados de *A. fumigatus s.s.* (Diaz-Guerra *et al.*, 2003; Mellado *et al.*, 2007; Pelaez *et al.*, 2015; Alastruey-Izquierdo *et al.*, 2018). Cuatro de estas sustituciones estaban asociadas a inserciones en tándem en el promotor del gen, de las que tres poseían el mecanismo de resistencia a azoles en *Aspergillus* más frecuente en todo el mundo tanto en aislados clínicos como ambientales, TR_{34}/L98H, y otra el TR_{46}/Y121F/T289A (Rivero-Menendez *et al.*, 2016). Estos cuatro aislados presentaron resistencia cruzada a los compuestos azólicos. Las dos cepas restantes albergaron la alteración puntual G54V en Cyp51A y fueron resistentes a itraconazol y posaconazol, algo concordante con los perfiles de sensibilidad generalmente descritos para las mutaciones en esta posición de la secuencia aminoacídica (Diaz-Guerra *et al.*, 2003; Nascimento *et al.*, 2003).

No se encontraron sustituciones en el gen *cyp51A* de los otros 17 aislados con distintos patrones de resistencia de *A. fumigatus s.s.*, lo que evidencia la importancia de investigar otros mecanismos de resistencia. Sin embargo, diez de estas cepas solo fueron resistentes a isavuconazol con una CMI de 2 mg/L. Esto trae a colación un tema actualmente en debate, ya que el punto de corte clínico propuesto por EUCAST para este azol, 1 mg/L, está dando lugar a la clasificación de cepas de *A. fumigatus s.s.* como resistentes sin presentar mutaciones en su *cyp51A* que confirmen tal resistencia ni ser resistentes a otros azoles (Howard *et al.*, 2013; Buil *et al.*, 2018; Jorgensen *et al.*, 2019). A pesar de que este punto de corte fue propuesto tras evaluar el efecto de una serie de dosis de este antifúngico en modelos murinos de aspergilosis diseminada causada tanto por cepas salvajes como mutantes para *cyp51A* (Seyedmousavi *et al.*, 2015), otro trabajo más reciente demuestra que el uso de dosis escaladas resulta efectivo para tratar a pacientes infectados con cepas de *A. fumigatus s.s.* con CMI de 2 mg/L a isavuconazol

(Buil *et al.*, 2018), valor que además es el establecido como ECOFF para este antifúngico. Si este punto de corte epidemiológico se aplicase a las cepas de este estudio, solo siete de las que no poseen alteraciones en *cyp51A* serían clasificadas como resistentes a azoles, de las cuales cinco son resistentes al resto de compuestos de esta clase, una resistente a voriconazol y a posaconazol y otra solo a este último. Hasta que se dilucide si es necesario proponer un nuevo punto de corte clínico más elevado, EUCAST recomienda realizar en duplicado las pruebas de sensibilidad para este antifúngico y secuenciar su *cyp51A* en caso de tener una CMI de 2 mg/L, además de tener en cuenta los valores de CMI de otros compuestos de esta familia, como el itraconazol o el voriconazol, como marcadores de resistencia a azoles (Arendrup *et al.*, 2016). No obstante, existen otros mecanismos de resistencia asociados a estos antifúngicos que pueden explicar estos patrones de sensibilidad, como las modificaciones en HapE (Camps *et al.*, 2012b), la sobrexpresión de *cyp51B* (Bueid *et al.*, 2013) o la de los genes implicados en la activación de las bombas de flujo (Fraczek *et al.*, 2013).

El estudio de Cyp51A de los aislados resistentes de *A. terreus s.s.* reveló la existencia en dos de ellos de la mutación D344N, en uno de ellos combinada con M217I. La asociación de mutaciones en estas dos posiciones ha sido ya descrita, aunque con la sustitución en la metionina en posición 217 por valina en vez de por isoleucina (Zoran *et al.*, 2018). La alteración M217I ha sido asociada con resistencia a itraconazol y valores de CMI altos a voriconazol y posaconazol cuando aparece sola (Arendrup *et al.*, 2012a), perfil similar al de esta cepa con mutaciones combinadas. Nunca antes se había descrito la D344N como única alteración en una cepa de *A. terreus s.s.* resistente, aunque es necesario realizar más trabajos para confirmar el papel de esta mutación y de las detectadas en M217 en el desarrollo de la resistencias a azoles en esta especie.

Al igual que ocurrió para *A. fumigatus s.s.*, el punto de corte clínico propuesto para el isavuconazol para *A. terreus s.s.* (1 mg/L) fue el responsable de la clasificación como resistentes de siete cepas con una CMI a este antifúngico de 2 mg/L que no presentan ninguna alteración aparente en la diana de los azoles, las cuales pasan a ser catalogadas como sensibles a los azoles al aplicar el ECOFF para isavuconazol de 2 mg/L establecido para esta especie. Otras dos cepas con una CMI de 2 mg/L para este antifúngico albergaron la mutación D344N y otra una alteración no descrita anteriormente, A249G, que no fue encontrada en cepas sensibles a azoles.

Las dos cepas de *A. flavus* resistentes a itraconazol y con valores de CMI elevados al resto de compuestos azólicos albergaban una serie de mutaciones en Cyp51C que también se han encontrado en cepas sensibles (Sharma *et al.*, 2018; Choi *et al.*, 2019) pero, a su vez, cada una de ellas poseía una mutación nueva, una en esta misma enzima (H349R) y otra en Cyp51A (P220L). Se precisa de más estudios que aseguren si estas dos alteraciones son responsables de la resistencia a azoles en estas cepas o si, por el contrario, habría que considerar la existencia de algún otro mecanismo complementario aún no estudiado que explique su baja sensibilidad,

como la sobreexpresión de los genes *cyp51* o de aquellos que codifican las bombas de flujo (Paul *et al.*, 2015; Sharma *et al.*, 2018).

La instauración de este programa de vigilancia ha permitido constatar la existencia de resistencia antifúngica en las principales especies de *Aspergillus* causantes de infecciones invasoras en España, además de estudiar el principal mecanismo de resistencia a azoles en aislados clínicos españoles de *A. flavus* y *A. terreus* por primera vez. Puesto que los aislados del programa presentan un sesgo debido tanto a su envío voluntario por parte de los hospitales como a los criterios de inclusión utilizados, ya que se incluyen tanto cepas con CMIs elevadas como cepas aisladas de infecciones que no respondían a tratamiento antifúngico, estos resultados no permiten estimar la prevalencia de especies ni las tasas de resistencia. Sin embargo, los resultados obtenidos indican la importancia de vigilar la aparición de resistencia y los mecanismos de resistencia asociados, lo que en un futuro puede contribuir al desarrollo de medidas de control o al diseño de estrategias que permitan controlar la resistencia a azoles en *Aspergillus* a nivel nacional.

4.3. Desarrollo, estandarización y validación de una metodología basada en el análisis de curvas de fusión de alta resolución para detectar la resistencia a azoles en *Aspergillus fumigatus*

El considerable número de cepas de *A. fumigatus* resistentes a azoles halladas dentro del programa de vigilancia se correlaciona, como ya se ha mencionado anteriormente, con el progresivo aumento de casos descritos en países de todo el mundo. La tasa de mortalidad de los pacientes infectados con cepas de *A. fumigatus* resistentes a esta clase de antifúngicos es notoriamente superior a la de aquellos infectados con una cepa sensible (88% frente a 30% - 50%, respectivamente) (Van der Linden *et al.*, 2011), motivo por el que es importante realizar una detección temprana de la resistencia (Nivoix *et al.*, 2008b; Patterson *et al.*, 2009). Dado que las metodologías para la determinación de la sensibilidad antifúngica no permiten la obtención de resultados en un tiempo corto, no son realizadas de forma rutinaria en algunos laboratorios clínicos y, en ocasiones, no pueden ser llevadas a cabo por no disponer de un cultivo, el desarrollo de técnicas alternativas que faciliten la rápida categorización de los aislados de *A. fumigatus* como sensibles o resistentes a azoles podría ser de gran utilidad para el inicio de una correcta terapia antifúngica. Las principales alteraciones descritas en Cyp51A se han asociado con un perfil de resistencia a estos compuestos específico (Rivero-Menendez *et al.*, 2016), por lo que la detección de las mismas puede orientar acerca del tratamiento antifúngico más adecuado sin necesidad de realizar pruebas de sensibilidad antifúngica.

Los métodos moleculares de PCR a tiempo real o cuantitativa posibilitan la determinación de variables alélicas en genes asociados a la resistencia, evitando la demora asociada a la amplificación por PCR convencional y posterior análisis de la secuenciación del amplicón obtenido, algo imprescindible de cara al inicio precoz de una terapia antifúngica adecuada en

caso de encontrar aislados resistentes. A pesar de que se han descrito varios sistemas de PCR a tiempo real (Garcia-Effron *et al.*, 2008b; Xu *et al.*, 2010) para la detección paralela de resistencia a azoles en *A. fumigatus* y mutaciones en *cyp51A* que la provoquen, estos poseen las desventajas de requerir el uso de diferentes paneles de ensayo o diversas sondas marcadas con fluoróforos para discernir entre cepas sensibles y resistentes y comprobar la presencia de las distintas mutaciones, además de no discriminar entre los distintos cambios aminoacídicos descritos en cada posición. También se desarrolló un método basado en la combinación del análisis de curvas de fusión y el uso de sondas tipo FRET para detectar algunas de las mutaciones usuales en *cyp51A* (Klaassen *et al.*, 2010). Sin embargo, en estos estudios solo se detectan alguna de las alteraciones puntuales más habitualmente encontradas en este gen, ya que estas metodologías no permiten detectar nuevas mutaciones.

La técnica de HRM ha demostrado una buena resolución a la hora de diferenciar las cepas con mutaciones en el codón 54 de Cyp51A de las cepas salvajes de esta especie (Tuohy *et al.*, 2010), además de identificar mutaciones que generan resistencia a azoles en *Candida* spp. (Loeffler *et al.*, 2000) y, aunque deriva de la PCR cuantitativa, resulta más simple y barata que esta por no necesitar sondas marcadas, convirtiéndola en una mejor opción para este tipo de ensayos.

Por ello, el desarrollo, estandarización y validación de una metodología de HRM que permite detectar las principales mutaciones en el gen *cyp51A* asociadas a resistencia a azoles en *A. fumigatus* constituye el tercer capítulo de esta tesis.

Esta técnica se basa en un ensayo único que posibilita la distinción entre cepas con un gen *cyp51A* salvaje (es decir, sensibles a los azoles o con otro mecanismo de resistencia a estos compuestos diferente al principal), y cepas resistentes con mutaciones en este gen. A su vez, dentro de estas, diferencia las alteraciones en las posiciones G54, Y121, M220 y G448 de la proteína y las inserciones en tándem de distintos tamaños en el promotor del gen (TR_{34}, TR_{46} y TR_{53}) en cepas clínicas y ambientales de esta especie. Para la puesta a punto del método, se utilizó un conjunto de cepas previamente caracterizadas fenotípica y molecularmente que incluía sensibles y resistentes a azoles portadoras de los distintos cambios de aminoácidos descritos en las principales posiciones de Cyp51A (**Tabla 6**). Una vez estandarizada la amplificación de fragmentos para cada mutación por PCR cuantitativa, se normalizaron los parámetros para el análisis de las curvas de fusión obtenidas con el fin de conseguir la agrupación en clústeres de las cepas con el mismo mecanismo de resistencia. Tras ello, se validó el sistema utilizando un panel ciego de 80 cepas de *A. fumigatus* sensibles y resistentes a azoles con distintas mutaciones en *cyp51A*.

Tabla 6. Características fenotípicas y genotípicas de las cepas utilizadas para estandarizar y validar la metodología desarrollada.

Alteración Cyp51A/promotor	Número de cepas estandarización (n=30)	Número de cepas validación (n=80)	Fenotipo triazoles			Mutaciones puntuales
			ITC	VRC	PSC	
Salvaje	4	42	S	S	S	Ninguna
G54R	1	1	R	S	R	GGG a **A**GG
G54W	2	2	R	S	R	GGG a **T**GG
G54V	3	3	R	S	R	GGG a G**T**G
G54E	3	3	R	S	R	GGG a G**A**G
Y121F	2[a]	2	S	R	S	TAT a T**T**T
M220K	2	2	R	S[b]	R	ATG a A**A**G
M220T	1	1	R	S[b]	S[b]	ATG a A**C**G
M220I	1	1	R	S[b]	R	ATG a AT**A**
M220V	2	2	R	S[b]	S[b]	ATG a **G**TG
G448S	1	1	S[b]	R	S[b]	GGT a **A**GT
TR_{34}	6	18	R	R	R	[c]
TR_{46}	2[a]	2	R[d]	R	R[d]	[c]
TR_{53}	2	2	R	R	S[b]	[c]

[a] Mismas cepas Y121F y TR_{46}; [b] Sensibilidad reducida; [c] Inserción en tándem en el promotor de *cyp51A*; TR_{34}: GAATCACGCGGTCCGATGTGTGCTGAGCCGAAT; TR_{46}: GAATCACGCGGTCCGATGTGTGCTGAGCCGAA-TGAAAGTTGTCTA; TR_{53}: GAATCACGCGGTCCGATGTGTGCTGAGCCGAATGAAAGTTGTCTAATGTCTA; [d] CMIs variables.

No obstante, la técnica desarrollada tiene algunas limitaciones. Aunque se consiguió establecer grupos bien diferenciados entre las cepas con las sustituciones M220I, M220T y M220V, la temperatura de fusión de las cepas con la mutación M220K fue la misma que la de las cepas salvajes, por lo que ambas se juntaron en el mismo clúster. Tras probar sin éxito la modificación de la concentración de $MgCl_2$ o la incorporación, previamente sugerida, de aditivos como betaina o dimetilsulfóxido a la reacción de PCR (Song *et al.*, 2015), se diseñaron unos cebadores para la amplificación específica de las cepas con M220K para conseguir la correcta discriminación de esta sustitución. A su vez, los distintos cambios aminoacídicos que se producen en la glicina en posición 54 no pudieron ser detectados con la suficiente sensibilidad, aunque se diferenciaron con claridad de las cepas sin alteraciones en esta posición. Por otra parte, el reducido número de cepas disponibles para comprobar la detección de algunas de las mutaciones pone de manifiesto la importancia de confirmar la buena resolución de la técnica con mayor cantidad de ellas.

Tal y como se mencionó anteriormente, el desarrollo de técnicas que puedan ser ejecutadas directamente a partir de muestras clínicas resulta de gran utilidad en el caso de no poder disponer de un cultivo. Sin embargo, la baja concentración de hongo en las muestras clínicas representa un problema a la hora de poner a punto este tipo de metodologías. A pesar de que se ha propuesto el uso de un kit comercial de PCR a tiempo real para la detección directa de los

mecanismos asociados a resistencia a los azoles TR_{34}/L98H y TR_{46}/Y121F/T289A de *A. fumigatus* en lavados broncoalveolares a través del análisis de curvas de fusión (AsperGenius®, PathoNostics) (Chong *et al.*, 2015; Chong *et al.*, 2016), este solo funciona correctamente cuando la carga fúngica en la muestra es alta, puesto que *cyp51A* es un gen unicopia en esta especie. Esto puede requerir una amplificación previa del gen por PCR anidada para conseguir una mayor sensibilidad (Denning *et al*., 2011; Zhao *et al.*, 2013; Zhao *et al.*, 2016), lo que retrasa el tiempo de detección y encarece la técnica. La metodología de HRM es muy sensible a cambios en la concentración del ADN, por lo que necesita disponer de una cantidad exacta de ADN fúngico para asegurar su reproducibilidad. Aun así, es necesario comprobar si el método desarrollado es capaz de detectar las diferentes alteraciones en *cyp51A* de *A. fumigatus* a partir de muestra clínica.

En definitiva, esta herramienta desarrollada, estandarizada y validada basada en la tecnología HRM permite la rápida y precisa detección de las cepas resistentes a azoles de *A. fumigatus*, además de ser capaz de identificar cualquiera de las mutaciones en *cyp51A* más frecuentemente asociadas al desarrollo de este fenómeno. Es sensible y específica, y podría utilizarse para realizar pruebas de cribado que permitan detectar alteraciones que puedan relacionarse con un perfil de sensibilidad a azoles en unas pocas horas. Además, resulta fácilmente adaptable a cada situación, pudiendo comprobar solo las sustituciones que se deseen en cada caso o incluir cebadores para la detección de nuevas mutaciones que puedan desarrollarse en el futuro.

4.4. Estudio de la actividad *in vitro* del manogepix y la olorofima, comparada con la de otros antifúngicos en uso, frente a especies fúngicas emergentes y difíciles de tratar

En todos los trabajos incluidos en esta tesis se hace patente el gran reto que supone para la micología clínica la resistencia a los antifúngicos actualmente aprobados para el uso clínico. A su vez, muchas de estas terapias disponibles producen toxicidad y abundantes efectos secundarios y, además, pueden interaccionar con otros fármacos que estén siendo administrados al paciente de forma simultánea. Por ello, uno de los principales desafíos radica en desarrollar nuevos tratamientos antifúngicos que solventen las limitaciones que los vigentes poseen.

Dentro de este marco, se ha advertido que algunos de los nuevos compuestos que comparten diana con una determinada clase de antifúngicos de las que se encuentran en uso pueden dar lugar a la adquisición *in vitro* de resistencia y a la aparición de mutaciones en los genes que codifican dichas dianas en cepas sensibles (Locke *et al.*, 2016; Jimenez-Ortigosa *et al.*, 2017), lo que ha revalorizado aquellos con nuevos mecanismos de acción. Uno de los objetivos fundamentales en esta búsqueda de nuevas terapias es el de asegurar su efectividad frente a patógenos fúngicos multiresistentes, ya que estos poseen opciones terapéuticas muy limitadas en la actualidad (**Figura 4**). Como se ha comentado previamente, en los estudios FILPOP y

FILPOP2 se identificaron un notable número de cepas que responden a estas características, pertenecientes a *Scedosporium*/*Lomentospora* spp. (4,7% y 7,9% de las cepas identificadas en cada estudio, respectivamente), *Fusarium* spp. (1,2% y 2,6%), orden Mucorales (2,5% y 1%) y especies crípticas de *Aspergillus* (12% y 10%) (Alastruey-Izquierdo *et al.*, 2013; Alastruey-Izquierdo *et al.*, 2018).

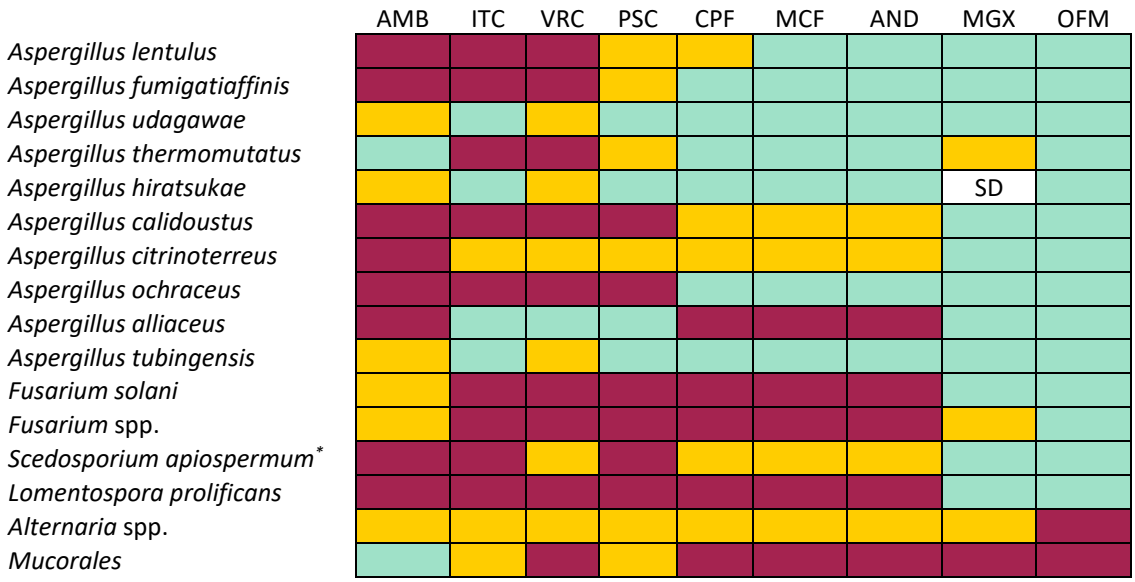


Figura 4. Perfil de sensibilidad de las especies multirresistentes, utilizando como criterio de clasificación los puntos de corte propuestos por EUCAST para *A. fumigatus*. En magenta se señalan los antifúngicos a los que las especies presentan un perfil de sensibilidad reducido, en amarillo aquellos frente a los que las especies poseen un perfil de sensibilidad variable y en verde aquellos a los que son sensibles. Datos adaptados de Alcazar-Fuoli *et al.*, 2008; Alastruey-Izquierdo *et al.*, 2009a; Alastruey-Izquierdo *et al.*, 2011; Lackner *et al.*, 2012; Alastruey-Izquierdo *et al.*, 2014; Guinea *et al.*, 2015; Al-Hatmi *et al.*, 2016b; Jorgensen *et al.*, 2018; Ramirez-Garcia *et al.*, 2018, y de los estudios incluidos en esta tesis Rivero-Menendez *et al.*, 2019a y Rivero-Menendez *et al.*, 2019b. SD: sin datos. *Complejo de especies.

Por ello, el último capítulo de esta tesis se centra en el estudio de la actividad *in vitro* de los dos agentes antifúngicos dirigidos a nuevas dianas que se encuentran en desarrollo frente a un amplio conjunto de hongos emergentes causantes de infecciones que en muchos casos son multirresistentes a los tratamientos disponibles. Concretamente, el manogepix fue probado frente a 200 cepas pertenecientes a 20 especies de los géneros *Scedosporium*/*Lomentospora*, *Fusarium*, *Aspergillus*, *Alternaria* y del orden Mucorales, y la olorofima frente a 150 cepas de especies crípticas de *Aspergillus* de seis complejos distintos. En ambos casos se determinó la sensibilidad antifúngica de las cepas mediante las dos metodologías de referencia, EUCAST y CLSI. Además, se comparó su actividad con la de otros antifúngicos de las distintas familias actualmente en uso: anfotericina B, micafungina en representación de las equinocandinas y voriconazol y/o posaconazol de los triazoles. No se probó el voriconazol en el ensayo del manogepix debido a su total ineficacia frente a las especies de Mucorales. Para ello, se calcularon la media geométrica de los valores de CMI/CME de las cepas de una misma especie

para cada antifúngico, además de la CMI/CME_{50} y la CMI/CME_{90}, definidos como el valor de CMI o CME que inhibe un 50% o un 90% de las cepas probadas, respectivamente.

El manogepix, mediante la inhibición de la proteína Gwt1 que afecta a la integridad de la pared celular fúngica y altera otras de las características principales de los hongos, ha demostrado ser eficaz contra las principales levaduras que originan infecciones tanto *in vitro* como en modelos murinos, incluso frente a cepas de *C. albicans* y *C. glabrata* resistentes a equinocandinas y *C. glabrata* multirresistentes (Miyazaki *et al.*, 2011; Pfaller *et al.*, 2011b; Pfaller *et al.*, 2011c; Wiederhold *et al.*, 2015; Arendrup *et al.*, 2018; Berkow *et al.*, 2018; Hager *et al.*, 2018; Shaw *et al.*, 2018; Zhao *et al.*, 2018a; Zhao *et al.*, 2018b; Pfaller *et al.*, 2019). En este trabajo, su actividad frente a las especies crípticas de *Aspergillus* que muestran una sensibilidad reducida a alguno de los tratamientos en uso ha sido comprobada, al demostrar unos valores de CME_{50} y CME_{90} inferiores a los de CMI_{50} y CMI_{90} de anfotericina B y posaconazol para todas las especies probadas, ya que *A. alliaceus* es poco sensible a los polienos, mientras que *A. thermomutatus*, *A. lentulus* y *A. udagawae* dieron lugar a CMIs altas también a azoles. La actividad del nuevo compuesto resultó similar a la de la micafungina para estas especies aunque en el caso de *A. calidoustus*, cuyas cepas mostraron CMEs elevadas también a esta candina, el manogepix fue considerablemente más eficaz. Además, en estudios previos se observó su actividad *in vitro* frente a otras especies de *Aspergillus*, incluyendo *A. fumigatus* resistentes a anfotericina B o azoles (Miyazaki *et al.*, 2011; Pfaller *et al.*, 2011d; Pfaller *et al.*, 2019), los cuales también han sido tratados con éxito en modelos *in vivo* con formulación oral de esta droga (Gebremariam *et al.*, 2019; Lepak *et al.*, 2019; Zhao *et al.*, 2019). A su vez, el manogepix mostró una mejor eficacia que cualquiera del resto de compuestos al ser probado contra *L. prolificans* y las especies principales del género *Scedosporium*, *S. apiospermum*, *S. boydii* y *S. aurantiacum*. Estos resultados son semejantes a los de otros de estudios *in vitro* e *in vivo* (Castanheira *et al.*, 2012; Alkhazraji *et al.*, 2019).

En cambio, este nuevo compuesto mostró una CME_{90} ≥8 mg/L frente a las especies de Mucorales y de *Fusarium* probadas, aunque alguna cepa presentó de forma puntual valores de CME algo menores. Destacaron entre estas las de *F. oxysporum*, ya que seis y ocho de las diez cepas exhibieron una mayor sensibilidad al manogepix por EUCAST y CLSI, con CME_{50}s de 0,25 mg/L y 0,015 mg/L, respectivamente, algo semejante a lo recogido en un estudio anterior frente a cepas de este complejo de especies (Castanheira *et al.*, 2012). Aunque no han sido probadas en este trabajo, este fármaco también ha mostrado actividad frente a las especies de los complejos de *F. fujikuroi* y *F. solani* (Castanheira *et al.*, 2012), además de contra fusariosis diseminada provocada por *F. solani s.s.* en modelo murino (Hata *et al.*, 2011). Del mismo modo, el manogepix también fue algo más efectivo frente a cuatro cepas *de F. verticillioides*, siendo su actividad superior que la del resto de compuestos probados tanto frente a las cepas de esta especie como a las de *F. oxysporum*. Entre los mucorales, solo dos y tres cepas de *M. circinelloides* mostraron mayor sensibilidad al manogepix por EUCAST y CLSI, respectivamente, al igual que tres de *Rhizomucor pusillus* utilizando la metodología europea. A pesar de que este

compuesto ha demostrado previamente una discreta eficacia *in vitro* contra especies de este orden no analizadas en este estudio, como *Mucor racemosus*, *Rhizopus arrhizus* o *Rhizopus microsporus* var. *rhizopodiformis*, o frente a *Cunninghamella bertholletiae* (Miyazaki *et al.*, 2011), que sí fue probada aquí, y en un modelo *in vivo* de mucormicosis causada por *R. delemar* (Gebremariam *et al.*, 2019), esta droga no resultó activa frente a la mayor parte de cepas de especies de Mucorales en este estudio. Tal y como se ha descrito (Alastruey-Izquierdo *et al.*, 2009a; Alastruey-Izquierdo *et al.*, 2009b), la anfotericina B fue el antifúngico más efectivo frente a estas, seguida del posaconazol. Por ello, el tratamiento de elección en la actualidad frente a las mucormicosis es la anfotericina B liposomal, además del isavuconazol (Cornely *et al.*, 2014; Marty *et al.*, 2016), mientras que se recomienda el posaconazol como terapia de rescate o profilaxis (Tissot *et al.*, 2017). Por último, el manogepix también demostró una actividad escasa frente a *A. alternata*, con una CME_{90} de 4 mg/L por ambas metodologías, siendo moderada la sensibilidad de estas cepas al resto de antifúngicos estudiados. A su vez, en otros trabajos, este compuesto resultó activo contra los hongos dimórficos *C. immitis* y *C. posadasii* (Viriyakosol *et al.*, 2019).

La otra droga con capacidad antifúngica probada en esta tesis fue la olorofima, orotomida que inhibe la síntesis de pirimidina comprometiendo, así, la síntesis de ácidos nucleicos, de la pared y la membrana celular fúngica. Esta ha demostrado buena eficacia *in vitro* contra un amplio espectro de hongos patogénicos, como *H. capsulatum*, *B. dermatitidis* y distintas especies de *Penicillium*, *Scedosporium*/*Lomentospora* o *Fusarium* (Wiederhold *et al.*, 2017; Biswas *et al.*, 2018a; Jorgensen *et al.*, 2018), además de *C. immitis* y *C. posadasii*, las cuales también han sido tratadas con éxito en un modelo murino (Wiederhold *et al.*, 2018a). Aunque es inactivo frente a Mucorales y levaduras (Oliver *et al.*, 2016; Jorgensen *et al.*, 2018), también presenta eficiencia en el laboratorio y en modelo animal frente a las principales especies del género *Aspergillus*, como *A. terreus*, *A. flavus*, *A. nidulans* o *A. fumigatus*, incluyendo cepas de esta última resistentes a azoles con o sin mutaciones en *cyp51A* (Oliver *et al.*, 2016; Buil *et al.*, 2017; Negri *et al.*, 2017; Lackner *et al.*, 2018; Seyedmousavi *et al.*, 2019). Sin embargo, pocos estudios recogen resultados de su actividad frente a las especies crípticas de este género.

Al probar la olorofima frente a las cinco especies crípticas más habitualmente identificadas en muestras clínicas del complejo de *A. fumigatus*: *A. lentulus*, *A. thermomutatus, A. fumigatiaffinis*, *A. udagawae* y *Aspergillus hiratsukae*, esta exhibió una CMI_{90} de entre 0,015 mg/L y 0,03 mg/L, siendo el antifúngico con CMIs más bajas de todos los ensayados. Del mismo modo, este nuevo fármaco fue activo frente a las especies multirresistentes del complejo *A. ustus*: *A. keveii*, *A. insuetus* y *A. calidoustus*. La CMI_{90} de la olorofima frente a 20 cepas de esta última especie fue de 0,12 mg/L para CLSI y de 0,25 mg/L para EUCAST, en consonancia con estudios anteriores (Buil *et al.*, 2017). Estos resultados son prometedores de cara a conseguir un tratamiento efectivo frente a estas especies crípticas que muestran resistencia intrínseca a una o más de las familias de antifúngicos disponibles y cuya incidencia en el ámbito clínico se

está incrementando (Alcazar-Fuoli *et al.*, 2008; Egli *et al.*, 2012; Zbinden *et al.*, 2012; Yoshida *et al.*, 2015; Seroy *et al.*, 2017).

Las especies crípticas del complejo *A. terreus*, *A. citrinoterreus*, *Aspergillus aureoterreus*, *Aspergillus hortai* y *Aspergillus carneus*, caracterizadas por exhibir baja sensibilidad a la anfotericina B y, en ocasiones, moderada a azoles (Escribano *et al.*, 2012; Kathuria *et al.*, 2015; Risslegger *et al.*, 2017), mostraron una media geométrica de sus CMIs frente a olorofima de 0,015 mg/L. Solo se obtuvieron resultados similares con la micafungina, mientras que el voriconazol, el posaconazol y la anfotericina B presentaron valores de CMI más elevados. En otro estudio en el que se analizaron cepas del complejo se alcanzaron un resultados semejantes (Lackner *et al.*, 2018).

Aspergillus ochraceus es la especie de la sección Circumdati que ha sido aislada con mayor frecuencia de individuos inmunodeprimidos, seguida de *A. sclerotiorum*, aunque se desconoce aún su función exacta como patógenos (Arabatzis *et al.*, 2011; Gheith *et al.*, 2014; Siqueira *et al.*, 2017). Los escasos estudios sobre su sensibilidad *in vitro* indican que se tratan de especies con CMIs muy elevadas a anfotericina B y variables a los azoles (Arabatzis *et al.*, 2011; Siqueira *et al.*, 2017), por lo que la actividad mostrada por la olorofima frente a ellas, con una media geométrica de las CMIs de cada especie menor de 0,02 mg/L, supone que deba ser considerada como una opción terapéutica que podría explorarse en el futuro.

Aspergillus alliaceus y *A. tubingensis*, de los complejos de *A. flavus* y *A. niger*, respectivamente, presentan una sensibilidad variable a los azoles y, además, *A. alliaceus* exhibe CMIs elevadas a anfotericina B (Balajee *et al.*, 2007; Ozhak-Baysan *et al.*, 2010; Perez-Cantero *et al.*, 2019). La olorofima ha demostrado ser efectiva *in vitro* frente a *A. tubingensis* en otros trabajos (Buil *et al.*, 2017; Jorgensen *et al.*, 2018), algo corroborado en este estudio con una CMI_{50} de 0,06 mg/L. Los reducidos valores de CMI obtenidos de este nuevo fármaco contra *A. alliaceus* también lo convierten en un potencial antifúngico para tratar las infecciones que esta especie causa.

Algunas especies crípticas de *Aspergillus* fueron ensayadas tanto frente al manogepix como a la olorofima, lo que permitió comparar la actividad de ambos compuestos, aunque es importante resaltar que se determinan CMEs en manogepix y, en cambio, CMIs en olorofima, por lo que los resultados no son directamente equiparables. En general, todas ellas mostraron valores de CMI/CME bajas a los dos, observándose solo una ligera diferencia en la mejor eficiencia del manogepix frente a *A. thermomutatus* (CME_{90} de 0,03 mg/L, mientras que la olorofima CMI_{90} de 0,25 mg/L por EUCAST y de 0,12 mg/L por CLSI) y de la olorofima frente a *A. alliaceus* (CMI_{90} de 0,03 mg/L por EUCAST y de 0,015 mg/L por CLSI, y el manogepix CME_{90} de 0,12 mg/L por EUCAST y de 0,03 mg/L por CLSI).

Al contrastar los resultados obtenidos por las dos metodologías utilizadas para determinar la sensibilidad antifúngica en ambos estudios, se observó que CLSI dio lugar, en general, a valores de CMI/CME más bajos que EUCAST. Aunque en la mayor parte de cepas se trata de una

diferencia de una o dos diluciones, otras no fueron capaces de crecer a las 24 horas de ser inoculadas en las placas de CLSI o mostraron una discrepancia de CMIs/CMEs aún mayor, como las de las especies de *Scedosporium*/*Lomentospora* o *Fusarium* probadas frente al manogepix y sus antifúngicos comparadores. Estas diferencias pueden deberse al lento crecimiento de estas cepas, además de a las características que distinguen a ambos métodos, como la concentración de cepa inoculada en cada pocillo de la placa o la concentración de glucosa en el medio utilizado, menor en ambos casos en CLSI. Sin embargo, en un trabajo anterior en el que se compararon las dos técnicas de referencia para probar el manogepix frente *Fusarium* y *Scedosporium* (Castanheira *et al.*, 2012) se encontraron menores diferencias que en este estudio.

Estos dos trabajos confirman la alentadora actividad *in vitro* del manogepix y la olorofima frente a un gran número de patógenos fúngicos que se caracterizan por originar infecciones que son difíciles de tratar con los antifúngicos actualmente disponibles para uso clínico. El manogepix ha demostrado ser efectivo contra las especies de *Scedosporium*/*Lomentospora* y las crípticas de *Aspergillus*, incluso frente a aquellas multirresistentes. A su vez, la olorofima ha evidenciado su actividad *in vitro* frente a un gran número de especies crípticas de *Aspergillus*. Estos resultados invitan a seguir investigando acerca de la utilidad de estos nuevos compuestos con capacidad antifúngica, y sería interesante realizar estudios multicéntricos para asegurar su reproducibilidad, además de trasladar las pruebas a modelos murinos de infección que garanticen la correlación de los resultados *in vitro* - *in vivo*.

4.5. Aplicabilidad y perspectivas futuras

En esta tesis se han estudiado en profundidad múltiples aspectos de la resistencia a los antifúngicos que permiten progresar en la caracterización de este fenómeno tanto desde el punto de vista epidemiológico como molecular.

En primer lugar, se ha ahondado en el estudio de la resistencia a equinocandinas en *C. glabrata*, ya que se ha observado que esta especie es capaz de adquirirla con especial facilidad y rapidez, habiendo aumentado el número de casos clínicos en diversas áreas geográficas en los últimos años. A pesar de que la prevalencia de las cepas resistentes a equinocandinas de esta especie no es elevada hasta la fecha en España, las conclusiones generadas de este trabajo permiten disponer de un mayor conocimiento acerca de cómo se desarrolla este fenómeno tanto a nivel clínico como en el laboratorio, el cual puede ser de gran utilidad en caso de que su detección comience a ser frecuente en nuestro país. Con el fin de continuar con este trabajo, se ha secuenciado el genoma de todas las cepas incluidas en él, además de del resto de las recibidas en el Laboratorio de Referencia e Investigación en Micología que fueron categorizadas como resistentes. Por tanto, nuestro trabajo futuro relacionado con esta investigación pasa por realizar el análisis de estos genomas, basado en la comparativa de los genomas resistentes frente a un genoma de referencia sensible a equinocandinas, o frente a las cepas sensibles disponibles del mismo paciente, para determinar el número de variantes existentes entre ellos

y en qué posición del genoma se encuentran. A su vez, la secuenciación de estos genomas ha permitido corroborar el poder de discriminación de esta técnica para estudiar la diversidad genética de *C. glabrata* (Biswas *et al.*, 2018b), al ser capaz de genotipar todas las cepas con mejor resolución que el MLST y el MLP.

El programa de vigilancia de la resistencia antifúngica establecido en el Laboratorio de Referencia de Micología del Centro Nacional de Microbiología del Instituto de Salud Carlos III ha permitido demostrar que la resistencia a los triazoles en las especies de *Aspergillus* es una realidad que debe ser supervisada en España, además de estudiar los principales mecanismos de resistencia basados en la presencia de mutaciones en las isoenzimas Cyp51. El programa de vigilancia sigue vigente con el fin de proveer a los laboratorios clínicos de un servicio adicional para la evaluación de la resistencia antifúngica. Sin embargo, en un futuro sería de gran utilidad realizar cortes puntuales en los que se analicen las tasas de resistencia reales presentes en el país para determinar si este fenómeno está aumentando, lo que puede ayudar a desarrollar medidas para su control a nivel nacional.

Además, se ha desarrollado una herramienta basada en la técnica de HRM que permite la detección temprana y simultánea de las principales mutaciones puntuales descritas en el gen *cyp51A* y de los mecanismos de tipo tándem en su promotor asociados a la resistencia a los azoles en *A. fumigatus*, fenómeno al alza en todo el mundo y observado entre las cepas de esta especie identificadas en el programa de vigilancia. Esta técnica evita el retraso en la obtención de resultados ligada a los métodos de determinación de la sensibilidad antifúngica y a la secuenciación de Sanger y permite conocer el perfil de resistencia asociado a las alteraciones en *cyp51A* que posee cada cepa, lo que facilita el inicio de una terapia antifúngica apropiada en un tiempo reducido. A su vez, puede constituir una alternativa más económica a los métodos basados en el uso de sondas marcadas con distintos fluoróforos, con una sensibilidad similar o superior. Como pasos futuros, habría que comprobar su utilidad para detectar estas mutaciones de forma directa a partir de muestras clínicas, además de integrar en ella los nuevos mecanismos de resistencia descritos. A su vez, podría valorarse el desarrollo de una herramienta de características similares para la detección de las principales alteraciones en los genes *cyp51* de otras especies de *Aspergillus*, en caso de confirmarse la implicación en la resistencia a azoles de las mutaciones encontradas en las cepas de A. *terreus* y A. *flavus* en esta tesis.

Por último, la confirmación de la actividad *in vitro* de dos nuevos compuestos con capacidad antifúngica, el manogepix y la olorofima, frente a algunas de las especies emergentes que presentan baja sensibilidad a los antifúngicos actualmente disponibles para el uso clínico constituye un gran avance frente al problema que supone la resistencia antifúngica. Los buenos resultados obtenidos invitan a realizar futuros estudios que aseguren su eficacia en modelos murinos de infección causada por estos patógenos fúngicos con el fin de conseguir su traslado a la clínica lo antes posible.

5. Conclusiones

5. CONCLUSIONES

1. *Candida glabrata* desarrolla resistencia secundaria a las equinocandinas tras la exposición a las mismas, tanto en el tratamiento de pacientes hospitalarios como en las condiciones *in vitro* establecidas en el laboratorio.

2. La detección de la resistencia a equinocandinas en cepas de *C. glabrata* debe realizarse mediante el estudio de la presencia de mutaciones en las regiones *hot-spot* de sus genes *FKS* y la determinación de su perfil de sensibilidad a micafungina y anidulafungina.

3. El desarrollo *in vitro* de la resistencia a equinocandinas en aislados clínicos de *C. glabrata* no se correlaciona con genotipos específicos.

4. La exposición directa *in vitro* a la concentración más baja de micafungina y anidulafungina considerada como resistente por EUCAST puede generar resistencia a equinocandinas en aislados clínicos de *C. glabrata* tras 48 horas desde su inicio, siendo necesarias concentraciones superiores a 2 mg/L de ambas para evitar su desarrollo.

5. La detección de aislados clínicos de *Aspergillus* spp. resistentes a azoles en España hace patente la importancia de vigilar este fenómeno con el fin de detectar el mecanismo de resistencia más prevalente a estos antifúngicos y desarrollar estrategias adaptadas que permitan su control.

6. La técnica desarrollada basada en el análisis de curvas de fusión de alta resolución (HRM) permite detectar de forma rápida cualquiera de las principales mutaciones en el gen *cyp51A* y/o su promotor asociadas a resistencia a los azoles en *Aspergillus fumigatus*, posibilitando el inicio temprano de una terapia antifúngica apropiada.

7. El manogepix resulta eficaz frente a las especies crípticas de *Aspergillus* y a las especies de *Scedosporium* y *Lomentospora*, pero no tiene actividad *in vitro* frente a las especies del orden Mucorales y solo es moderadamente activo frente a *Fusarium solani* y *Alternaria alternata*.

8. La olorofima es más activa *in vitro* que los antifúngicos de uso clínico frente a las especies crípticas de *Aspergillus*, lo que incita a estudiar en profundidad su eficacia como potencial tratamiento de las infecciones causadas por estas especies, algunas con escasa opción terapéutica.

6. Referencias

6. REFERENCIAS

Abbes S, Sellami H, Sellami A, Hadrich I, Amouri I, Mahfoudh N, *et al.* 2012. *Candida glabrata* strain relatedness by new microsatellite markers. *Eur J Clin Microbiol Infect Dis*, 31:83-91.

Adler-Moore JP, Proffitt RT. 2008. Amphotericin B lipid preparations: what are the differences? *Clin Microbiol Infect*, 14:25-36.

Al-Hatmi AM, Hagen F, Menken SB, Meis JF, de Hoog GS. 2016a. Global molecular epidemiology and genetic diversity of *Fusarium*, a significant emerging group of human opportunists from 1958 to 2015. *Emerg Microbes Infect*, 5:e124.

Al-Hatmi AM, Meis JF, de Hoog GS. 2016b. *Fusarium*: molecular diversity and intrinsic drug resistance. *PLoS Pathog*, 12:e1005464.

Al-Hatmi AMS, Bonifaz A, Ranque S, Sybren de Hoog G, Verweij PE, Meis JF. 2018. Current antifungal treatment of fusariosis. *Int J Antimicrob Agents*, 51:326-332.

Alanio A, Sitterle E, Liance M, Farrugia C, Foulet F, Botterel F, *et al.* 2011. Low prevalence of resistance to azoles in *Aspergillus fumigatus* in a French cohort of patients treated for haematological malignancies. *J Antimicrob Chemother*, 66:371-374.

Alanio A, Desnos-Ollivier M, Garcia-Hermoso D, Bretagne S. 2017. Investigating clinical issues by genotyping of medically important fungi: why and how? *Clin Microbiol Rev*, 30:671-707.

Alastruey-Izquierdo A, Castelli MV, Cuesta I, Zaragoza O, Monzon A, Mellado E, *et al.* 2009a. *In vitro* activity of antifungals against Zygomycetes. *Clin Microbiol Infect*, 15:71-76.

Alastruey-Izquierdo A, Castelli MV, Cuesta I, Monzon A, Cuenca-Estrella M, Rodriguez-Tudela JL. 2009b. Activity of posaconazole and other antifungal agents against Mucorales strains identified by sequencing of internal transcribed spacers. *Antimicrob Agents Chemother*, 53:1686-1689.

Alastruey-Izquierdo A, Cuesta I, Ros L, Mellado E, Rodriguez-Tudela JL. 2011. Antifungal susceptibility profile of clinical *Alternaria* spp. identified by molecular methods. *J Antimicrob Chemother*, 66:2585-2587.

Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M. 2012. Current section and species complex concepts in *Aspergillus*: recommendations for routine daily practice. *Ann N Y Acad Sci*, 1273:18-24.

Alastruey-Izquierdo A, Mellado E, Pelaez T, Peman J, Zapico S, Alvarez M, *et al.* 2013. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). *Antimicrob Agents Chemother,* 57:3380-3387.

Alastruey-Izquierdo A, Alcazar-Fuoli L, Cuenca-Estrella M. 2014. Antifungal susceptibility profile of cryptic species of *Aspergillus*. *Mycopathologia*, 178:427-33.

Alastruey-Izquierdo A, Melhem MS, Bonfietti LX, Rodriguez-Tudela JL. 2015. Susceptibility test for fungi: clinical and laboratorial correlations in medical mycology. *Rev Inst Med Trop Sao Paulo*, 57:57-64.

Alastruey-Izquierdo A, Alcazar-Fuoli L, Rivero-Menendez O, Ayats J, Castro C, Garcia-Rodriguez J, *et al.* 2018. Molecular identification and susceptibility testing of molds isolated in a prospective surveillance of triazole resistance in Spain (FILPOP2 study). *Antimicrob Agents Chemother*, 62:e00358-18.

Albarrag AM, Anderson MJ, Howard SJ, Robson GD, Warn PA, Sanglard D, *et al.* 2011. Interrogation of related clinical pan-azole-resistant *Aspergillus fumigatus* strains: G138C, Y431C, and G434C single nucleotide polymorphisms in *cyp51A*, upregulation of *cyp51A*, and integration and activation of transposon Atf1 in the *cyp51A* promoter. *Antimicrob Agents Chemother*, 55:5113-5121.

Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A, Cuenca-Estrella M, Rodriguez-Tudela JL. 2008. *Aspergillus* section Fumigati: antifungal susceptibility patterns and sequence-based identification. *Antimicrob Agents Chemother*, 52:1244-1251.

Alexander BD, Johnson MD, Pfeiffer CD, Jimenez-Ortigosa C, Catania J, Booker R, *et al.* 2013. Increasing echinocandin resistance in *Candida glabrata*: clinical failure correlates with presence of *FKS* mutations and elevated minimum inhibitory concentrations. *Clin Infect Dis*, 56:1724-1732.

Alkhazraji S, Gebremariam T, Alqarihi A, Lin L, Gu Y, Shaw K, *et al*. 2019. APX001 protects immunosupressed mice from scedosporiosis. Poster oral O0734 en *29th European Congress of Clinical Microbiology and Infectious Diseases* (ECCMID), Amsterdam, Holanda.

Almirante B, Rodriguez D, Park BJ, Cuenca-Estrella M, Planes AM, Almela M, *et al.* 2005. Epidemiology and predictors of mortality in cases of *Candida* bloodstream infection: results from population-based surveillance, Barcelona, Spain, from 2002 to 2003. *J Clin Microbiol*, 43:1829-1835.

Allen D, Wilson D, Drew R, Perfect J. 2015. Azole antifungals: 35 years of invasive fungal infection management. E*xpert Rev Anti Infect Ther*, 13:787-798.

Andes DR, Safdar N, Baddley JW, Playford G, Reboli AC, Rex JH, *et al.* 2012. Impact of treatment strategy on outcomes in patients with candidemia and other forms of invasive candidiasis: a patient-level quantitative review of randomized trials. *Clin Infect Dis*, 54:1110-1122.

Andes D. 2013. Optimizing antifungal choice and administration. *Curr Med Res Opin*, 29:13-18.

Arabatzis M, Kambouris M, Kyprianou M, Chrysaki A, Foustoukou M, Kanellopoulou M, *et al.* 2011. Polyphasic identification and susceptibility to seven antifungals of 102 *Aspergillus* isolates recovered from immunocompromised hosts in Greece. *Antimicrob Agents Chemother*, 55:3025-3030.

Arendrup MC, Garcia-Effron G, Buzina W, Mortensen KL, Reiter N, Lundin C, *et al.* 2009. Breakthrough *Aspergillus fumigatus* and *Candida albicans* double infection during caspofungin treatment: laboratory characteristics and implication for susceptibility testing. *Antimicrob Agents Chemother*, 53:1185-1193.

Arendrup MC, Mavridou E, Mortensen KL, Snelders E, Frimodt-Moller N, Khan H, *et al.* 2010. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. *PLoS One,* 5:e10080.

Arendrup MC, Jensen RH, Grif K, Skov M, Pressler T, Johansen HK, *et al.* 2012a. *In vivo* emergence of *Aspergillus terreus* with reduced azole susceptibility and a Cyp51A M217I alteration. *J Infect Dis*, 206:981-985.

Arendrup MC, Perlin DS, Jensen RH, Howard SJ, Goodwin J, Hope W. 2012b. Differential *in vivo* activities of anidulafungin, caspofungin, and micafungin against *Candida glabrata* isolates with and without *FKS* resistance mutations. *Antimicrob Agents Chemother,* 56:2435-2442.

Arendrup MC, Perlin DS. 2014. Echinocandin resistance: an emerging clinical problem? *Curr Opin Infect Dis*, 27:484-492.

Arendrup MC, Meletiadis J, Mouton JW, Guinea J, Cuenca-Estrella M, Lagrou K, *et al.* 2016. EUCAST technical note on isavuconazole breakpoints for *Aspergillus*, itraconazole breakpoints for *Candida* and updates for the antifungal susceptibility testing method documents. *Clin Microbiol Infect*, 22:571 e1-4.

Arendrup MC, Chowdhary A, Astvad KMT, Jorgensen KM. 2018. APX001A *in vitro* activity against contemporary blood isolates and *Candida auris* determined by the EUCAST reference method. *Antimicrob Agents Chemother*, 62:e01225-18.

Astvad KMT, Johansen HK, Roder BL, Rosenvinge FS, Knudsen JD, Lemming L, *et al.* 2018. Update from a 12-year nationwide fungemia surveillance: increasing intrinsic and acquired resistance causes concern. *J Clin Microbiol*, 56: e01564-17.

Baddley JW, Marr KA, Andes DR, Walsh TJ, Kauffman CA, Kontoyiannis DP, *et al.* 2009. Patterns of susceptibility of *Aspergillus* isolates recovered from patients enrolled in the Transplant-Associated Infection Surveillance Network. *J Clin Microbiol*, 47:3271-3275.

Bader O, Weig M, Reichard U, Lugert R, Kuhns M, Christner M, *et al.* 2013. *cyp51A*-based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. A*ntimicrob Agents Chemother*, 57:3513-3517.

Bain JM, Tavanti A, Davidson AD, Jacobsen MD, Shaw D, Gow NA, *et al.* 2007. Multilocus sequence typing of the pathogenic fungus *Aspergillus fumigatus*. *J Clin Microbiol*, 45:1469-1477.

Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA. 2005a. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. *Eukaryot Cell*, 4:625-632.

Balajee SA, Gribskov J, Brandt M, Ito J, Fothergill A, Marr KA. 2005b. Mistaken identity: *Neosartorya pseudofischeri* and its anamorph masquerading as *Aspergillus fumigatus*. *J Clin Microbiol,* 43:5996-5999.

Balajee SA, Nickle D, Varga J, Marr KA. 2006. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. *Eukaryot Cell*, 5:1705-1712.

Balajee SA, Lindsley MD, Iqbal N, Ito J, Pappas PG, Brandt ME. 2007. Nonsporulating clinical isolate identified as *Petromyces alliaceus* (anamorph *Aspergillus alliaceus*) by morphological and sequence-based methods. *J Clin Microbiol,* 45:2701-2703.

Balajee SA, Borman AM, Brandt ME, Cano J, Cuenca-Estrella M, Dannaoui E, *et al.* 2009a. Sequence-based identification of *Aspergillus*, *Fusarium*, and Mucorales species in the clinical mycology laboratory: where are we and where should we go from here? *J Clin Microbiol*, 47:877-884.

Balajee SA, Kano R, Baddley JW, Moser SA, Marr KA, Alexander BD, *et al.* 2009b. Molecular identification of *Aspergillus* species collected for the transplant-associated infection surveillance network. *J Clin Microbiol*, 47:3138-3141.

Balashov SV, Gardiner R, Park S, Perlin DS. 2005. Rapid, high-throughput, multiplex, real-time PCR for identification of mutations in the *cyp51A* gene of *Aspergillus fumigatus* that confer resistance to itraconazole. *J Clin Microbiol*, 43:214-222.

Balashov SV, Park S, Perlin DS. 2006. Assessing resistance to the echinocandin antifungal drug caspofungin in *Candida albicans* by profiling mutations in *FKS1*. *Antimicrob Agents Chemother*, 50:2058-2063.

Beardsley J, Denning DW, Chau NV, Yen NT, Crump JA, Day JN. 2015. Estimating the burden of fungal disease in Vietnam. *Mycoses*, 58:101-106.

Bellete B, Raberin H, Morel J, Flori P, Hafid J, Manhsung RT. 2010. Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma. *Med Mycol*, 48:197-200.

Ben-Ami R, Lewis RE, Kontoyiannis DP. 2008. Immunocompromised hosts: immunopharmacology of modern antifungals. *Clin Infect Dis*, 47:226-235.

Bergamasco MD, Garnica M, Colombo AL, Nucci M. 2013. Epidemiology of candidemia in patients with hematologic malignancies and solid tumours in Brazil. *Mycoses*, 56:256-263.

Berkow EL, Lockhart SR. 2018. Activity of novel antifungal compound APX001A against a large collection of *Candida auris*. *J Antimicrob Chemother*, 73:3060-3062.

Bernhardt A, Sedlacek L, Wagner S, Schwarz C, Wurstl B, Tintelnot K. 2013. Multilocus sequence typing of *Scedosporium apiospermum* and *Pseudallescheria boydii* isolates from cystic fibrosis patients. *J Cyst Fibros*, 12:592-598.

Beyda ND, Lewis RE, Garey KW. 2012. Echinocandin resistance in *Candida* species: mechanisms of reduced susceptibility and therapeutic approaches. *Ann Pharmacother*, 46:1086-1096.

Beyda ND, John J, Kilic A, Alam MJ, Lasco TM, Garey KW. 2014. *FKS* mutant *Candida glabrata*: risk factors and outcomes in patients with candidemia. *Clin Infect Dis*, 59:819-825.

Biswas C, Chen SC, Halliday C, Martinez E, Rockett RJ, Wang Q, *et al.* 2017. Whole genome sequencing of *Candida glabrata* for detection of markers of antifungal drug resistance. *J Vis Exp*, 130: e56714.

Biswas C, Law D, Birch M, Halliday C, Sorrell TC, Rex J, *et al.* 2018a. *In vitro* activity of the novel antifungal compound F901318 against Australian *Scedosporium* and *Lomentospora* fungi. *Med Mycol*, 56:1050-1054.

Biswas C, Marcelino VR, Van Hal S, Halliday C, Martinez E, Wang Q, *et al.* 2018b. Whole genome sequencing of Australian *Candida glabrata* isolates reveals genetic diversity and novel sequence types. *Front Microbiol*, 9:2946.

Bitar D, Van Cauteren D, Lanternier F, Dannaoui E, Che D, Dromer F, *et al.* 2009. Increasing incidence of zygomycosis (mucormycosis), France, 1997-2006. *Emerg Infect Dis*, 15:1395-1401.

Bizerra FC, Jimenez-Ortigosa C, Souza AC, Breda GL, Queiroz-Telles F, Perlin DS, *et al.* 2014. Breakthrough candidemia due to multidrug-resistant *Candida glabrata* during prophylaxis with a low dose of micafungin. *Antimicrob Agents Chemother*, 58:2438-2440.

Bongomin F, Gago S, Oladele RO, Denning DW. 2017. Global and multi-national prevalence of fungal diseases-estimate precision. *J Fungi*, 3:57.

Bordallo-Cardona MA, Escribano P, de la Pedrosa EG, Marcos-Zambrano LJ, Canton R, Bouza E, *et al.* 2017. *In vitro* exposure to increasing micafungin concentrations easily promotes echinocandin resistance in *Candida glabrata* isolates. *Antimicrob Agents Chemother*, 61:e01542-16.

Bordallo-Cardona MA, Escribano P, Marcos-Zambrano LJ, Diaz-Garcia J, de la Pedrosa EG, Canton R, *et al.* 2018a. Low and constant micafungin concentrations may be sufficient to lead to resistance mutations in *FKS2* gene of *Candida glabrata*. *Med Mycol*, 56:903-906.

Bordallo-Cardona MA, Marcos-Zambrano LJ, Sanchez-Carrillo C, de la Pedrosa EGG, Canton R, Bouza E, *et al.* 2018b. Mutant Prevention Concentration and Mutant Selection Window of micafungin and anidulafungin in clinical *Candida glabrata* isolates. *Antimicrob Agents Chemother*, 62: e01982-17.

Bordallo-Cardona MA, Marcos-Zambrano LJ, Sanchez-Carrillo C, Bouza E, Munoz P, Escribano P, *et al.* 2018c. Resistance to echinocandins in *Candida* can be detected by performing the Etest directly on blood culture samples. *Antimicrob Agents Chemother*, 62:e00162-18.

Bordallo-Cardona MA, Agnelli C, Gomez-Nunez A, Sanchez-Carrillo C, Bouza E, Munoz P, *et al.* 2019. *MSH2* gene point mutations are not antifungal resistance markers in *Candida glabrata*. *Antimicrob Agents Chemother*, 63:e01876-18.

Borst A, Theelen B, Reinders E, Boekhout T, Fluit AC, Savelkoul PH. 2003. Use of amplified fragment length polymorphism analysis to identify medically important *Candida* spp., including *C. dubliniensis*. *J Clin Microbiol*, 41:1357-1362.

Borst A, Raimer MT, Warnock DW, Morrison CJ, Arthington-Skaggs BA. 2005. Rapid acquisition of stable azole resistance by *Candida glabrat*a isolates obtained before the clinical introduction of fluconazole. *Antimicrob Agents Chemother*, 49:783-787.

Bougnoux ME, Tavanti A, Bouchier C, Gow NA, Magnier A, Davidson AD, *et al.* 2003. Collaborative consensus for optimized multilocus sequence typing of *Candida albicans*. *J Clin Microbiol*, 41:5265-5266.

Brajtburg J, Powderly WG, Kobayashi GS, Medoff G. 1990. Amphotericin B: current understanding of mechanisms of action. *Antimicrob Agents Chemother*, 34:183-188.

Brisse S, Pannier C, Angoulvant A, de Meeus T, Diancourt L, Faure O, *et al.* 2009. Uneven distribution of mating types among genotypes of *Candida glabrata* isolates from clinical samples. *Eukaryot Cell*, 8:287-295.

Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. *Sci Transl Med*, 4:165rv13.

Brown GD, Netea MG. 2012. Exciting developments in the immunology of fungal infections. *Cell Host Microbe*, 11:422-424.

Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, *et al.* 2010. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. *J Antimicrob Chemother*, 65:2116-2118.

Buied A, Moore CB, Denning DW, Bowyer P. 2013. High-level expression of *cyp51B* in azole-resistant clinical *Aspergillus fumigatus* isolates. *J Antimicrob Chemother*, 68:512-514.

Buil JB, Rijs A, Meis JF, Birch M, Law D, Melchers WJG, *et al.* 2017. *In vitro* activity of the novel antifungal compound F901318 against difficult-to-treat *Aspergillus* isolates. *J Antimicrob Chemother*, 72:2548-2552.

Buil JB, Bruggemann RJM, Wasmann RE, Zoll J, Meis JF, Melchers WJG, *et al.* 2018. Isavuconazole susceptibility of clinical *Aspergillus fumigatus* isolates and feasibility of isavuconazole dose escalation to treat isolates with elevated MICs. *J Antimicrob Chemother*, 73:134-142.

Byun SA, Won EJ, Kim MN, Lee WG, Lee K, Lee HS, *et al.* 2018. Multilocus Sequence Typing (MLST) genotypes of *Candida glabrata* bloodstream isolates in Korea: association with

antifungal resistance, mutations in mismatch repair gene (*MSH2*), and clinical outcomes. *Front Microbiol*, 9:1523.

Campoy S, Adrio JL. 2017. Antifungals. *Biochem Pharmacol*, 133:86-96.

Camps SM, van der Linden JW, Li Y, Kuijper EJ, van Dissel JT, Verweij PE, *et al.* 2012a. Rapid induction of multiple resistance mechanisms in *Aspergillus fumigatus* during azole therapy: a case study and review of the literature. *Antimicrob Agents Chemother*, 56:10-16.

Camps SM, Dutilh BE, Arendrup MC, Rijs AJ, Snelders E, Huynen MA, *et al.* 2012b. Discovery of a HapE mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing. *PLoS One*, 7:e50034.

Cannon RD, Lamping E, Holmes AR, Niimi K, Baret PV, Keniya MV, *et al.* 2009. Efflux-mediated antifungal drug resistance. *Clin Microbiol Rev*, 22:291-321.

Canton R, Morosini MI. 2011. Emergence and spread of antibiotic resistance following exposure to antibiotics. *FEMS Microbiol Rev*, 35:977-991.

Carrete L, Ksiezopolska E, Gomez-Molero E, Angoulvant A, Bader O, Fairhead C, *et al.* 2019. Genome comparisons of *Candida glabrata* serial clinical isolates reveal patterns of genetic variation in infecting clonal populations. *Front Microbiol*, 10:112.

Castanheira M, Duncanson FP, Diekema DJ, Guarro J, Jones RN, Pfaller MA. 2012. Activities of E1210 and comparator agents tested by CLSI and EUCAST broth microdilution methods against *Fusarium* and *Scedosporium* species identified using molecular methods. *Antimicrob Agents Chemother*, 56:352-357.

Castanheira M, Woosley LN, Messer SA, Diekema DJ, Jones RN, Pfaller MA. 2014. Frequency of *FKS* mutations among *Candida glabrata* isolates from a 10-year global collection of bloodstream infection isolates. Antimicrob Agents Chemother, 58:577-580.

Castanheira M, Messer SA, Rhomberg PR, Pfaller MA. 2016. Antifungal susceptibility patterns of a global collection of fungal isolates: results of the SENTRY Antifungal Surveillance Program (2013). *Diagn Microbiol Infect Dis*, 85:200-204.

Chakrabarti A, Singh R. 2014. Mucormycosis in India: unique features. *Mycoses*, 57:85-90.

Chang YL, Yu SJ, Heitman J, Wellington M, Chen YL. 2017. New facets of antifungal therapy. *Virulence*, 8:222-236.

Choi MJ, Won EJ, Joo MY, Park YJ, Kim SH, Shin MG, *et al.* 2019. Microsatellite typing and resistance mechanism analysis of voriconazole-resistant *Aspergillus flavus* isolates in South Korean hospitals. *Antimicrob Agents Chemother*, 63(2): e01610-e01618.

Chong GL, van de Sande WW, Dingemans GJ, Gaajetaan GR, Vonk AG, Hayette MP, *et al.* 2015. Validation of a new *Aspergillus* real-time PCR assay for direct detection of *Aspergillus* and azole resistance of *Aspergillus fumigatus* on bronchoalveolar lavage fluid. *J Clin Microbiol*, 53:868-874.

Chong GM, van der Beek MT, von dem Borne PA, Boelens J, Steel E, Kampinga GA, *et al.* 2016. PCR-based detection of *Aspergillus fumigatus* Cyp51A mutations on bronchoalveolar lavage: a multicentre validation of the AsperGenius assay(R) in 201 patients with haematological disease suspected for invasive aspergillosis. *J Antimicrob Chemother*, 71:3528-3535.

C**howdhary A, Sharma C, Meis JF.** 2017. *Candida auris*: A rapidly emerging cause of hospital-acquired multidrug-resistant fungal infections globally. *PLoS Pathog*, 13:e1006290.

Cervera C. 2012. Candidemia and invasive candidiasis in the adult: clinical forms and treatment. *Enferm Infecc Microbiol Clin*, 30:483-491.

Cleveland AA, Farley MM, Harrison LH, Stein B, Hollick R, Lockhart SR, *et al.* 2012. Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008-2011. *Clin Infect Dis*, 55:1352-1361.

Cleveland AA, Harrison LH, Farley MM, Hollick R, Stein B, Chiller TM, *et al.* 2015. Declining incidence of candidemia and the shifting epidemiology of *Candida* resistance in two US metropolitan areas, 2008-2013: results from population-based surveillance. *PLoS One*, 10:e0120452.

Cornely OA, Maertens J, Winston DJ, Perfect J, Ullmann AJ, Walsh TJ, *et al.* 2007. Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. *N Engl J Med*, 356:348-359.

Cornely OA, Arikan-Akdagli S, Dannaoui E, Groll AH, Lagrou K, Chakrabarti A, *et al.* 2014. ESCMID and ECMM joint clinical guidelines for the diagnosis and management of mucormycosis 2013. *Clin Microbiol Infect*, 20:5-26.

Costa-de-Oliveira S, Marcos Miranda I, Silva RM, Pinto ESA, Rocha R, Amorim A, *et al.* 2011. *FKS2* mutations associated with decreased echinocandin susceptibility of *Candida glabrata* following anidulafungin therapy. *Antimicrob Agents Chemother*, 55:1312-1314.

Coste A, Turner V, Ischer F, Morschhauser J, Forche A, Selmecki A, *et al.* 2006. A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. *Genetics*, 172:2139-2156.

Cowen LE, Sanglard D, Howard SJ, Rogers PD, Perlin DS. 2014. Mechanisms of antifungal drug resistance. *Cold Spring Harb Perspect Med*, 5:a019752.

Cuenca-Estrella M, Rodriguez-Tudela JL. 2010. The current role of the reference procedures by CLSI and EUCAST in the detection of resistance to antifungal agents *in vitro*. *Expert Rev Anti Infect Ther*, 8:267-276.

D'Enfert C, Diaquin M, Delit A, Wuscher N, Debeaupuis JP, Huerre M, *et al.* 1996. Attenuated virulence of uridine-uracil auxotrophs of *Aspergillus fumigatus*. *Infect Immun*, 64:4401-4405.

da Matta DA, Souza ACR, Colombo AL. 2017. Revisiting species distribution and antifungal susceptibility of *Candida* bloodstream isolates from Latin American medical centers. *J Fungi*, 3:24.

Dagenais TR, Keller NP. 2009. Pathogenesis of *Aspergillus fumigatus* in invasive aspergillosis. *Clin Microbiol Rev*, 22:447-465.

Dannaoui E, Borel E, Monier MF, Piens MA, Picot S, Persat F. 2001. Acquired itraconazole resistance in *Aspergillus fumigatus*. *J Antimicrob Chemother*, 47:333-340.

Dannaoui E, Desnos-Ollivier M, Garcia-Hermoso D, Grenouillet F, Cassaing S, Baixench MT, *et al.* 2012. *Candida* spp. with acquired echinocandin resistance, France, 2004-2010. *Emerg Infect Dis*, 18:86-90.

Darling BA, Milder EA. 2018. Invasive aspergillosis. *Pediatr Rev*, 39:476-478.

Das I, Nightingale P, Patel M, Jumaa P. 2011. Epidemiology, clinical characteristics, and outcome of candidemia: experience in a tertiary referral center in the UK. *Int J Infect Dis*, 15:e759-e763.

de Gontijo FA, Pascon RC, Fernandes L, Machado J, Jr., Alspaugh JA, Vallim MA. 2014. The role of the *de novo* pyrimidine biosynthetic pathway in *Cryptococcus neoformans* high temperature growth and virulence. *Fungal Genet Biol*, 70:12-23.

Delliere S, Healey K, Gits-Muselli M, Carrara B, Barbaro A, Guigue N, *et al.* 2016. Fluconazole and echinocandin resistance of *Candida glabrata* correlates better with antifungal drug exposure rather than with *MSH2* mutator genotype in a French cohort of patients harboring low rates of resistance. *Front Microbiol*, 7:2038.

Denning DW, Park S, Lass-Florl C, Fraczek MG, Kirwan M, Gore R, *et al.* 2011. High-frequency triazole resistance found In nonculturable *Aspergillus fumigatus* from lungs of patients with chronic fungal disease. *Clin Infect Dis,* 52:1123-1129.

Denning DW. 2015. The ambitious '95-95 by 2025' roadmap for the diagnosis and management of fungal diseases. *Thorax*, 70:613-614.

Denning DW, Bromley MJ. 2015. Infectious Disease. How to bolster the antifungal pipeline. *Science*, 347:1414-1416.

Desnos-Ollivier M, Moquet O, Chouaki T, Guerin AM, Dromer F. 2011. Development of echinocandin resistance in *Clavispora lusitaniae* during caspofungin treatment. *J Clin Microbiol*, 49:2304-2306.

Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL. 2003. A point mutation in the 14alpha-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother*, 47:1120-1124.

Diekema DJ, Messer SA, Hollis RJ, Jones RN, Pfaller MA. 2003. Activities of caspofungin, itraconazole, posaconazole, ravuconazole, voriconazole, and amphotericin B against 448 recent clinical isolates of filamentous fungi. *J Clin Microbiol*, 41:3623-3626.

Diekema D, Arbefeville S, Boyken L, Kroeger J, Pfaller M. 2012. The changing epidemiology of healthcare-associated candidemia over three decades. *Diagn Microbiol Infect Dis*, 73:45-48.

Dodgson AR, Pujol C, Denning DW, Soll DR, Fox AJ. 2003. Multilocus sequence typing of *Candida glabrata* reveals geographically enriched clades. *J Clin Microbiol*, 41:5709-5717.

Drlica K. 2003. The mutant selection window and antimicrobial resistance. *J Antimicrob Chemother*, 52:11-17.

Dudiuk C, Macedo D, Leonardelli F, Theill L, Cabeza MS, Gamarra S, *et al.* 2017. Molecular confirmation of the relationship between *Candida guilliermondii* Fks1p naturally occurring amino acid substitutions and its intrinsic reduced echinocandin susceptibility. *Antimicrob Agents Chemother*, 61:e02644-16.

Egli A, Fuller J, Humar A, Lien D, Weinkauf J, Nador R, *et al.* 2012. Emergence of *Aspergillus calidoustus* infection in the era of posttransplantation azole prophylaxis. *Transplantation*, 94:403-410.

Enache-Angoulvant A, Bourget M, Brisse S, Stockman-Pannier C, Diancourt L, Francois N, *et al.* 2010. Multilocus microsatellite markers for molecular typing of *Candida glabrata*: application to analysis of genetic relationships between bloodstream and digestive system isolates. *J Clin Microbiol*, 48:4028-4034.

Escribano P, Recio S, Pelaez T, Bouza E, Guinea J. 2011. *Aspergillus fumigatus* strains with mutations in the *cyp51A* gene do not always show phenotypic resistance to itraconazole, voriconazole, or posaconazole. *Antimicrob Agents Chemother*, 55:2460-2462.

Escribano P, Pelaez T, Recio S, Bouza E, Guinea J. 2012. Characterization of clinical strains of *Aspergillus terreus* complex: molecular identification and antifungal susceptibility to azoles and amphotericin B. *Clin Microbiol Infect,* 18:E24-26.

Escribano P, Pelaez T, Munoz P, Bouza E, Guinea J. 2013. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? *Antimicrob Agents Chemother*, 57:2815-2820.

Espinel-Ingroff A, Turnidge J. 2016. The role of epidemiological cutoff values (ECVs/ECOFFs) in antifungal susceptibility testing and interpretation for uncommon yeasts and moulds. *Rev Iberoam Micol,* 33:63-75.

EUCAST Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing. 2017a. EUCAST E.DEF 9.3.1. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9_3_1_Mould_testing__definitive.pdf.

EUCAST Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing. 2017b. EUCAST E.DEF 7.3.1. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts.http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_7_3_1_Yeast_testing__definitive.pdf.

Farmakiotis D, Tarrand JJ, Kontoyiannis DP. 2014. Drug-resistant *Candida glabrata* infection in cancer patients. *Emerg Infect Dis*, 20:1833-1840.

Farmakiotis D, Kontoyiannis DP. 2016. Mucormycoses. *Infect Dis Clin North Am*, 30:143-163.

Fekkar A, Meyer I, Brossas JY, Dannaoui E, Palous M, Uzunov M, *et al.* 2013. Rapid emergence of echinocandin resistance during *Candida kefyr* fungemia treatment with caspofungin. *Antimicrob Agents Chemother*, 57:2380-2382.

Fernandez-Ruiz M, Aguado JM, Almirante B, Lora-Pablos D, Padilla B, Puig-Asensio M, *et al.* **2**014. Initial use of echinocandins does not negatively influence outcome in *Candida parapsilosis* bloodstream infection: a propensity score analysis. *Clin Infect Dis*, 58:1413-1421.

Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, *et al.* 2012. Gain-of-function mutations in *UPC2* are a frequent cause of *ERG11* upregulation in azole-resistant clinical isolates of *Candida albicans*. *Eukaryot Cell*, 11:1289-1299.

Foulet F, Nicolas N, Eloy O, Botterel F, Gantier JC, Costa JM, *et al.* 2005. Microsatellite marker analysis as a typing system for *Candida glabrata*. *J Clin Microbiol*, 43:4574-4579.

Fraczek MG, Bromley M, Buied A, Moore CB, Rajendran R, Rautemaa R, *et al.* 2013. The *cdr1B* efflux transporter is associated with non-*cyp51a*-mediated itraconazole resistance in *Aspergillus fumigatus*. *J Antimicrob Chemother*, 68:1486-1496.

Franz R, Michel S, Morschhauser J. 1998. A fourth gene from the *Candida albicans* CDR family of ABC transporters. *Gene*, 220:91-98.

Gago S, Zaragoza O, Cuesta I, Rodriguez-Tudela JL, Cuenca-Estrella M, Buitrago MJ. 2011. High-resolution melting analysis for identification of the *Cryptococcus neoformans-Cryptococcus gattii* complex. *J Clin Microbiol*, 49:3663-3666.

Garavito MF, Narvaez-Ortiz HY, Zimmermann BH. 2015. Pyrimidine metabolism: dynamic and versatile pathways in pathogens and cellular development. *J Genet Genomics*, 42:195-205.

Garcia-Effron G, Katiyar SK, Park S, Edlind TD, Perlin DS. 2008a. A naturally occurring proline-to-alanine amino acid change in Fks1p in *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* accounts for reduced echinocandin susceptibility. *Antimicrob Agents Chemother*, 52:2305-2312.

Garcia-Effron G, Dilger A, Alcazar-Fuoli L, Park S, Mellado E, Perlin DS. 2008b. Rapid detection of triazole antifungal resistance in *Aspergillus fumigatus*. *J Clin Microbiol*, 46:1200-1206.

Garcia-Effron G, Park S, Perlin DS. 2009a. Correlating echinocandin MIC and kinetic inhibition of *fks1* mutant glucan synthases for *Candida albicans*: implications for interpretive breakpoints. *Antimicrob Agents Chemother*, 53:112-122.

Garcia-Effron G, Lee S, Park S, Cleary JD, Perlin DS. 2009b. Effect of *Candida glabrata FKS1* and *FKS2* mutations on echinocandin sensitivity and kinetics of 1,3-beta-D-glucan synthase: implication for the existing susceptibility breakpoint. *Antimicrob Agents Chemother*, 53:3690-3699.

Garcia-Rubio R, Cuenca-Estrella M, Mellado E. 2017. Triazole resistance in *Aspergillus* species: an emerging problem. *Drugs*, 77:599-613.

Garcia-Rubio R, Monzon S, Alcazar-Fuoli L, Cuesta I, Mellado E. 2018. Genome-wide comparative analysis of *Aspergillus fumigatus* strains: the reference genome as a matter of concern. *Genes*, 9:363.

Garcia-Vidal C, Alastruey-Izquierdo A, Aguilar-Guisado M, Carratala J, Castro C, Fernandez-Ruiz M, *et al.* 2018. Executive summary of clinical practice guideline for the management of invasive diseases caused by *Aspergillus*: 2018 Update by the GEMICOMED-SEIMC/REIPI. *Enferm Infecc Microbiol Clin*, S0213-005X(18)30200-3.

Gautier M, Normand AC, Ranque S. 2016. Previously unknown species of *Aspergillus*. *Clin Microbiol Infect*, 22:662-669.

Gebremariam T, Alkhazraji S, Alqarihi A, Jeon HH, Gu Y, Kapoor M, *et al.* 2019. APX001 is effective in the treatment of murine invasive pulmonary aspergillosis. *Antimicrob Agents Chemother*, 63:e01713-18.

Gheith S, Saghrouni F, Bannour W, Ben Youssef Y, Khelif A, Normand AC, *et al.* 2014. Characteristics of invasive aspergillosis in neutropenic haematology patients (Sousse, Tunisia). *Mycopathologia*, 177:281-289.

Giacomazzi J, Baethgen L, Carneiro LC, Millington MA, Denning DW, Colombo AL, *et al.* 2016. The burden of serious human fungal infections in Brazil. *Mycoses*, 59:145-150.

Girmenia C. 2009. New generation azole antifungals in clinical investigation. *Expert Opin Investig Drugs*, 18:1279-1295.

Grau S, Luque S, Campillo N, Samso E, Rodriguez U, Garcia-Bernedo CA, *et al.* 2015. Plasma and peritoneal fluid population pharmacokinetics of micafungin in post-surgical patients with severe peritonitis. *J Antimicrob Chemother*, 70:2854-2861.

Grenouillet F, Millon L, Bart JM, Roussel S, Biot I, Didier E, *et al.* 2007. Multiple-locus variable-number tandem-repeat analysis for rapid typing of *Candida glabrata*. *J Clin Microbiol*, 45:3781-3784.

Grossman NT, Pham CD, Cleveland AA, Lockhart SR. 2015. Molecular mechanisms of fluconazole resistance in *Candida parapsilosis* isolates from a U.S. surveillance system. *Antimicrob Agents Chemother*, 59:1030-1037.

Guarro J. 2013. Fusariosis, a complex infection caused by a high diversity of fungal species refractory to treatment. *Eur J Clin Microbiol Infect Dis*, 32:1491-1500.

Guinea J, Zaragoza O, Escribano P, Martin-Mazuelos E, Peman J, Sanchez-Reus F, *et al.* 2014. Molecular identification and antifungal susceptibility of yeast isolates causing fungemia collected in a population-based study in Spain in 2010 and 2011. *Antimicrob Agents Chemother*, 58:1529-1537.

Guinea J, Sandoval-Denis M, Escribano P, Pelaez T, Guarro J, Bouza E. 2015. *Aspergillus citrinoterreus*, a new species of section Terrei isolated from samples of patients with nonhematological predisposing conditions. *J Clin Microbiol*, 53:611-617.

Guinea J, Verweij PE, Meletiadis J, Mouton JW, Barchiesi F, Arendrup MC, *et al.* 2019. How to: EUCAST recommendations on the screening procedure E.Def 10.1 for the detection of azole resistance in *Aspergillus fumigatus* isolates using four-well azole-containing agar plates. *Clin Microbiol Infect*, 25:681-687.

Ha YE, Peck KR, Joo EJ, Kim SW, Jung SI, Chang HH, *et al.* 2012. Impact of first-line antifungal agents on the outcomes and costs of candidemia. *Antimicrob Agents Chemother*, 56:3950-3956.

Hadrich I, Makni F, Neji S, Cheikhrouhou F, Bellaaj H, Elloumi M, *et al.* 2012. Amphotericin B *in vitro* resistance is associated with fatal *Aspergillus flavus* infection. *Med Mycol*, 50:829-834.

Hager CL, Larkin EL, Long L, Zohra Abidi F, Shaw KJ, Ghannoum MA. 2018. *In vitro* and *in vivo* evaluation of the antifungal activity of APX001A/APX001 against *Candida auris*. *Antimicrob Agents Chemother*, 62:e02319-17.

Hagiwara D, Takahashi H, Watanabe A, Takahashi-Nakaguchi A, Kawamoto S, Kamei K, *et al.* 2014. Whole-genome comparison of *Aspergillus fumigatus* strains serially isolated from patients with aspergillosis. *J Clin Microbiol*, 52:4202-4209.

Hakem R. 2008. DNA-damage repair; the good, the bad, and the ugly. *EMBO J*, 27:589-605.

Hamill RJ. 2013. Amphotericin B formulations: a comparative review of efficacy and toxicity. *Drugs*, 73:919-934.

Hare RK, Gertsen JB, Astvad KMT, Degn KB, Lokke A, Stegger M, *et al.* 2019. *In vivo* selection of a unique tandem repeat mediated azole resistance mechanism (TR120) in *Aspergillus fumigatus cyp51A*, Denmark. *Emerg Infect Dis*, 25:577-580.

Hashimoto A, Hagiwara D, Watanabe A, Yahiro M, Yikelamu A, Yaguchi T, *et al.* 2017. Drug sensitivity and resistance mechanism in *Aspergillus* section Nigri strains from Japan. *Antimicrob Agents Chemother*, 61: e02583-16.

Hata K, Horii T, Miyazaki M, Watanabe NA, Okubo M, Sonoda J, *et al.* 2011. Efficacy of oral E1210, a new broad-spectrum antifungal with a novel mechanism of action, in murine models of candidiasis, aspergillosis, and fusariosis. *Antimicrob Agents Chemother*, 55:4543-4551.

Healey KR, Jimenez Ortigosa C, Shor E, Perlin DS. 2016a. Genetic drivers of multidrug resistance in *Candida glabrata*. *Front Microbiol*, 7:1995.

Healey KR, Zhao Y, Perez WB, Lockhart SR, Sobel JD, Farmakiotis D, *et al.* 2016b. Prevalent mutator genotype identified in fungal pathogen *Candida glabrata* promotes multi-drug resistance. *Nat Commun*, 7:11128.

Healey KR, Nagasaki Y, Zimmerman M, Kordalewska M, Park S, Zhao Y, *et al.* 2017. The gastrointestinal tract is a major source of echinocandin drug resistance in a murine model of *Candida glabrata* colonization and systemic dissemination. *Antimicrob Agents Chemother,* 61:e01412-17.

Healey KR, Perlin DS. 2018. Fungal resistance to echinocandins and the MDR phenomenon in *Candida glabrata*. *J Fungi*, 4:105.

Heath CH, Slavin MA, Sorrell TC, Handke R, Harun A, Phillips M, *et al.* 2009. Population-based surveillance for scedosporiosis in Australia: epidemiology, disease manifestations and emergence of *Scedosporium aurantiacum* infection. *Clin Microbiol Infect*, 15:689-693.

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.

Hesstvedt L, Gaustad P, Andersen CT, Haarr E, Hannula R, Haukland HH, *et al.* 2015. Twenty-two years of candidaemia surveillance: results from a Norwegian national study. *Clin Microbiol Infect*, 21:938-945.

Hodiamont CJ, Dolman KM, Ten Berge IJ, Melchers WJ, Verweij PE, Pajkrt D. 2009. Multiple-azole-resistant *Aspergillus fumigatus* osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. *Med Mycol*, 47:217-220.

Hoffmann K, Pawlowska J, Walther G, Wrzosek M, de Hoog GS, Benny GL, *et al.* 2013. The family structure of the Mucorales: a synoptic revision based on comprehensive multigene-genealogies. *Persoonia*, 30:57-76.

Hou X, Xiao M, Chen SC, Kong F, Wang H, Chu YZ, *et al.* 2017. Molecular epidemiology and antifungal susceptibility of *Candida glabrata* in China (August 2009 to July 2014): a multi-center study. *Front Microbiol*, 8:880.

Hou X, Xiao M, Wang H, Yu SY, Zhang G, Zhao Y, *et al.* 2018. Profiling of *PDR1* and *MSH2* in *Candida glabrata* bloodstream isolates from a multicenter study in China. *Antimicrob Agents Chemother*, 62:e00153-18..

Howard SJ, Webster I, Moore CB, Gardiner RE, Park S, Perlin DS, *et al.* 2006. Multi-azole resistance in *Aspergillus fumigatus*. *Int J Antimicrob Agents*, 28:450-453.

Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, *et al.* 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg Infect Dis*, 15:1068-1076.

Howard SJ, Lass-Florl C, Cuenca-Estrella M, Gomez-Lopez A, Arendrup MC. 2013. Determination of isavuconazole susceptibility of *Aspergillus* and *Candida* species by the EUCAST method. *Antimicrob Agents Chemother*, 57:5426-31.

Hu W, Sillaots S, Lemieux S, Davison J, Kauffman S, Breton A, *et al.* 2007. Essential gene identification and drug target prioritization in *Aspergillus fumigatus*. *PLoS Pathog*, 3:e24.

Hull CM, Bader O, Parker JE, Weig M, Gross U, Warrilow AG, *et al.* 2012. Two clinical isolates of *Candida glabrata* exhibiting reduced sensitivity to amphotericin B both harbor mutations in *ERG2*. A*ntimicrob Agents Chemother*, 56:6417-6421.

Iatta R, Nuccio F, Immediato D, Mosca A, De Carlo C, Miragliotta G, *et al.* 2016. Species distribution and *in vitro* azole susceptibility of *Aspergillus* section Nigri isolates from clinical and environmental settings. *J Clin Microbiol*, 54:2365-2372.

Jabeen K, Farooqi J, Mirza S, Denning D, Zafar A. 2017. Serious fungal infections in Pakistan. *Eur J Clin Microbiol Infect Dis*, 36:949-956.

Jacobsen MD, Boekhout T, Odds FC. 2008. Multilocus sequence typing confirms synonymy but highlights differences between *Candida albicans* and *Candida stellatoidea*. *FEMS Yeast Res*, 8:764-770.

Jensen RH, Justesen US, Rewes A, Perlin DS, Arendrup MC. 2014. Echinocandin failure case due to a previously unreported *FKS1* mutation in *Candida krusei*. *Antimicrob Agents Chemother*, 58:3550-3552.

Jensen RH, Johansen HK, Soes LM, Lemming LE, Rosenvinge FS, Nielsen L, *et al.* 2015. Posttreatment antifungal resistance among colonizing *Candida* isolates in candidemia patients: results from a systematic multicenter study. *Antimicrob Agents Chemother*, 60:1500-1508.

Jimenez-Ortigosa C, Paderu P, Motyl MR, Perlin DS. 2014. Enfumafungin derivative MK-3118 shows increased *in vitro* potency against clinical echinocandin-resistant *Candida* species and *Aspergillus* species isolates. *Antimicrob Agents Chemother*, 58(2):1248-1251.

Jimenez-Ortigosa C, Perez WB, Angulo D, Borroto-Esoda K, Perlin DS. 2017. *De novo* acquisition of resistance to SCY-078 in *Candida glabrata* involves *FKS* mutations that both overlap and are distinct from those conferring echinocandin resistance. *Antimicrob Agents Chemother*, 61(9): e00833-17.

Jorgensen KM, Astvad KMT, Hare RK, Arendrup MC. 2018. EUCAST determination of olorofim (F901318) susceptibility of mold species, method validation, and MICs. *Antimicrob Agents Chemother*, 62: e00487-18.

Jorgensen KM, Astvad KMT, Hare RK, Arendrup MC. 2019. EUCAST susceptibility testing of isavuconazole: MIC data for contemporary clinical mould and yeast isolates. *Antimicrob Agents Chemother*, 63:e00073-19.

Kale-Pradhan PB, Morgan G, Wilhelm SM, Johnson LB. 2010. Comparative efficacy of echinocandins and nonechinocandins for the treatment of *Candida parapsilosis* Infections: a meta-analysis. *Pharmacotherapy*, 30:1207-1213.

Kanafani ZA, Perfect JR. 2008. Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. *Clin Infect Dis*, 46:120-128.

Kathuria S, Sharma C, Singh PK, Agarwal P, Agarwal K, Hagen F, *et al.* 2015. Molecular epidemiology and *in vitro* antifungal susceptibility of *Aspergillus terreus* species complex isolates in Delhi, India: evidence of genetic diversity by amplified fragment length polymorphism and microsatellite typing. *PLoS One*, 10:e0118997.

Katiyar SK, Alastruey-Izquierdo A, Healey KR, Johnson ME, Perlin DS, Edlind TD. 2012. Fks1 and Fks2 are functionally redundant but differentially regulated in *Candida glabrata*: implications for echinocandin resistance. *Antimicrob Agents Chemother*, 56:6304-6309.

Klaassen CH, de Valk HA, Curfs-Breuker IM, Meis JF. 2010. Novel mixed-format real-time PCR assay to detect mutations conferring resistance to triazoles in *Aspergillus fumigatus* and prevalence of multi-triazole resistance among clinical isolates in the Netherlands. *J Antimicrob Chemother*, 65:901-905.

Klimko N, Kozlova Y, Khostelidi S, Shadrivova O, Borzova Y, Burygina E, *et al.* 2015. The burden of serious fungal diseases in Russia. *Mycoses*, 58:58-62.

Klingspor L, Saaedi B, Ljungman P, Szakos A. 2015a. Epidemiology and outcomes of patients with invasive mould infections: a retrospective observational study from a single centre (2005-2009). *Mycoses*, 58:470-477.

Klingspor L, Tortorano AM, Peman J, Willinger B, Hamal P, Sendid B, *et al.* 2015b. Invasive *Candida* infections in surgical patients in intensive care units: a prospective, multicentre survey initiated by the European Confederation of Medical Mycology (ECMM) (2006-2008). *Clin Microbiol Infect*, 21(1):87.e1-87.e10.

Kocsube S, Perrone G, Magista D, Houbraken J, Varga J, Szigeti G, *et al.* 2016. *Aspergillus* is monophyletic: evidence from multiple gene phylogenies and extrolites profiles. *Stud Mycol*, 85:199-213.

Krcmery V, Jr., Spanik S, Kunova A, Trupl J. 1997. Breakthrough fungemia appearing during empiric therapy with amphotericin B. *Chemotherapy*, 43:367-370.

Kriengkauykiat J, Ito JI, Dadwal SS. 2011. Epidemiology and treatment approaches in management of invasive fungal infections. *Clin Epidemiol*, 3:175-191.

Krishnan BR, James KD, Polowy K, Bryant BJ, Valdya A, Smith S, *et al*. 2017. CD101, a novel echinocandin with exceptional stability properties and enhanced aqueous solubility. *J Antibiot (Tokyo)*, 70(2):130-135.

Krishnan-Natesan S, Chandrasekar PH, Alangaden GJ, Manavathu EK. 2008. Molecular characterisation of *cyp51A* and *cyp51B* genes coding for P450 14alpha-lanosterol demethylases A (CYP51Ap) and B (CYP51Bp) from voriconazole-resistant laboratory isolates of *Aspergillus flavus*. *Int J Antimicrob Agents*, 32:519-524.

Kurtz MB, Heath IB, Marrinan J, Dreikorn S, Onishi J, Douglas C. 1994. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-beta-D-glucan synthase. *Antimicrob Agents Chemother*, 38:1480-1489.

Lackner M, de Hoog GS, Verweij PE, Najafzadeh MJ, Curfs-Breuker I, Klaassen CH, *et al.* 2012. Species-specific antifungal susceptibility patterns of *Scedosporium* and *Pseudallescheria* species. *Antimicrob Agents Chemother*, 56:2635-2642.

Lackner M, Hagen F, Meis JF, Gerrits van den Ende AH, Vu D, Robert V, *et al.* 2014. Susceptibility and diversity in the therapy-refractory genus *Scedosporium*. *Antimicrob Agents Chemother*, 58:5877-5885.

Lackner M, Birch M, Naschberger V, Grassle D, Beckmann N, Warn P, *et al.* 2018. Dihydroorotate dehydrogenase inhibitor olorofim exhibits promising activity against all clinically relevant species within *Aspergillus* section Terrei. *J Antimicrob Chemother*, 73:3068-3073.

Lass-Florl C, Griff K, Mayr A, Petzer A, Gastl G, Bonatti H, *et al.* 2005. Epidemiology and outcome of infections due to *Aspergillus terreus*: 10-year single centre experience. *Br J Haematol*, 131:201-207.

Lass-Florl C, Cuenca-Estrella M. 2017. Changes in the epidemiological landscape of invasive mould infections and disease. *J Antimicrob Chemother*, 72:i5-i11.

Latge JP. 1999. *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev*, 12:310-350.

Laverdiere M, Lalonde RG, Baril JG, Sheppard DC, Park S, Perlin DS. 2006. Progressive loss of echinocandin activity following prolonged use for treatment of *Candida albicans* oesophagitis. *J Antimicrob Chemother*, 57:705-708.

Le Pape P, Lavergne RA, Morio F, Alvarez-Moreno C. 2016. Multiple fungicide-driven alterations in azole-resistant *Aspergillus fumigatus*, Colombia, 2015. *Emerg Infect Dis*, 22:156-157.

Lee CZ, Liou GY, Yuan GF. 2004. Comparaison of *Aspergillus flavus* and *Aspergillus oryzae* by amplified fragment length polymorphism. *Bot Bull Acad Sin*, 45:61-68.

Lee A, Prideaux B, Lee M, Zimmerman M, Padreu P, Barat S, *et al*. 2019. Penetration of ibrexafungerp (formerly SCY-078) versus micafungin at the site of infection in an intra-abdominal candidiasis mouse model. Poster oral O0740 en *29th European Congress of Clinical Microbiology and Infectious Diseases* (ECCMID), Amsterdam, Holanda.

Lehmann PF, Lin D, Lasker BA. 1992. Genotypic identification and characterization of species and strains within the genus *Candida* by using random amplified polymorphic DNA. *J Clin Microbiol*, 30:3249-3254.

Lemke A, Kiderlen AF, Kayser O. 2005. Amphotericin B. *Appl Microbiol Biotechnol*, 68:151-162.

Lepak A, Zhao M, Andes D. 2019. In vivo pharmacodynamic evaluation of the novel antifungal agent APX001 in a murine model of invasive pulmonary aspergillosis against wild-type and Cyp51 mutant Aspergillus fumigatus strains. Poster oral O1066 en *29th European Congress of Clinical Microbiology and Infectious Diseases* (ECCMID), Amsterdam, Holanda.

Lescar J, Meyer I, Akshita K, Srinivasaraghavan K, Verma C, Palous M, *et al.* 2014. *Aspergillus fumigatus* harbouring the sole Y121F mutation shows decreased susceptibility to voriconazole but maintained susceptibility to itraconazole and posaconazole. *J Antimicrob Chemother*, 69:3244-3247.

Lewis RE. 2011. Current concepts in antifungal pharmacology. *Mayo Clin Proc*, 86:805-817.

Lewis JS 2nd, Wiederhold NP, Wickes BL, Patterson TF, Jorgensen JH. 2013. Rapid emergence of echinocandin resistance in *Candida glabrata* resulting in clinical and microbiologic failure. *Antimicrob Agents Chemother*, 57:4559-4561.

Lin CY, Chen YC, Lo HJ, Chen KW, Li SY. 2007. Assessment of *Candida glabrata* strain relatedness by pulsed-field gel electrophoresis and multilocus sequence typing. *J Clin Microbiol*, 45:2452-2459.

Lin E, Moua T, Limper AH. 2017. Pulmonary mucormycosis: clinical features and outcomes. *Infection*, 45:443-448.

Liu W, Sun Y, Chen W, Liu W, Wan Z, Bu D, *et al.* 2012. The T788G mutation in the *cyp51C* gene confers voriconazole resistance in *Aspergillus flavus* causing aspergillosis. *Antimicrob Agents Chemother*, 56:2598-2603.

Locke JB, Almaguer AL, Zuill DE, Bartizal K. 2016. Characterization of *in vitro* resistance development to the novel echinocandin CD101 in *Candida* species. *Antimicrob Agents Chemother*, 60:6100-6107.

Lockhart SR, Etienne KA, Vallabhaneni S, Farooqi J, Chowdhary A, Govender NP, *et al.* 2017. Simultaneous emergence of multidrug-resistant *Candida auris* on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. *Clin Infect Dis*, 64:134-140.

Loeffler J, Hagmeyer L, Hebart H, Henke N, Schumacher U, Einsele H. 2000. Rapid detection of point mutations by fluorescence resonance energy transfer and probe melting curves in *Candida* species. *Clin Chem*, 46:631-635.

Long B, Koyfman A. 2015. Mucormycosis: what emergency physicians need to know? *Am J Emerg Med*, 33:1823-1825.

Lott TJ, Frade JP, Lyon GM, Iqbal N, Lockhart SR. 2012. Bloodstream and non-invasive isolates of *Candida glabrata* have similar population structures and fluconazole susceptibilities. *Med Mycol*, 50:136-142.

Loudon KW, Burnie JP, Coke AP, Matthews RC. 1993. Application of polymerase chain reaction to fingerprinting *Aspergillus fumigatus* by random amplification of polymorphic DNA. *J Clin Microbiol*, 31:1117-1121.

Madureira A, Bergeron A, Lacroix C, Robin M, Rocha V, de Latour RP, *et al.* 2007. Breakthrough invasive aspergillosis in allogeneic haematopoietic stem cell transplant recipients treated with caspofungin. *Int J Antimicrob Agents*, 30:551-554.

Maertens JA, Raad, II, Marr KA, Patterson TF, Kontoyiannis DP, Cornely OA, *et al.* 2016. Isavuconazole versus voriconazole for primary treatment of invasive mould disease caused by *Aspergillus* and other filamentous fungi (SECURE): a phase 3, randomised-controlled, non-inferiority trial. *Lancet*, 387:760-769.

Marty FM, Ostrosky-Zeichner L, Cornely OA, Mullane KM, Perfect JR, Thompson GR 3rd, *et al.* 2016. Isavuconazole treatment for mucormycosis: a single-arm open-label trial and case-control analysis. *Lancet Infect Dis*, 16:828-837.

Mavridou E, Meletiadis J, Rijs A, Mouton JW, Verweij PE. 2015. The strength of synergistic interaction between posaconazole and caspofungin depends on the underlying azole resistance mechanism of *Aspergillus fumigatus*. *Antimicrob Agents Chemother*, 59:1738-1744.

McCarty TP, Pappas PG. 2016. Invasive candidiasis. *Infect Dis Clin North Am*, 30:103-124.

McLellan CA, Whitesell L, King OD, Lancaster AK, Mazitschek R, Lindquist S. 2012. Inhibiting GPI anchor biosynthesis in fungi stresses the endoplasmic reticulum and enhances immunogenicity. *ACS Chem Biol*, 7:1520-1528.

Meletiadis J, Siopi M, Kanioura L, Jorgensen KM, Perlin DS, Mouton JW, *et al.* 2019. Development and multicentre validation of an agar-based screening method for echinocandin susceptibility testing of *Aspergillus* species. *J Antimicrob Chemother*, 74:2247-2254.

Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. 2001. Identification of two different 14-alpha sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. *J Clin Microbiol*, 39:2431-2438.

Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 2004. Substitutions at methionine 220 in the 14alpha-sterol demethylase (Cyp51A) of *Aspergillus fumigatus* are responsible for resistance *in vitro* to azole antifungal drugs. *Antimicrob Agents Chemother*, 48:2747-2750.

Mellado E, Garcia-Effron G, Buitrago MJ, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 2005. Targeted gene disruption of the 14-alpha sterol demethylase (*cyp51A*) in *Aspergillus fumigatus* and its role in azole drug susceptibility. *Antimicrob Agents Chemother,* 49:2536-2538.

Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Melchers WJ, Verweij PE, Cuenca-Estrella M, *et al.* 2007. A new *Aspergillus fumigatus* resistance mechanism conferring *in vitro* cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. *Antimicrob Agents Chemother*, 51:1897-1904.

Mencarini J, Mantengoli E, Tofani L, Riccobono E, Fornaini R, Bartalesi F, *et al.* 2018. Evaluation of candidemia and antifungal consumption in a large tertiary care Italian hospital over a 12-year period. *Infection*, 46:469-476.

Mesa-Arango AC, Trevijano-Contador N, Roman E, Sanchez-Fresneda R, Casas C, Herrero E, *et al.* 2014. The production of reactive oxygen species is a universal action mechanism of amphotericin B against pathogenic yeasts and contributes to the fungicidal effect of this drug. *Antimicrob Agents Chemother*, 58:6627-6638.

Mio T, Adachi-Shimizu M, Tachibana Y, Tabuchi H, Inoue SB, Yabe T, *et al.* 1997. Cloning of the *Candida albicans* homolog of *Saccharomyces cerevisiae GSC1*/*FKS1* and its involvement in beta-1,3-glucan synthesis. *J Bacteriol*, 179:4096-4105.

Miyazaki M, Horii T, Hata K, Watanabe NA, Nakamoto K, Tanaka K, *et al.* 2011. *In vitro* activity of E1210, a novel antifungal, against clinically important yeasts and molds. *Antimicrob Agents Chemother*, 55:4652-4658.

Morales-Lopez S, Dudiuk C, Vivot W, Szusz W, Cordoba SB, Garcia-Effron G. 2017. Phenotypic and molecular evaluation of echinocandin susceptibility of *Candida glabrata*, *Candida bracarensis*, and *Candida nivariensis* strains isolated during 30 Years in Argentina. *Antimicrob Agents Chemother*, 61: e00170-17.

Morio F, Loge C, Besse B, Hennequin C, Le Pape P. 2010. Screening for amino acid substitutions in the *Candida albicans* Erg11 protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. *Diagn Microbiol Infect Dis*, 66:373-384.

Morio F, Pagniez F, Lacroix C, Miegeville M, Le Pape P. 2012. Amino acid substitutions in the *Candida albicans* sterol Delta5,6-desaturase (Erg3p) confer azole resistance: characterization of two novel mutants with impaired virulence. *J Antimicrob Chemother*, 67:2131-2138.

Morschhauser J, Barker KS, Liu TT, Bla BWJ, Homayouni R, Rogers PD. 2007. The transcription factor Mrr1p controls expression of the *MDR1* efflux pump and mediates multidrug resistance in *Candida albicans*. *PLoS Pathog*, 3:e164.

Mortensen KL, Jensen RH, Johansen HK, Skov M, Pressler T, Howard SJ, *et al.* 2011. *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *J Clin Microbiol*, 49:2243-2251.

Mushi MF, Gross U, Mshana SE, Bader O. 2018. High diversity of *Candida glabrata* in a tertiary hospital-Mwanza, Tanzania. *Med Mycol*, doi:10.1093/mmy/myy151.

Nakamura I, Yoshimura S, Masaki T, Takase S, Ohsumi K, Hashimoto M, *et al.* 2017a. ASP2397: a novel antifungal agent produced by *Acremonium persicinum* MF-347833. *J Antibiot*, 70:45-51.

Nakamura I, Kanasaki R, Yoshikawa K, Furukawa S, Fujie A, Hamamoto H, *et al*. 2017b. Discovery of a new antifungal agent ASP2397 using a silkworm model of *Aspergillus fumigatus* infection. *J Antibiot*, 70: 41-44.

Nascimento AM, Goldman GH, Park S, Marras SA, Delmas G, Oza U, *et al.* 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrob Agents Chemother*, 47:1719-1726.

Navarro-Rodriguez P, Martin-Vicente A, Lopez-Fernandez L, Guarro J, Capilla J. 2019. Expression of *ERG11* and efflux pump genes *CDR1*, *CDR2* and *SNQ2* in voriconazole susceptible and resistant *Candida glabrata* strains. *Med Mycol*, doi:10.1093/mmy/myz014.

Negri CE, Goncalves SS, Xafranski H, Bergamasco MD, Aquino VR, Castro PT, *et al.* 2014. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and *in vitro* susceptibility to triazoles. *J Clin Microbiol*, 52:3633-3640.

Negri CE, Johnson A, McEntee L, Box H, Whalley S, Schwartz JA, *et al.* 2018. Pharmacodynamics of the novel antifungal agent F901318 for acute sinopulmonary aspergillosis caused by *Aspergillus flavus*. *J Infect Dis*, 217:1118-1127.

Neofytos D, Treadway S, Ostrander D, Alonso CD, Dierberg KL, Nussenblatt V, *et al.* 2013. Epidemiology, outcomes, and mortality predictors of invasive mold infections among transplant recipients: a 10-year, single-center experience. *Transpl Infect Dis*, 15:233-242.

Nett JE, Andes DR. 2016. Antifungal agents: spectrum of activity, pharmacology, and clinical indications. *Infect Dis Clin North Am*, 30:51-83.

Nivoix Y, Leveque D, Herbrecht R, Koffel JC, Beretz L, Ubeaud-Sequier G. 2008a. The enzymatic basis of drug-drug interactions with systemic triazole antifungals. *Clin Pharmacokinet*, 47:779-792.

Nivoix Y, Velten M, Letscher-Bru V, Moghaddam A, Natarajan-Ame S, Fohrer C, *et al.* 2008b. Factors associated with overall and attributable mortality in invasive aspergillosis. *Clin Infect Dis*, 47:1176-1184.

Noble SM, Johnson AD. 2005. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot Cell*, 4:298-309.

Nucci M, Varon AG, Garnica M, Akiti T, Barreiros G, Trope BM, *et al.* 2013. Increased incidence of invasive fusariosis with cutaneous portal of entry, Brazil. *Emerg Infect Dis*, 19:1567-1572.

Nucci M, Anaissie EJ, Queiroz-Telles F, Martins CA, Trabasso P, Solza C, *et al.* 2003. Outcome predictors of 84 patients with hematologic malignancies and *Fusarium* infection. Cancer 98:315-319.

O'Donnell K, Sutton DA, Rinaldi MG, Sarver BAJ, Balajee SA, Schroers HJ, *et al*. 2010. Internet-accessible DNA sequence database for identifying Fusaria from human and animal infections. *J Clin Microbiol*, 48:3708-3718.

Odell ID, Cloud JL, Seipp M, Wittwer CT. 2005. Rapid species identification within the *Mycobacterium chelonae-abscessus* group by high-resolution melting analysis of hsp65 PCR products. *Am J Clin Pathol*, 123:96-101.

Oliver JD, Sibley GEM, Beckmann N, Dobb KS, Slater MJ, McEntee L, *et al.* 2016. F901318 represents a novel class of antifungal drug that inhibits dihydroorotate dehydrogenase. *Proc Natl Acad Sci USA*, 113:12809-12814.

Orasch C, Marchetti O, Garbino J, Schrenzel J, Zimmerli S, Muhlethaler K, *et al.* 2014. *Candida* species distribution and antifungal susceptibility testing according to European Committee on Antimicrobial Susceptibility Testing and new vs. old Clinical and Laboratory Standards Institute clinical breakpoints: a 6-year prospective candidaemia survey from the fungal infection network of Switzerland. *Clin Microbiol Infect*, 20:698-705.

Organización Mundial de la Salud. 2015. Global action plan on antimicrobial resistance. https://apps.who.int/iris/bitstream/handle/10665/193736/9789241509763_eng.pdf.

Ortoneda M, Capilla J, Pastor FJ, Pujol I, Guarro J. 2002. Efficacy of liposomal amphotericin B in treatment of systemic murine fusariosis. *Antimicrob Agents Chemother*, 46:2273-2275.

Oura M, Sternberg TH, Wright ET. 1955. A new antifungal antibiotic, amphotericin B. *Antibiot Annu*, 3:566-573.

Ozhak-Baysan B, Alastruey-Izquierdo A, Saba R, Ogunc D, Ongut G, Timuragaoglu A, *et al.* 2010. *Aspergillus alliaceus* and *Aspergillus flavus* co-infection in an acute myeloid leukemia patient. *Med Mycol*, 48:995-999.

Pagano L, Caira M, Candoni A, Offidani M, Fianchi L, Martino B, *et al.* 2006. The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. *Haematologica*, 91:1068-1075.

Pagano L, Caira M, Nosari A, Van Lint MT, Candoni A, Offidani M, *et al.* 2007. Fungal infections in recipients of hematopoietic stem cell transplants: results of the SEIFEM B-2004 study--Sorveglianza Epidemiologica Infezioni Fungine Nelle Emopatie Maligne. *Clin Infect Dis*, 45:1161-1170.

Pagano L, Akova M, Dimopoulos G, Herbrecht R, Drgona L, Blijlevens N. 2011. Risk assessment and prognostic factors for mould-related diseases in immunocompromised patients. *J Antimicrob Chemother*, 66:i5-14.

Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, *et al.* 2016. Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis*, 62:e1-50.

Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. 2018. Invasive candidiasis. *Nat Rev Dis Primers*, 4:18026.

Park S, Kelly R, Kahn JN, Robles J, Hsu MJ, Register E, *et al.* 2005. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical *Candida* sp. isolates. *Antimicrob Agents Chemother*, 49:3264-3273.

Pastor FJ, Guarro J. 2008. *Alternaria* infections: laboratory diagnosis and relevant clinical features. *Clin Microbiol Infect*, 14:734-746.

Patel GP, Simon D, Scheetz M, Crank CW, Lodise T, Patel N. 2009. The effect of time to antifungal therapy on mortality in candidemia associated septic shock. *Am J Ther*, 16:508-511.

Patterson TF. 2005. Advances and challenges in management of invasive mycoses. *Lancet*, 366:1013-1025.

Patterson TF. 2009. Risk stratification for invasive aspergillosis: early assessment of host susceptibility. *Med Mycol*, 47:S255-260.

Patterson TF. 2011. Aspergillosis. In Kauffman CA *et al*. Essentials of Clinical Microbiology. Springer, p-243-263.

Paul RA, Rudramurthy SM, Meis JF, Mouton JW, Chakrabarti A. 2015. A novel Y319H substitution in Cyp51C associated with azole resistance in *Aspergillus flavus*. *Antimicrob Agents Chemother*, 59:6615-6619.

Paul S, Singh P, A SS, Rudramurthy SM, Chakrabarti A, Ghosh AK. 2018. Rapid detection of fluconazole resistance in *Candida tropicalis* by MALDI-TOF MS. *Med Mycol*, 56:234-241.

Pegorie M, Denning DW, Welfare W. 2017. Estimating the burden of invasive and serious fungal disease in the United Kingdom. *J Infect*, 74:60-71.

Pelaez T, Gijon P, Bunsow E, Bouza E, Sanchez-Yebra W, Valerio M, *et al.* 2012. Resistance to voriconazole due to a G448S substitution in *Aspergillus fumigatus* in a patient with cerebral aspergillosis. *J Clin Microbiol*, 50:2531-2534.

Pelaez T, Monteiro MC, Garcia-Rubio R, Bouza E, Gomez-Lopez A, Mellado E. 2015. First detection of *Aspergillus fumigatus* azole-resistant strain due to Cyp51A TR46/Y121F/T289A in an azole-naive patient in Spain. *New Microbes New Infect*, 6:33-34.

Peman J, Canton E, Quindos G, Eraso E, Alcoba J, Guinea J, *et al.* 2012. Epidemiology, species distribution and *in vitro* antifungal susceptibility of fungaemia in a Spanish multicentre prospective survey. *J Antimicrob Chemother*, 67:1181-1187.

Peman J, Salavert M. 2014. Invasive fungal disease due to *Scedosporium*, *Fusarium* and Mucorales. *Rev Iberoam Micol*, 31:242-248.

Peman J, Quindos G. 2016. Current aspects of invasive diseases caused by *Candida* and other yeast fungi. *Rev Iberoam Micol*, 33:133-139.

Perez-Cantero A, Lopez-Fernandez L, Guarro J, Capilla J. 2019. New insights into the Cyp51 contribution to azole resistance in *Aspergillus* section Nigri. *Antimicrob Agents Chemother*, 63: e00543-19.

Perlin DS. 2007. Resistance to echinocandin-class antifungal drugs. *Drug Resist Updat*, 10:121-130.

Perlin DS. 2011. Current perspectives on echinocandin class drugs. *Future Microbiol*, 6:441-457.

Perlin DS. 2015. Mechanisms of echinocandin antifungal drug resistance. *Ann N Y Acad Sci*, 1354:1-11.

Pfaller MA, Diekema DJ. 2004. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *J Clin Microbiol*, 42:4419-4431.

Pfaller MA, Boyken L, Hollis RJ, Messer SA, Tendolkar S, Diekema DJ. 2005. *In vitro* activities of anidulafungin against more than 2,500 clinical isolates of *Candida* spp., including 315 isolates resistant to fluconazole. *J Clin Microbiol*, 43:5425-5427.

Pfaller MA, Messer SA, Moet GJ, Jones RN, Castanheira M. 2011a. *Candida* bloodstream infections: comparison of species distribution and resistance to echinocandin and azole antifungal agents in Intensive Care Unit (ICU) and non-ICU settings in the SENTRY Antimicrobial Surveillance Program (2008-2009). *Int J Antimicrob Agents*, 38:65-69.

Pfaller MA, Watanabe N, Castanheira M, Messer SA, Jones RN. 2011b. Pre-clinical development of antifungal susceptibility test methods for the testing of the novel antifungal agent E1210 versus *Candida*: comparison of CLSI and European Committee on Antimicrobial Susceptibility Testing methods. *J Antimicrob Chemother*, 66:2581-2584.

Pfaller MA, Hata K, Jones RN, Messer SA, Moet GJ, Castanheira M. 2011c. *In vitro* activity of a novel broad-spectrum antifungal, E1210, tested against *Candida* spp. as determined by CLSI broth microdilution method. *Diagn Microbiol Infect Dis*, 71:167-170.

Pfaller MA, Duncanson F, Messer SA, Moet GJ, Jones RN, Castanheira M. 2011d. *In vitro* activity of a novel broad-spectrum antifungal, E1210, tested against *Aspergillus* spp. determined by CLSI and EUCAST broth microdilution methods. *Antimicrob Agents Chemother*, 55:5155-5158.

Pfaller MA. 2012. Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. *Am J Med*, 125:S3-13.

Pfaller MA, Messer SA, Woosley LN, Jones RN, Castanheira M. 2013. Echinocandin and triazole antifungal susceptibility profiles for clinical opportunistic yeast and mold isolates collected from 2010 to 2011: application of new CLSI clinical breakpoints and epidemiological cutoff values for characterization of geographic and temporal trends of antifungal resistance. *J Clin Microbiol*, 51:2571-2581.

Pfaller MA, Huband MD, Flamm RK, Bien PA, Castanheira M. 2019. *In vitro* activity of APX001A (manogepix) and comparator agents against 1,706 Fungal isolates collected during an international surveillance program (2017). *Antimicrob Agents Chemother*, 63:e00840-19.

Pham CD, Iqbal N, Bolden CB, Kuykendall RJ, Harrison LH, Farley MM, *et al.* 2014a. Role of *FKS* mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance. *Antimicrob Agents Chemother*, 58:4690-4696.

Pham CD, Bolden CB, Kuykendall RJ, Lockhart SR. 2014b. Development of a Luminex-based multiplex assay for detection of mutations conferring resistance to echinocandins in *Candida glabrata*. *J Clin Microbiol*, 52:790-795.

Poikonen E, Lyytikainen O, Anttila VJ, Koivula I, Lumio J, Kotilainen P, *et al.* 2010. Secular trend in candidemia and the use of fluconazole in Finland, 2004-2007. *BMC Infect Dis*, 10:312.

Posch W, Heimdorfer D, Wilflingseder D, Lass-Florl C. 2017. Invasive candidiasis: future directions in non-culture based diagnosis. *Expert Rev Anti Infect Ther*, 15:829-838.

Presterl E, Daxbock F, Graninger W, Willinger B. 2007. Changing pattern of candidaemia 2001-2006 and use of antifungal therapy at the University Hospital of Vienna, Austria. *Clin Microbiol Infect*, 13:1072-1076.

Prigent G, Ait-Ammar N, Levesque E, Fekkar A, Costa JM, El Anbassi S, *et al.* 2017. Echinocandin resistance in *Candida* species isolates from liver transplant recipients. *Antimicrob Agents Chemother*, 61: e01229-16.

Prigitano A, Venier V, Cogliati M, De Lorenzis G, Esposto MC, Tortorano AM. 2014. Azole-resistant *Aspergillus fumigatus* in the environment of northern Italy, May 2011 to June 2012. *Euro Surveill*, 19:20747.

Puig-Asensio M, Padilla B, Garnacho-Montero J, Zaragoza O, Aguado JM, Zaragoza R, *et al.* 2014. Epidemiology and predictive factors for early and late mortality in *Candida* bloodstream infections: a population-based surveillance in Spain. *Clin Microbiol Infect*, 20:O245-54.

Quindos G. 2014. Epidemiology of candidaemia and invasive candidiasis. A changing face. *Rev Iberoam Micol*, 31:42-48.

Ramirez-Garcia A, Pellon A, Rementeria A, Buldain I, Barreto-Bergter E, Rollin-Pinheiro R, *et al.* 2018. *Scedosporium* and *Lomentospora*: an updated overview of underrated opportunists. *Med Mycol*, 56:102-125.

Ramos-Martinez A, Vicente-Lopez N, Sanchez-Romero I, Padilla B, Merino-Amador P, Garnacho-Montero J, *et al.* 2017. Epidemiology and prognosis of candidaemia in elderly patients. *Mycoses*, 60:808-817.

Reichert-Lima F, Lyra L, Pontes L, Moretti ML, Pham CD, Lockhart SR, *et al.* 2018. Surveillance for azoles resistance in *Aspergillus* spp. highlights a high number of amphotericin B-resistant isolates. *Mycoses*, 61:360-365.

Retallack DM, Woods JP. 1999. Molecular epidemiology, pathogenesis, and genetics of the dimorphic fungus *Histoplasma capsulatum*. *Microbes Infect*, 1:817-825.

Rex JH. 2008a. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard-second edition. Wayne PA. Clinical Laboratory Standards Institute, CLSI Document M38-A2.

Rex JH. 2008b. Reference method for broth dilution antifungal susceptibility testing of yeast; approved standard-third edition. Wayne PA. Clinical Laboratory Standards Institute, CLSI Document M27-A3.

Risslegger B, Zoran T, Lackner M, Aigner M, Sanchez-Reus F, Rezusta A, *et al.* 2017. A prospective international *Aspergillus terreus* survey: an EFISG, ISHAM and ECMM joint study. *Clin Microbiol Infect*, 23:776.e1-776.e5.

Rivero-Menendez O, Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M. 2016. Triazole resistance in *Aspergillus* spp.: a worldwide problem? *J Fungi*, 2: 21.

Rivero-Menendez O, Cueca-Estrella M, Alastruey-Izquierdo A. 2019a. *In vitro* activity of APX001A against rare moulds using EUCAST and CLSI methodologies. *J Antimicrob Chemother*, 74: 1295-1299.

Rivero-Menendez O, Cuenca-Estrella M, Alastruey-Izquierdo A. 2019b. *In vitro a*ctivity of olorofim (F901318) against clinical isolates of cryptic species of *Aspergillus* by EUCAST and CLSI methodologies. *J Antimicrob Chemother*, 74(6): 1586-1590.

Robinson RF, Nahata MC. 1999. A comparative review of conventional and lipid formulations of amphotericin B. *J Clin Pharm Ther*, 24:249-257.

Roden MM, Zaoutis TE, Buchanan WL, Knudsen TA, Sarkisova TA, Schaufele RL, *et al.* 2005. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. *Clin Infect Dis*, 41:634-653.

Rodriguez-Tudela JL, Alastruey-Izquierdo A, Gago S, Cuenca-Estrella M, Leon C, Miro JM, *et al.* 2015. Burden of serious fungal infections in Spain. *Clin Microbiol Infect*, 21:183-189.

Rogers PD, Vermitsky JP, Edlind TD, Hilliard GM. 2006. Proteomic analysis of experimentally induced azole resistance in *Candida glabrata*. *J Antimicrob Chemother*, 58:434-438.

Saegeman V, Maertens J, Ectors N, Meersseman W, Lagrou K. 2010. Epidemiology of mucormycosis: review of 18 cases in a tertiary care hospital. *Med Mycol*, 48:245-254.

Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH, *et al.* 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud Mycol*, 78:141-173.

Sanguinetti M, Posteraro B, Fiori B, Ranno S, Torelli R, Fadda G. 2005. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. *Antimicrob Agents Chemother*, 49:668-679.

Saracli MA, Fothergill AW, Sutton DA, Wiederhold NP. 2015. Detection of triazole resistance among *Candida* species by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). *Med Mycol*, 53:736-742.

Sasso M, Roger C, Lachaud L. 2017. Rapid emergence of *FKS* mutations in *Candida glabrata* isolates in a peritoneal candidiasis. *Med Mycol Case Rep*, 16:28-30.

Scorzoni L, de Paula ESAC, Marcos CM, Assato PA, de Melo WC, de Oliveira HC, *et al.* 2017. Antifungal therapy: new advances in the understanding and treatment of mycosis. *Front Microbiol*, 8:36.

Selmecki A, Forche A, Berman J. 2006. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science*, 313:367-370.

Seroy J, Antiporta P, Grim SA, Proia LA, Singh K, Clark NM. 2017. *Aspergillus calidoustus* case series and review of the literature. *Transpl Infect Dis*, 19:e12755.

Seyedmousavi S, Verweij PE, Mouton JW. 2015. Isavuconazole, a broad-spectrum triazole for the treatment of systemic fungal diseases. *Expert Rev Anti Infect Ther*, 13:9-27.

Seyedmousavi S, Chang YC, Law D, Birch M, Rex JH, Kwon-Chung KJ. 2019. Efficacy of olorofim (F901318) against *Aspergillus fumigatus*, *A. nidulans*, and *A. tanneri* in murine models of profound neutropenia and chronic granulomatous disease. *Antimicrob Agents Chemother*, 63: e00129-19.

Sharma C, Kumar N, Pandey R, Meis JF, Chowdhary A. 2016. Whole genome sequencing of emerging multidrug resistant *Candida auris* isolates in India demonstrates low genetic variation. *New Microbes New Infect*, 13:77-82.

Sharma C, Kumar R, Kumar N, Masih A, Gupta D, Chowdhary A. 2018. Investigation of multiple resistance mechanisms in voriconazole-resistant *Aspergillus flavus* clinical isolates from a chest hospital surveillance in Delhi, India. *Antimicrob Agents Chemother*, 62: e01928-17.

Shaw KJ, Schell WA, Covel J, Duboc G, Giamberardino C, Kapoor M, *et al.* 2018. *In vitro* and *in vivo* evaluation of APX001A/APX001 and other Gwt1 inhibitors against *Cryptococcus*. *Antimicrob Agents Chemother*, 62(8): e00523-18.

Sheehan DJ, Hitchcock CA, Sibley CM. 1999. Current and emerging azole antifungal agents. *Clin Microbiol Rev*, 12:40-79.

Shields RK, Nguyen MH, Press EG, Kwa AL, Cheng S, Du C, *et al.* 2012. The presence of an *FKS* mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to *Candida glabrata*. *Antimicrob Agents Chemother*, 56:4862-4869.

Shields RK, Nguyen MH, Press EG, Clancy CJ. 2014. Abdominal candidiasis is a hidden reservoir of echinocandin resistance. *Antimicrob Agents Chemother*, 58:7601-7605.

Shields RK, Kline EG, Healey KR, Kordalewska M, Perlin DS, Nguyen MH, *et al.* 2019. Spontaneous mutational frequency and *FKS* mutation rates vary by echinocandin agent against *Candida glabrata*. *Antimicrob Agents Chemother*, 63(1): e01692-18.

Silver PM, Oliver BG, White TC. 2004. Role of *Candida albicans* transcription factor Upc2p in drug resistance and sterol metabolism. *Eukaryot Cell*, 3:1391-1397.

Singh A, Healey KR, Yadav P, Upadhyaya G, Sachdeva N, Sarma S, *et al.* 2018. Absence of azole or echinocandin resistance in *Candida glabrata* isolates in India despite background prevalence of strains with defects in the DNA mismatch repair pathway. *Antimicrob Agents Chemother*, 62(6): e00195-18.

Sinko J, Sulyok M, Denning DW. 2015. Burden of serious fungal diseases in Hungary. *Mycoses*, 58:29-33.

Siqueira JP, Sutton DA, Gene J, Garcia D, Wiederhold N, Peterson SW, *et al.* 2017. Multilocus phylogeny and antifungal susceptibility of *Aspergillus* section Circumdati from clinical samples and description of *A. pseudosclerotiorum* sp. nov. *J Clin Microbiol,* 55:947-958.

Skiada A, Lass-Floerl C, Klimko N, Ibrahim A, Roilides E, Petrikkos G. 2018. Challenges in the diagnosis and treatment of mucormycosis. *Med Mycol*, 56:93-101.

Snelders E, Camps SM, Karawajczyk A, Schaftenaar G, Kema GH, van der Lee HA, *et al.* 2012. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS One*, 7:e31801.

Song C, Castellanos-Rizaldos E, Bejar R, Ebert BL, Makrigiorgos GM. 2015. DMSO increases mutation scanning detection sensitivity of High-Resolution Melting in clinical samples. *Clin Chem*, 61:1354-1362.

Squires K, Clancy C, Cheng S, Zhao Y, Perlin D, Nguyen MH. 2019. Rezafungin (RZA) is more effective than micafungin in treating of FKS-mutant Candida glabrata intra-abdominal candidiasis. Poster oral O0741 en *29th European Congress of Clinical Microbiology and Infectious Diseases* (ECCMID), Amsterdam, Holanda.

Stevens DA, Kan VL, Judson MA, Morrison VA, Dummer S, Denning DW, *et al.* 2000. Practice guidelines for diseases caused by *Aspergillus*. Infectious Diseases Society of America. *Clin Infect Dis*, 30:696-709.

Stover KR, Farley JM, Kyle PB, Cleary JD. 2014. Cardiac toxicity of some echinocandin antifungals. *Expert Opin Drug Saf*, 13:5-14.

Suwunnakorn S, Wakabayashi H, Kordalewska M, Perlin DS, Rustchenko E. 2018. *FKS2* and *FKS3* genes of opportunistic human pathogen *Candida albicans* influence echinocandin susceptibility. *Antimicrob Agents Chemother*, 62(4): e02299-17.

Tan J, Zhang J, Chen W, Sun Y, Wan Z, Li R, *et al.* 2015. The A395T mutation in *ERG11* gene confers fluconazole resistance in *Candida tropicalis* causing candidemia. *Mycopathologia*, 179:213-218.

Tavanti A, Davidson AD, Johnson EM, Maiden MC, Shaw DJ, Gow NA, *et al.* 2005. Multilocus sequence typing for differentiation of strains of *Candida tropicalis*. *J Clin Microbiol*, 43:5593-5600.

Tissot F, Agrawal S, Pagano L, Petrikkos G, Groll AH, Skiada A, *et al.* 2017. ECIL-6 guidelines for the treatment of invasive candidiasis, aspergillosis and mucormycosis in leukemia and hematopoietic stem cell transplant patients. *Haematologica*, 102:433-444.

Torres-Narbona M, Guinea J, Martinez-Alarcon J, Munoz P, Gadea I, Bouza E, *et al.* 2007. Impact of zygomycosis on microbiology workload: a survey study in Spain. *J Clin Microbiol*, 45:2051-2053.

Tsukahara K, Hata K, Nakamoto K, Sagane K, Watanabe NA, Kuromitsu J, *et al.* 2003. Medicinal genetics approach towards identifying the molecular target of a novel inhibitor of fungal cell wall assembly. *Mol Microbiol*, 48:1029-1042.

Tuohy MJ, Reja V, Park S, Perlin DS, Wnek M, Procop GW, *et al.* 2010. Use of a high-resolution melt assay to characterize codon 54 of the *cyp51A* gene of *Aspergillus fumigatus* on a Rotor-Gene 6000 instrument. *Antimicrob Agents Chemother*, 54:2248-2251.

Vale-Silva LA, Coste AT, Ischer F, Parker JE, Kelly SL, Pinto E, *et al.* 2012. Azole resistance by loss of function of the sterol Delta(5),(6)-desaturase gene (*ERG3*) in *Candida albicans* does not necessarily decrease virulence. *Antimicrob Agents Chemother*, 56:1960-1968.

Vallabhaneni S, Cleveland AA, Farley MM, Harrison LH, Schaffner W, Beldavs ZG, *et al.* 2015. Epidemiology and risk factors for echinocandin nonsusceptible *Candida glabrata* bloodstream infections: data from a large multisite population-based candidemia surveillance program, 2008-2014. *Open Forum Infect Dis*, 2:ofv163.

van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, *et al.* 2011. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007-2009. *Emerg Infect Dis*, 17:1846-1854.

van der Linden JW, Camps SM, Kampinga GA, Arends JP, Debets-Ossenkopp YJ, Haas PJ, *et al.* 2013. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clin Infect Dis*, 57:513-520.

van der Linden JW, Arendrup MC, Warris A, Lagrou K, Pelloux H, Hauser PM, *et al.* 2015. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. *Emerg Infect Dis*, 21:1041-1044.

van Ingen J, van der Lee HA, Rijs AJ, Snelders E, Melchers WJ, Verweij PE. 2015. High-level pan-azole-resistant aspergillosis. *J Clin Microbiol*, 53:2343-2345.

Vanden Bossche H, Koymans L, Moereels H. 1995. P450 inhibitors of use in medical treatment: focus on mechanisms of action. *Pharmacol Ther*, 67:79-100.

Vandeputte P, Larcher G, Berges T, Renier G, Chabasse D, Bouchara JP. 2005. Mechanisms of azole resistance in a clinical isolate of *Candida tropicalis*. *Antimicrob Agents Chemother*, 49:4608-4615.

Varga J, Houbraken J, Van Der Lee HA, Verweij PE, Samson RA. 2008. *Aspergillus calidoustus* sp. nov., causative agent of human infections previously assigned to *Aspergillus ustus*. *Eukaryot Cell*, 7:630-638.

Vella A, De Carolis E, Vaccaro L, Posteraro P, Perlin DS, Kostrzewa M, *et al.* 2013. Rapid antifungal susceptibility testing by matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis. *J Clin Microbiol*, 51:2964-2969.

Vella A, De Carolis E, Mello E, Perlin DS, Sanglard D, Sanguinetti M, *et al.* 2017. Potential use of MALDI-ToF mass spectrometry for rapid detection of antifungal resistance in the human pathogen *Candida glabrata*. *Sci Rep*, 7:9099.

Vermitsky JP, Earhart KD, Smith WL, Homayouni R, Edlind TD, Rogers PD. 2006. Pdr1 regulates multidrug resistance in *Candida glabrata*: gene disruption and genome-wide expression studies. *Mol Microbiol*, 61:704-722.

Verweij PE, Mellado E, Melchers WJ. 2007. Multiple-triazole-resistant aspergillosis. *N Engl J Med*, 356:1481-1483.

Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ. 2009. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? *Lancet Infect Dis*, 9:789-795.

Verweij PE, Ananda-Rajah M, Andes D, Arendrup MC, Bruggemann RJ, Chowdhary A, *et al.* 2015. International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. *Drug Resist Updat*, 21-22:30-40.

Vinh DC, Shea YR, Sugui JA, Parrilla-Castellar ER, Freeman AF, Campbell JW, *et al.* 2009. Invasive aspergillosis due to *Neosartorya udagawae*. *Clin Infect Dis,* 49:102-111.

Viriyakosol S, Kapoor M, Okamoto S, Covel J, Soltow QA, Trzoss M, *et al.* 2019. APX001 and other Gwt1 inhibitor prodrugs are effective in experimental *Coccidioides immitis* pneumonia. *Antimicrob Agents Chemother*, 63:e01715-18.

Watanabe NA, Miyazaki M, Horii T, Sagane K, Tsukahara K, Hata K. 2012. E1210, a new broad-spectrum antifungal, suppresses *Candida albicans* hyphal growth through inhibition of glycosylphosphatidylinositol biosynthesis. *Antimicrob Agents Chemother*, 56:960-971.

Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD. 2016. Azole antifungal resistance in *Candida albicans* and emerging non-albicans *Candida* species. *Front Microbiol*, 7:2173.

White TC, Marr KA, Bowden RA. 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin Microbiol Rev*, 11:382-402.

Wiederhold NP, Lewis RE. 2003. The echinocandin antifungals: an overview of the pharmacology, spectrum and clinical efficacy. *Expert Opin Investig Drugs,* 12:1313-1333.

Wiederhold NP, Najvar LK, Fothergill AW, McCarthy DI, Bocanegra R, Olivo M, *et al.* 2015. The investigational agent E1210 is effective in treatment of experimental invasive candidiasis caused by resistant *Candida albicans*. *Antimicrob Agents Chemother*, 59:690-692.

Wiederhold NP, Gil VG, Gutierrez F, Lindner JR, Albataineh MT, McCarthy DI, *et al.* 2016. First detection of TR34/L98H and TR46/Y121F/T289A Cyp51 mutations in *Aspergillus fumigatus* isolates in the United States. *J Clin Microbiol*, 54:168-171.

Wiederhold NP, Law D, Birch M. 2017a. Dihydroorotate dehydrogenase inhibitor F901318 has potent *in vitro* activity against *Scedosporium* species and *Lomentospora prolificans*. *J Antimicrob Chemother*, 72:1977-1980.

Wiederhold NP, Najvar LK, Jaramillo R, Olivo M, Catano G, Sullivan SM, *et al*. 2017b. The novel VL-2397 demonstrates efficacy in an *in vivo* model of invasive candidiasis caused by wild-type and multi-drug resistant *Candida glabrata*. Poster en *Microbe 2017 - American Society for Microbiology*, Nueva Orleans, Estados Unidos.

Wiederhold NP, Najvar LK, Jaramillo R, Olivo M, Birch M, Law D, *et al.* 2018a. The orotomide olorofim is efficacious in an experimental model of central nervous system coccidioidomycosis. *Antimicrob Agents Chemother*, 62(9): e00999-18.

Wiederhold NP. 2018b. The antifungal arsenal: alternative drugs and future targets. *Int J Antimicrob Agents*, 51:333-339.

Willger SD, Puttikamonkul S, Kim KH, Burritt JB, Grahl N, Metzler LJ, *et al.* 2008. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. *PLoS Pathog*, 4:e1000200.

Xu H, Chen W, Li L, Wan Z, Li R, Liu W. 2010. Clinical itraconazole-resistant strains of *Aspergillus fumigatus*, isolated serially from a lung aspergilloma patient with pulmonary tuberculosis, can be detected with real-time PCR method. *Mycopathologia*, 169:193-199.

Ying C, Zhang H, Tang Z, Chen H, Gao J, Yue C. 2016. Antifungal susceptibility and molecular typing of 115 *Candida albicans* isolates obtained from vulvovaginal candidiasis patients in 3 Shanghai maternity hospitals. *Med Mycol*, 54:394-399.

Yoshida H, Seki M, Umeyama T, Urai M, Kinjo Y, Nishi I, *et al.* 2015. Invasive pulmonary aspergillosis due to Aspergillus lentulus: Successful treatment of a liver transplant patient. *J Infect Chemother*, 21:479-481.

Zaki SM, Denning DW. 2017. Serious fungal infections in Egypt. *Eur J Clin Microbiol Infect Dis*, 36:971-974.

Zbinden A, Imhof A, Wilhelm MJ, Ruschitzka F, Wild P, Bloemberg GV, *et al.* 2012. Fatal outcome after heart transplantation caused by *Aspergillus lentulus*. *Transpl Infect Dis*, 14:E60-3.

Zhang J, Snelders E, Zwaan BJ, Schoustra SE, Meis JF, van Dijk K, *et al.* 2017. A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. *MBio*, 8:e00791-17.

Zhao X, Drlica K. 2001. Restricting the selection of antibiotic-resistant mutants: a general strategy derived from fluoroquinolone studies. *Clin Infect Dis*, 33:S147-56.

Zhao Y, Stensvold CR, Perlin DS, Arendrup MC. 2013. Azole resistance in *Aspergillus fumigatus* from bronchoalveolar lavage fluid samples of patients with chronic diseases. *J Antimicrob Chemother*, 68:1497-1504.

Zhao Y, Garnaud C, Brenier-Pinchart MP, Thiebaut-Bertrand A, Saint-Raymond C, Camara B, *et al.* 2016. Direct molecular diagnosis of aspergillosis and Cyp51A profiling from respiratory samples of French patients. *Front Microbiol*, 7:1164.

Zhao M, Lepak AJ, VanScoy B, Bader JC, Marchillo K, Vanhecker J, *et al.* 2018a. *In vivo* pharmacokinetics and pharmacodynamics of APX001 against *Candida* spp. in a neutropenic disseminated candidiasis mouse model. *Antimicrob Agents Chemother*, 62: e02542-17.

Zhao Y, Lee MH, Paderu P, Lee A, Jimenez-Ortigosa C, Park S, *et al.* 2018b. Significantly improved pharmacokinetics enhances *in vivo* efficacy of APX001 against echinocandin- and multidrug-resistant *Candida* isolates in a mouse model of invasive candidiasis. *Antimicrob Agents Chemother*, 62: e00425-18.

Zhao M, Lepak AJ, Marchillo K, Vanhecker J, Sanchez H, Ambrose PG, *et al.* 2019. APX001 pharmacokinetic/pharmacodynamic target determination against *Aspergillus fumigatus* in an *in vivo* model of invasive pulmonary aspergillosis. *Antimicrob Agents Chemother*, 63: e02372-18.

Zimbeck AJ, Iqbal N, Ahlquist AM, Farley MM, Harrison LH, Chiller T, *et al.* 2010. *FKS* mutations and elevated echinocandin MIC values among *Candida glabrata* isolates from U.S. population-based surveillance. *Antimicrob Agents Chemother*, 54:5042-5047.

Zoran T, Sartori B, Sappl L, Aigner M, Sanchez-Reus F, Rezusta A, *et al.* 2018. Azole-resistance in *Aspergillus terreus* and related species: an emerging problem or a rare phenomenon? *Front Microbiol*, 9:516.

7. Anexos

7. ANEXOS

Otras publicaciones

Delliere S, **Rivero-Menendez O**, Gautier C, Garcia-Hermoso D, Alastruey-Izquierdo A, Alanio A. 2019. Emerging mould infections: Get prepared to meet unexpected fungi in your patient. *Med Mycol*. doi: 10.1093/mmy/myz039.

Garcia-Hermoso D, Valenzuela-Lopez N, **Rivero-Menendez O**, Alastruey-Izquierdo A, Guarro J, Cano-Lira JF, Stchigel AM; French Mycoses Study Group. 2019. Diversity of coelomycetous fungi in human infections: A 10-y experience of two European reference centres. *Fungal Biol*, 123(4):341-349. doi: 10.1016/j.funbio.2019.02.001.

Valenzuela-Lopez N, Cano-Lira JF, Stchigel AM, **Rivero-Menendez O**, Alastruey-Izquierdo A, Guarro J. 2018. *Neocucurbitaria keratinophila*: An emerging opportunistic fungus causing superficial mycosis in Spain. *Med Mycol*, 57(6):733-738. doi: 10.1093/mmy/myy132.

Alastruey-Izquierdo A, Alcazar-Fuoli L, **Rivero-Menendez O**, Ayats J, Castro C, García-Rodríguez J, Goterris-Bonet L, Ibáñez-Martínez E, Linares-Sicilia MJ, Martin-Gomez MT, Martín-Mazuelos E, Pelaez T, Peman J, Rezusta A, Rojo S, Tejero R, Anza DV, Viñuelas J, Zapico MS, Cuenca-Estrella M; the FILPOP2 Project from GEMICOMED (SEIMC) and REIPI. 2018. Molecular identification and susceptibility testing of molds Isolated in a prospective surveillance of triazole resistance in Spain (FILPOP2 Study). *Antimicrob Agents Chemother*, 62(9):e00358-18. doi: 10.1128/AAC.00358-18.

Rivero-Menendez O, Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M. 2016. Triazole resistance in *Aspergillus* spp.: a worldwide problem? *J Fungi*, 2(3):21. doi: 10.3390/jof2030021.

Forastiero A, Garcia-Gil V, **Rivero-Menendez O**, Garcia-Rubio R, Monteiro MC, Alastruey-Izquierdo A, Jordan R, Agorio I, Mellado E. 2015. Rapid development of *Candida krusei* echinocandin resistance during caspofungin therapy. *Antimicrob Agents Chemother*, 59(11):6975-6982. doi: 10.1128/AAC.01005-15.

ISHAM
INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2019, 0, 1–7
doi: 10.1093/mmy/myz039
Advance Access Publication Date: 0 2019
Review Article

Review Article

Emerging mould infections: Get prepared to meet unexpected fungi in your patient

Sarah Dellière[1], Olga Rivero-Menendez[2], Cécile Gautier[3], Dea Garcia-Hermoso[3], Ana Alastrey-Izquierdo[2] and Alexandre Alanio[1,3,4,*]

[1]Université de Paris, Laboratoire de Parasitologie-Mycologie, AP-HP, Groupe Hospitalier Saint-Louis-Lariboisière-Fernand-Widal, Paris, France, [2]Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain, [3]Institut Pasteur, Molecular Mycology Unit, CNRS UMR2000, National Reference Center for Invasive Mycoses and Antifungals (NRCMA), Paris, France and [4]Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA

*To whom correspondence should be addressed. Alexandre Alanio, MD, PhD, Molecular Mycology unit, Institut Pasteur, 25 rue du Dr Roux 75724 Paris Cedex 15. Tel: +33140613255; Fax: +33145688420; E-mail: alexandre.alanio@pasteur.fr

Received 18 December 2018; Revised 1 March 2019; Accepted 5 April 2019; Editorial Decision 29 March 2019

Abstract

Invasive fungal diseases are increasing issues in modern medicine, where the human immunodeficiency virus (HIV) pandemic and the wider use of immunosuppressive drugs generate an ever-growing number of immunocompromised patients with an increased susceptibility to uncommon fungal pathogens. In the past decade, new species have been reported as being responsible for disseminated and invasive fungal diseases in humans. Among them, the following genera are rare but seem emerging issues: *Scopulariopsis*, *Hormographiella*, *Emergomyces*, *Westerdykella*, *Trametes*, *Actinomucor*, *Saksenaea*, *Apophysomyces*, and *Rhytidhysteron*. Delay in diagnosis, which is often the case in these infections, jeopardizes patients' prognosis and leads to increased mortality. Here we summarize the clinical and biological presentation and the key features to identify these emerging pathogens and we discuss the available antifungal classes to treat them. We focused on Pubmed to recover extensively reported human invasive cases and articles regarding the nine previously cited fungal organisms. Information concerning patient background, macroscopic and microscopic description and pictures of these fungal organisms, histological features in tissues, findings with commonly used antigen tests in practice, and hints on potential efficient antifungal classes were gathered. This review's purpose is to help clinical microbiologists and physicians to suspect, identify, diagnose, and treat newly encountered fungi in hospital settings.

Key words: invasive fungal disease, emerging fungi, *Emergomyces*, *Scopulariopsis*, *Hormographiella*, *Actinomucor*, *Apophysomyces*, *Rhytidhysteron*, *Saksenaea*, *Trametes*, *Westerdykella*.

Introduction

Invasive fungal infections (IFIs) are increasing due to the growing use of immunosuppressive chemotherapy and the persistence of the AIDS pandemic in specific areas. In addition, the improvement of diagnostic means may concur to that trend. *Candida* spp. and *Cryptococcus* spp. are the most frequent isolated yeasts in human IFIs, while *Aspergillus* spp., *Fusarium* spp. and *Scedosporium* spp. are the most isolated moulds.[1] However, the part attributed to other fungal organisms is increasing and emerging uncommon species responsible for IFIs are more frequently described around the world. In this review, we describe unusual emerging moulds responsible for IFIs with a focus on the fungus—morphology, environmental niche, location, mode of transmission—and on the infection (clinical presentation and treatments available). These genera and species where selected

for their scarcity and their recent emergence during the past two decades.

Actinomucor spp.

The genus *Actinomucor* belongs to the order *Mucorales* and is commonly isolated from soil, from the rhizosphere of plants and from mouse and rabbit dung.[2] *Actinomucor elegans* is involved in traditional fermentation of sufu, a cheese-like soybean very common in Asia.[3] The only species of the genus *Actinomucor* is *Actinomucor elegans*. divided in var. *elegans*, var. *kuwaitiensis,* and var. *taiwanensis*, considered synonyms (http://www.indexfungorum.org). *A. elegans* has white to cream floccose cultures growing rapidly at 30°C. Microscopically they are verticillately branched sporangiophores with terminate sporangia in various lengths. The smooth or spiny sporangia wall encloses spherical to ovoidal sporangiospores.[4] Sequencing of the ITS1-5.8S-ITS2 region of rDNA is currently used to identify accurately species of this genus.

Only five cases of superficial and invasive mucormycosis involving *Actinomucor* have been described in the literature since 2001. Two cases were associated with maxillary sinusitis in an immunocompetent 11-year-old female[5] and in an allogeneic transplant patient with a lymphoblastic lymphoma;[6] a third case was a necrotic foot lesion in a diabetic patient.[7] Disseminated infection was diagnosed in two cases following a trauma during combat in Iran[8] and in a diabetic patient with refractory aplasic anemia.[9] Out of five patients, two were cured with surgery debridement and amphotericin B administration,[5,6] two died despite debridement and broad spectrum antifungal,[8,9] and one was lost to follow-up.[7]

Apophysomyces mexicanus

Among the subphylum *Mucoromycotina*, the *Apophysomyces* species complex[10] encompasses five species: *A. elegans*, *A. variabilis*, *A. trapeziformis*, *A. ossiformis,* and the most recently proposed *A. mexicanus*.[11] The members of this complex are mainly soil thermophilic microorganisms with tropical to subtropical distribution and have been implicated in cases of human mucormycosis mainly in immunocompetent individuals.[12,13] The most common clinical presentations are cutaneous and subcutaneous localizations following trauma, burn, invasive procedures or natural disasters.[14] In the reported case of *A. mexicanus*, patient presented with a necrotizing fasciitis rapidly spreading despite the administration of amphotericin B and fluconazole followed by an extensive surgical debridement. Diagnosis of was achieved by identifying coenocytic hyphae during the histopathological examination of the necrotic tissue and the growth of a whitish fungal colony characteristic of mucoralean fungi. The species of *Apophysomyces* produce fast-growing white to gray colonies and genus specific morphological features such as the prominent bell-shaped, vase-shaped or funnel-shaped apophyses. While smooth walled sporangiophores (including the apophyse) are a common feature of this genus, the verrucose texture of the wall in *A. mexicanus* constitutes its distinctive characteristic.[11] A molecular approach with the amplification of the ITS1-5.8S-ITS2 region of rDNA is required to assure definite species-level identification. The biggest case series of *Apophysomyces* sp. reports 13 cases after cutaneous injury during a tornado in Missouri in May 2011.[15] Five patients (38%) died, however; a focus on patients treated appropriately according to guidelines (i.e., amphotericin B in combination with surgical debridement)[16] shows a treatment efficiency of 85% with only two deaths.[15]

Saksenaea erythrospora

Among the subphylum *Mucoromycotina* the genus *Saksenaea* comprises three species: *S. vasiformis* species complex, *S. erythrospora* and *S. oblongispora*.[10] These cosmopolitan mucoralean organisms produce white-gray expanding colonies with a maximum temperature of 44°C. Sporulation may be promoted by subculturing the fungus on water agar 1% or Czapek medium at 30° to 37°C.[4] The multispored flask-shaped sporangium is characteristic of the genus. Species recognition is based mainly on differences in length of sporangiophores and sporangia; shape and size of sporangiophores and maximum growth temperatures. Accurate species identification also relies on sequencing of the ITS1-5.8S-ITS2 region and the D1-D2 domains of the large-subunit ribosomal DNA. Mucormycosis due to *Saksenaea* spp. have been mostly associated with cutaneous infections in healthy and immunocompetent subjects following traumatic implantation with soil and water contamination, inhalation of spores, insect stings, or the use of indwelling catheters.[17,18] Up to now, nine cases of the newly described species *S. erythrospora* have been reported. The majority of infections were observed in immunocompetent individuals (except for one diabetic patient)[19] and were associated with trauma (sailing accident, combat trauma, intramuscular injection or application of medicated adhesive tape),[19–21] medical tourism activities (esthetical surgery) or invasive rhinosinusitis.[22,23] Clinical presentations included necrotic or ulcerative skin and soft tissue lesions, necrotizing fasciitis, or orbital cellulitis. No specific management has been recommended in recent guidelines for *Saksenaea* sp. compared to other mucormycosis. As first line therapy, a combination of surgical debridement when feasible and the administration of a lipid formulation of amphotericin B, which offers a better chance of survival to the patient, have been recommended.[16,24]

Hormographiella aspergillata

H. aspergillata is the asexual form of *Coprinopsis cinerea*, a common basidiomycete found in compost.[25] This fungus has been

Table 1. Macroscopic and microscopic features of emerging fungal species in IFIs.

	Macroscopic aspect in culture		Microscopic aspect in culture		Microscopic aspect in tissue	
Genus	Description	Image	Description	Image	Description	Image
Actinomucor spp.	White to cream floccose colonies	Fig. 1A	Verticillately branched sporangiophores; smooth or spiny sporangia	Fig. 1B	Intravasculaire and parenchymal invasive, ribbon-like, branching fungal elements characteristics of the member of *Mucorales* species	[8,9]
Apophysomyces mexicanus	White to gray rapid growing colonies	ND	Verrucose-walled sporangiophores and apophyse	[11]	Wide coenocytic mycelia	[11]
Saksenaea erythrospora	White to gray expanding colonies	[19,21]	Multispored flask-shaped sporangium	Fig. 1F	Broad aseptate hyphae with wide angle branching	[19,21]
Hormographiella aspergillata	White to cream cotton-like colonies	[27,28]	Septate conidiophores/clusters of smooth-walled hyaline and cylindrical arthroconidia	[27,28]	Hyaline hyphae with acute branching Necrotic tissue	[27,28]
Trametes polyzona	White and cottony colonies	[31]	Hyaline septate hyphae and rectangular arthroconidia	[30]	Septate fungal hyphae Neutrophil tissue infiltration	[31]
Emergomyces africanus	Mycelial phase (25–30°C) Light brown wrinkled colony with powdery segment Yeast phase (37°C) Smooth cream-to-beige color colony	Fig. 1C	Mycelial phase: Septate hyaline hyphae with numerous smooth-walled oval conidia borne on thin pedicles. Yeast phase: Small thin-walled, globose-to-oval yeasts (2–4 μm in diameter)	Fig. 1D	Round to ovoid yeasts with single budding nuclei and thin walls simulating *Histoplasma capsulatum*	[54]
Rhytidhysteron rufulum	Dark green to grey velvety colonies	Fig. 1E	Septate irregularly branched dematiaceus hyphae Ascomata apothecioid, Ascospores transversely 3-septate, with no longitudinal septa	[39,41]	Thick-walled brown, spherical, sclerotic bodies with or without septation	[39,41]
Scopulariopsis (*Microascus*) spp.	Powdery pale brown colonies	Fig. 1G	Septate hyphae, annellidic conidiogenous cells in chains and smooth or rough-walled subglobose conidia with a flattened base.	Fig. 1H	Irregularly shaped hyphae with swollen thick-walled structure Angioinvasive Necrotic tissue formation May be melanized	[43,45]
Westerdykella dispersa	Dark-brown colonies with dark globose cleistothecium	Fig. 1I	Reddish-brown, smooth, cylindrical to slightly reniform ascospores Pycnidia with ostioles and subglobose to pyriform, hyaline conidia	Fig. 1J Fig. 1K	septate hyphal angioinvasive	[48]

ND, not described.

reported in 15 cases in the past two decades and majorly arises in neutropenic patients who underwent allogeneic stem cell transplantation as part of acute leukemia treatment.[26,27] Clinical presentation is an invasive pulmonary infection in all cases but one has been described to disseminate to the central nervous system (CNS), eyes, skin, sinuses, and blood (endocarditis).[28] Diagnosis is driven by the presence of nodular infiltrates and the halo sign on pulmonary computed tomography (CT). Galactomannan is constantly negative and bronchoalveolar lavage (BAL) is always negative Table(2).[29] Thoracoscopic surgery and lung biopsy allowed to have final diagnosis in all cases allowing the growth of *H. aspergillata* in all cases in which it was performed.[26,29] The morphological characteristics of the fungus are described in Table 1. This fungus responds poorly to all antifungal and mortality rate of all cases published is 73%.[25,26] *In vitro* susceptibility shows intrinsic resistance to echinocandins and variable minimal inhibitory concentration (MIC) to second generation azoles and amphotericin B (AmB), but these data cannot be translated into clinical practice without caution. The addition of nebulized liposomal-AmB (L-AmB) to IV L-AmB is the treatment combination that showed the best improvement of a patient condition so far.[26]

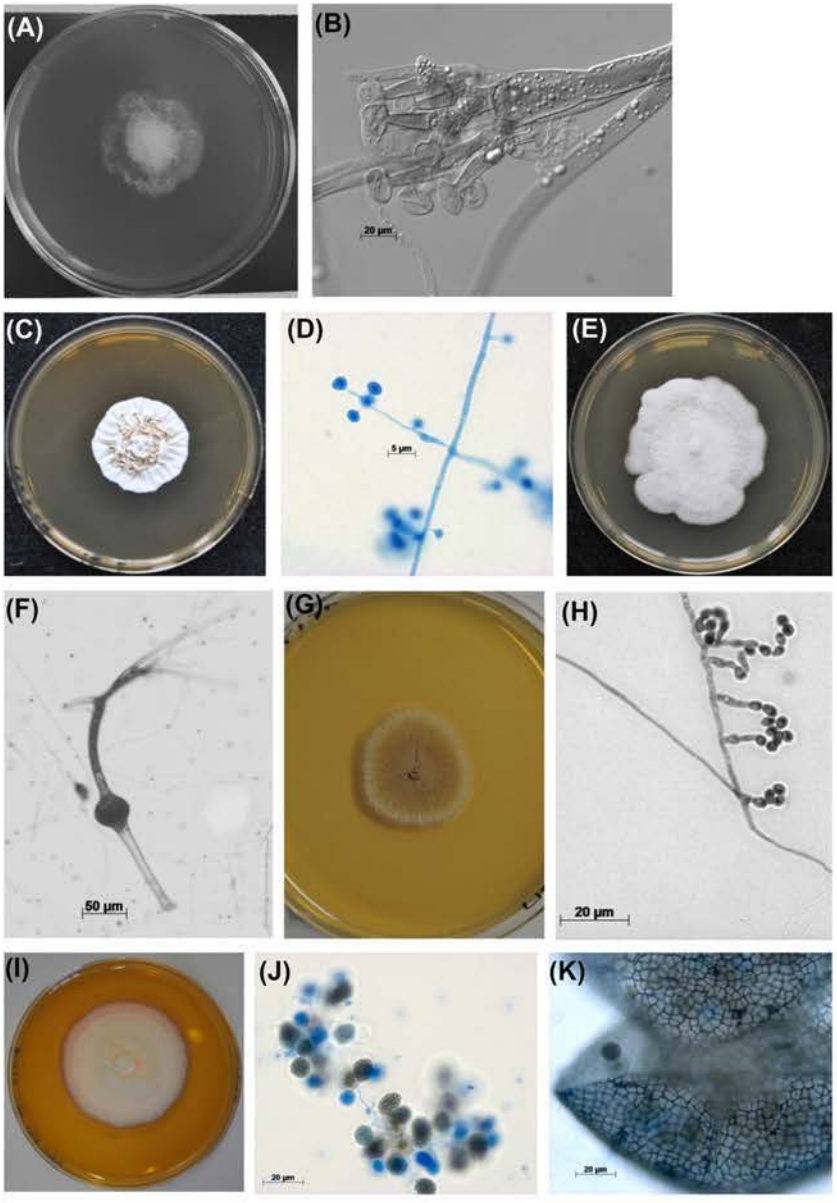


Figure 1. *Actinomucor elegans* (A) floccose colony on MEA 2% after 3 days of incubation at 30°C (B) microscopic aspect of sporangiophores terminating in columellae. *Emergomyces africanus* (C) restricted colony on MEA 2% after 10 days at 24°C (D) microscopy of slide culture on MEA 2% after 7 days at 24°C; solitary conidiophores and subspherical conidia. *Rhytidhysteron rufulum* (E) velvety colony on MEA 2% after 15 days at 21°C. *Saksenaea erythrospora* (F) sporangiophore, flask-shaped sporangia and rhizoïds after 15 days on Czapek agar at 30°C. *Scopulariopsis brevicaulis* (G) brown powdery colony on MEA 2% after 10 days at 30°C. *Microascus cirrosus* (H) annelated conidiophores and conidia after 7 days on MEA 2% at 30°C. *Westerdykella dispersa* (I) expanding colony on MEA 2% after 7 days at 30°C (J) reddish brown cylindrical ascospores (K) dark globose cleistothecium and reddish brown cylindrical ascospores. MEA2%: Malt extract agar 2%.

Trametes polyzona

Trametes polyzona is a basidomycete that belongs to the order *Polyporales*. It is a saprotrophic fungi that has been isolated in Africa, South and Central America, and Asia being particularly prevalent in tropical countries and playing a major role as a decomposer in the tropical forest ecosystem.[30] The first report in human infections was in 2016 from two cases of pneumonia[31] in Reunion island. Patients suffered from chronic debilitating diseases that lead to fungal colonization of the respiratory tract. They were initially treated with caspofungin or fluconazole and later with liposomal amphotericin B. Histopathological confirmation was obtained in one case. The isolates recovered from both cases did not sporulate in standard culture and were identified by sequencing informative targets. Antifungal susceptibility testing revealed low MICs (<0.5 mg/l) to amphotericin B and azoles but high MICs (>8 mg/l) for caspofungin and terbinafine.

Table 2. Results of routinely used biological fungal markers in invasive fungal disease of emergent fungi.

	ß-D-glucan Number of positive patient (*n*)	GM serum Number of positive patient (*n*)	GM BAL Number of positive patient (*n*)	Urine Histoplasma Ag Number of positive patient (*n*)	Reference
Actinomucor spp.	NA	1 (1)	NA	NA	6
Apophysomyces mexicanus	NA	NA	NA	NA	
Saksaenae erythrospora	NA	NA	NA	NA	
Hormographiella aspergillata	0 (1)	0 (7)	0 (4)	NA	26-29
Trametes polyzona	NA	NA	NA	NA	
Emergomyces africanus	NA	NA	NA	3 (10)	54
Rhytidhysteron rufulum	NA*	NA**	NA	NA	
Scopulariopsis (*Microascus*) spp.	0 (1)	0 (2)	0 (1)	NA	45,55
Westerdykella dispersa	NA	0 (1)	NA	NA	48

Ag, antigen; BAL, broncho-alveolar lavage; GM, galactomannan; NA, no assessment.
*1(1) in an unpublished case in 2018; **0 (1) in an unpublished case in 2018.

Emergomyces spp.

For more than 50 years, the genus *Emmonsia* was known to cause a rare disease called adiaspiromycosis.[32] While the incidence of adiaspiromycosis does not seem to increase, an outbreak of *Emmonsia*-like fungi has been causing severe disseminated infection in South African human immunodeficiency virus (HIV) patients in the past 10 years.[33] Considering that these fungi do not produce adiaspores in tissue, display thermal dimorphism and generate disseminated disease in immunocompromised patients, a new genus called *Emergomyces* has been proposed supported by molecular investigations.[34] *Emergomyces* accommodates former *Emmonsia pasteuriana*, and the new species *Emergomyces africanus* responsible for the South African outbreak[34] and *Emergomyces orientalis* only described in a case in China.[35] *Emergomyces pasteuriana*, ex-*Emmonsia pasteuriana*, has been reported in five cases since 1998,[36] while *E. africanus* has been responsible for at least 86 reported cases in South Africa since 2008.[35] Both present as a disseminated infections with skin lesions in AIDS patients with a CD4 cell count $<100/mm^3$. Clinical presentation of *E. africanus*, whose infection has been recently well described, associates fever, weight loss, anemia, and widespread nonspecific skin lesions from erythematous papules to ulcers and crusted plaques. Infection is fatal in about 30% of cases.[33,35] Dissemination is documented by lesions on chest X-ray resembling tuberculosis and elevated liver enzymes. Culture of skin biopsy isolates the fungus in all cases, whose characteristics are described in Table 1. Isolation of the fungus from blood or bone marrow is significantly associated with death.[35] Despite the lack of guidelines on the management of emergomycosis, the fungus seems to respond well and rapidly to amphotericin B deoxycholate for 14 days followed by itraconazole maintenance therapy.[33,35]

Rhytidhysteron rufulum

Rhytidhysteron rufulum is a melanized ascomycete genus that has being recognized for many years as a plan pathogen. Recent molecular studies transferred the genus from *Patellariaceae* to the *Hysteriaceae* family.[37] *Rhydhysteron rufulum* was first isolated from a human infection in 2009 in a case of chromoblastomycosis in India.[38] Seven more phaeophyphomycosis cases have been described in the literature since then.[38–42] Five were immunocompetent patients, one renal transplant and two diabetic patients. Diagnosis was always based on the isolation of the fungus and sequencing since the isolates do not sporulate in culture. All cases were found in the lower limbs. Patient's treatment included itraconazole alone or in combination with terbinafine and/or lesion excision. One case was also treated with amphotericin B in addition to lesion excision, itraconazole and terbinafine.[39] Four of the cases got cured, one (chromoblastomycosis) did not, and three patients were lost to follow-up.

Scopulariopsis (Microascus) spp.

The ascomyceteous genus *Scopulariopsis* includes hyaline and melanized species that are ubiquitous and found in soil, air, wood, dung, and animal remains.[43] Most common species involved in human pathogenicity are *Scopulariopsis brevicaulis*, *Scopulariopsis gracilis*, *Scopulariopsis brumptii* (recently

renamed as *Microascus gracilis* and *Microascus paisii* respectively), *Scopulariopsis candida*, *Microascus cirrosus* and *Microascus cinereus*.[43] They are occasionally involved in human infections ranging from superficial infections (onychomychosis, keratitis, otomycosis) to invasive infections with high mortality rates. The latter include endocarditis, sinusitis, brain abscess, pulmonary, deep cutaneous and disseminated cases with poor outcome, mostly seen in immunocompromised patients.[43] *Scopulariopsis* spp. grows well on routine laboratory media and are recovered and identified easily from clinical samples (Table 1). Fungal biological markers are also usually negative in disseminated infection (Table 2). Infections by *Scopulariopsis* spp. lack recommendation guidelines related to high *in vitro* MICs (minimum inhibitory concentrations) to all current antifungal agents.[44] Furthermore, many cases report discrepancies between MIC *in vitro* and clinical response suggesting that some antifungals, mainly amphotericin B and voriconazole, may be active in patients, as observed in fusariosis with voriconazole.[44,45] Surgery combined with antifungal is usually advised to give patients the highest chance of survival.[44,45]

Westerdykella dispersa

Westerdykella spp. are saprobic fungi with a worldwide distribution. It belongs to the family *Sporomiaceae* (order *Pleosporales*). It has been described as the causative agent of systemic infection in dogs.[46] Two cases of human infections have been reported until now.[47,48] Sue et al. reported the infection in a neutropenic patient with acute lymphoblastic leukemia.[48] Infectious complication around a peripherally inserted central catheter occurred. The patient was successfully treated with a combination of voriconazole and liposomal amphotericin B in addition to repeated debridements. The second case was isolated from a critically burned patient.[47] Three other isolates were recovered from lower extremity wounds or tissue in the United States identified as *Westerdykella*, one of them as *W. dispersa* and the other two as *W. reniformis*.[48] However, their virulence in these cases has not been acknowledged.

Discussion

New mould species are increasingly reported as responsible for IFIs in humans. Some belong to newly described genus such as *Emergomyces* spp., but some are well-known fungi never encountered in human disease before like *H. aspergillata*. On one hand, the use of antifungal therapy necessarily gives rise to the emergence of naturally resistant species. In addition, antifungal prophylaxis with azoles in at-risk patients has been shown to select for resistant species such as Mucorales or *Scopulariopsis* spp.[49,50] In patients empirically treated with echinocandins, the most common antifungal class for the treatment of candidemia, invasive mould infection may also arise.[27] New classes of antifungals are needed to treat and prevent these naturally resistant emerging species in immunocompromised patients. On the other hand, introduction and improvement of biomolecular techniques to identify mould and yeast species may create a reporting bias, since these techniques, following recommendations on fungal barcoding, are able to detect more species that are misidentified by classical methods previously used.[51,52]

In the light of new diagnostic methods, including fungal biomarkers and polymerase chain reaction (PCR) techniques, microscopic identification skills tend to be overlooked. However, a thorough direct examination of patient samples not only can catch and orientate towards a fungal group (yeast, hyalohyphomycetes, Mucorales) but also remains the fastest result to help initiate early treatment as a mean to improve prognosis. Furthermore, a direct observation of fungal elements in tissue is a strong evidence to prove an invasive fungal infection according to the EORTC criteria.[53] Considering that infections by emerging fungi are difficult cases to diagnose, they may therefore be under reported.

Acknowledgments

We thank Françoise Dromer and Stéphane Bretagne for their continuous support.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References

1. Vallabhaneni S, Mody RK, Walker T, Chiller T. The global burden of fungal diseases. *Infect Dis Clin N Am*. 2016; 30: 1–11.
2. Karimi K, Arzanlou M, Ahari AB, Ghazi MM. Phenotypic and molecular characterization of the causal agent of chafer beetle mortality in the wheat fields f the Kurdistan province, Iran. *J Plant Protect Res*. 2015; 55: 227–234.
3. Han B-Z, Kuijpers AFA, Thanh NV, Nout MJR. Mucoraceous moulds involved in the commercial fermentation of Sufu Pehtze. *Antonie van Leeuwenhoek*. 2004; 85: 253–257.
4. Garcia-Hermoso D, Dromer F, Alanio A, Lortholary O. Agents of systemic and subcutaneous mucormycosis and entomophthoromycosis. In: *Manual of Clinical Microbiology*, 11th edn. Washington, DC: American Society of Microbiology, 2015: 2087–2108.
5. Davel G, Featherston P, Fernandez A et al. Maxillary sinusitis caused by *Actinomucor elegans*. *J Clin Microbiol*. 2001; 39: 740–742.
6. Dorin J, D'Aveni M, Debourgogne A et al. Update on *Actinomucor elegans*, a mucormycete infrequently detected in human specimens: how combined microbiological tools contribute efficiently to a more accurate medical care. *Int J Med Microbiol*. 2017; 307: 435–442.
7. Khan ZU, Ahmad S, Mokaddas E, Chandy R, Cano J, Guarro J. Actinomucor elegans var. kuwaitiensis isolated from the wound of a diabetic patient. *Antonie van Leeuwenhoek*. 2008; 94: 343–352.
8. Tully CC, Romanelli AM, Sutton DA, Wickes BL, Hospenthal DR. Fatal *Actinomucor elegans* var. *kuwaitiensis* infection following combat trauma. *J Clin Microbiol*. 2009; 47: 3394–3399.
9. Mahmud A, Lee R, Munfus-McCray D et al. *Actinomucor elegans* as an emerging cause of mucormycosis. *J Clin Microbiol*. 2012; 50: 1092–1095.
10. Alvarez E, Stchigel AM, Cano J et al. Molecular phylogenetic diversity of the emerging mucoralean fungus *Apophysomyces*: proposal of three new species. *Revista Iberoamericana de Micología*. 2010; 27: 80–89.

11. Bonifaz A, Stchigel AM, Guarro J et al. Primary cutaneous mucormycosis produced by the new species *Apophysomyces mexicanus*. *J Clin Microbiol*. 2014; 52: 4428–4431.
12. Garcia-Covarrubias L, Bartlett R, Barratt DM, Wassermann ARJ. Rhinoorbitocerebral mucormycosis attributable to *Apophysomyces elegans* in an immunocompetent individual: case report and review of the literature. *J Trauma*. 2001; 50: 353–357.
13. Chakrabarti A, Shivaprakash MR, Curfs-Breuker I, Baghela A, Klaassen CH, Meis JF. *Apophysomyces elegans*: epidemiology, amplified fragment length polymorphism typing, and in vitroantifungal susceptibility pattern. *J Clin Microbiol*. 2010; 48: 4580–4585.
14. Gomes MZR, Lewis RE, Kontoyiannis DP. Mucormycosis caused by unusual mucormycetes, non-*Rhizopus*, -*Mucor*, and -*Lichtheimia* species. *Clin Microbiol Rev*. 2011; 24: 411–445.
15. Neblett-Fanfair R, Benedict K, Bos J et al. Necrotizing cutaneous mucormycosis after a tornado in Joplin, Missouri, in 2011. *N Engl J Med*. 2012; 367: 2214–2225.
16. Cornely OA, Arikan-Akdagli S, Dannaoui E et al. ESCMID† and ECMM‡ joint clinical guidelines for the diagnosis and management of mucormycosis 2013. *Clin Microbiol Infect*. *2014*; 20: 5–26.
17. Chakrabarti A, Kumar P, Padhye AA et al. Primary cutaneous zygomycosis due to *Saksenaea vasiformis* and *Apophysomyces elegans*. *Clin Infect Dis*. 1997; 24: 580–582.
18. Spellberg B, Edwards J, Ibrahim A. Novel perspectives on mucormycosis: pathophysiology, presentation, and management. *Clin Microbiol Rev*. 2005; 18: 556–569.
19. Chander J, Singla N, Kaur M et al. *Saksenaea erythrospora*, an emerging mucoralean fungus causing severe necrotizing skin and soft tissue infections: a study from a tertiary care hospital in north India. *Infect Dis*. 2016; 49: 170–177.
20. Relloso S, Romano V, Landaburu MF et al. *Saksenaea erythrospora* infection following a serious sailing accident. *J Med Microbiol*. 2014; 63: 317–321.
21. Hospenthal DR, Chung KK, Lairet K et al. *Saksenaea erythrospora* infection following combat trauma. *J Clin Microbiol*. 2011; 49: 3707–3709.
22. Rodríguez JY, Rodríguez GJ, Morales-López SE, Cantillo CE, Le Pape P, Álvarez-Moreno CA. *Saksenaea erythrospora* infection after medical tourism for esthetic breast augmentation surgery. *Int J Infect Dis*. 2016; 49: 107–110.
23. Bradoo R, Baveja S, Tendolkar U et al. Rhinosinusitis caused by *Saksenaea erythrospora* in an immunocompetent patient in India: a first report. *JMM Case Reports*. 2015; 2: 4410.
24. Lortholary O, Fernández-Ruiz M, Perfect JR. The current treatment landscape: other fungal diseases (cryptococcosis, fusariosis and mucormycosis). *J Antimicrob Chemother*. 2016; 71: ii31–ii36.
25. Chowdhary A, Kathuria S, Agarwal K, Meis JF. Recognizing filamentous basidiomycetes as agents of human disease: a review. *Med Mycol*. 2014; 52: 782–797.
26. Godet C, Cateau E, Rammaert B et al. Nebulized liposomal amphotericin B for treatment of pulmonary infection caused by *Hormographiella aspergillata*: case report and literature review. *Mycopathologia*. 2017; 182: 709–713.
27. Suarez F, Olivier G, Garcia-Hermoso D et al. Breakthrough *Hormographiella aspergillata* infections arising in neutropenic patients treated empirically with caspofungin. *J Clin Microbiol*. 2010; 49: 461–465.
28. Nanno S, Nakane T, Okamura H et al. Disseminated *Hormographiella aspergillata* infection with involvement of the lung, brain, and small intestine following allogeneic hematopoietic stem cell transplantation: case report and literature review. *Transplant Infect Dis*. 2016; 18: 611–616.
29. Conen A, Weisser M, Hohler D, Frei R, Stern M. *Hormographiella aspergillata*: an emerging mould in acute leukaemia patients? *Clin Microbiol Infect*. 2011; 17: 273–277.
30. Zmitrovich IV, Ezhov ON, Wasser SP. A survey of species of genus *Trametes* fr. (higher Basidiomycetes) with estimation of their medicinal source potential. *Int J Med Mushrooms*. 2012; 14: 307–319.
31. Gauthier A, Jaubert J, Traversier N et al. *Trametes polyzona*, an emerging filamentous basidiomycete in Réunion Island. *Mycoses*. 2017; 60: 412–415.
32. Schwartz IS, Kenyon C, Feng P et al. 50 Years of *Emmonsia* disease in humans: the dramatic emergence of a cluster of novel fungal pathogens. *PLoS Pathogens*. 2015; 11: e1005198–e1005198.
33. Kenyon C, Bonorchis K, Corcoran C et al. A dimorphic fungus causing disseminated infection in South Africa. *N Engl J Med*. 2013; 369: 1416–1424.
34. Dukik K, Muñoz JF, Jiang Y et al. Novel taxa of thermally dimorphic systemic pathogens in the *Ajellomycetaceae* (Onygenales). *Mycoses*. 2017; 60: 296–309.
35. Maphanga TG, Britz E, Zulu TG et al. In vitro antifungal susceptibility of yeast and mold phases of isolates of dimorphic fungal pathogen *Emergomyces africanus* (formerly Emmonsia sp.) from HIV-infected South African patients. *J Clin Microbiol*. 2017; 55: 1812–1820.
36. Malik R, Capoor MR, Vanidassane I et al. Disseminated *Emmonsia pasteuriana* infection in India: a case report and a review. *Mycoses*. 2015; 59: 127–132.
37. Boehm EWA, Mugambi GK, Miller AN et al. A molecular phylogenetic reappraisal of the *Hysteriaceae*, *Mytilinidiaceae* and *Gloniaceae* (*Pleosporomycetidae*, *Dothideomycetes*) with keys to world species. *Stud Mycol*. 2009; 64: 49–83.
38. Chowdhary A, Guarro J, Randhawa HS et al. A rare case of chromoblastomycosis in a renal transplant recipient caused by a non-sporulating species of *Rhytidhysteron*. *Med Mycol*. 2008; 46: 163–166.
39. Mahajan VK, Sharma V, Prabha N et al. A rare case of subcutaneous phaeohyphomycosis caused by a *Rhytidhysteron* species: a clinico-therapeutic experience. *Int J Dermatol*. 2014; 53: 1485–1489.
40. Mishra K, Das S, Goyal S et al. Subcutaneous mycoses caused by *Rhytidhysteron* species in an immunocompetent patient. *Med Mycol Case Rep*. 2014; 5: 32–34.
41. Chander J, Singla N, Kundu R, Handa U, Chowdhary A. Phaeohyphomycosis caused by *Rhytidhysteron rufulum* and review of literature. *Mycopathologia*. 2016; 182: 1–5.
42. Mudhigeti N, Patnayak R, Kalawat U, Yeddula SRC. Subcutaneous *Rhytidhysteron* infection: a case report from South India with literature review. *Cureus*. 2018; 10: 1–9.
43. Iwen PC, Schutte SD, Florescu DF, Noel-Hurst RK, Sigler L. Invasive *Scopulariopsis brevicaulis* infection in an immunocompromised patient and review of prior cases caused by *Scopulariopsis* and *Microascus* species. *Med Mycol*. 2012; 50: 561–569.
44. Paredes K, Capilla J, Mayayo E, Guarro J. Virulence and resistance to antifungal therapies of *Scopulariopsis* species. *Antimicrob Agents Chemother*. 2016; 60: 2063–2068.
45. Gavril D, Woerther P-L, Ben Lakhdar A et al. Invasive cutaneous infection due to *Scopulariopsis brevicaulis* unsuccessfully treated with high-dose micafungin in a neutropenic patient. *Infection*. 2017; 45: 361–363.
46. Armentano RA, American KCJOT, 2013. Disseminated mycotic infection caused by *Westerdykella* species in a German Shepherd dog. *Am Vet Med Assoc*. 2013; 242: 381–387.
47. Lipovy B, Kocmanova I, Holoubek J et al. The first isolation of *Westerdykella dispersa* in a critically burned patient. *Folia Microbiol*. 2018; 63: 1–4.
48. Sue PK, Gurda GT, Lee R et al. First report of *Westerdykella dispersa* as a cause of an angioinvasive fungal infection in a neutropenic host. *J Clin Microbiol*. 2014; 52: 4407–4411.
49. Lamoth F, Chung SJ, Damonti L, Alexander BD. Changing epidemiology of invasive mold infections in patients receiving azole Prophylaxis. *Clin Infect Dis*. 2017; 64: 1619–1621.
50. Danion F, Aguilar C, Catherinot E et al. Mucormycosis: new developments into a persistently devastating infection. *Semin Respir Crit Care Med*. 2015; 36: 692–705.
51. Schoch CL, Seifert KA, Huhndorf S et al. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Sci U S A*. 2012; 109: 6241–6246.
52. Stielow JB, Lévesque CA, Seifert KA et al. One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Pers - Int Mycol J*. 2015; 35: 242–263.
53. De Pauw B, Walsh TJ, Donnelly JP et al. Revised definitions of invasive fungal disease from the European organization for research and treatment of cancer/invasive fungal infections cooperative group and the national institute of allergy and infectious diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin Infect Dis*. 2008; 46: 1813–1821.
54. Schwartz IS, Kenyon C, Lehloenya R et al. AIDS-related endemic mycoses in Western Cape, South Africa, and clinical mimics: a cross-sectional study of adults with advanced HIV and recent-onset, widespread skin lesions. *Open Forum Infect Dis*. 2017; 4: ofx186.
55. Rose SR, Vallabhajosyula S, Velez MG et al. The utility of bronchoalveolar lavage beta-D-glucan testing for the diagnosis of invasive fungal infections. *J Infect*. 2014; 69: 278–283.

Fungal Biology 123 (2019) 341–349

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

Diversity of coelomycetous fungi in human infections: A 10-y experience of two European reference centres

Dea Garcia-Hermoso [a, 1], Nicomedes Valenzuela-Lopez [b, c, 1], Olga Rivero-Menendez [d], Ana Alastruey-Izquierdo [d], Josep Guarro [b], José F. Cano-Lira [b, *], Alberto M. Stchigel [b], the French Mycoses Study Group[2]

[a] Institut Pasteur, CNRS, National Reference Center for Invasive Mycoses and Antifungals (NRCMA), Molecular Mycology Unit, UMR2000, Paris, France
[b] Mycology Unit, Medical School and IISPV, University Rovira i Virgili, C/ Sant Llorenç 21, 43201, Reus, Spain
[c] Microbiology Unit, Medical Technology Department, Faculty of Health Science, University of Antofagasta, Chile
[d] Mycology Reference Laboratory, Spanish National Center for Microbiology, Instituto de Salud Carlos III, Madrid, Spain

ARTICLE INFO

Article history:
Received 5 November 2018
Received in revised form
30 January 2019
Accepted 8 February 2019
Available online 14 February 2019

Corresponding Editor: Steven Bates

Keywords:
Antifungal testing
Coelomycetes
Colletotrichum
Medicopsis
Mycosis
Phoma

ABSTRACT

The coelomycetous fungi are difficult to properly identify from their phenotypic characterization and their role as etiologic agents of human infections is not clear. We studied the species distribution of these fungi among clinical isolates that had been collected and stored over a ten-year period in two European reference laboratories (France and Spain). We identified phenotypically and molecularly 97 isolates by sequencing the D1-D2 fragment of the 28S nrRNA (LSU) gene and we provided the *in vitro* antifungal susceptibility pattern of seven antifungals against 46 isolates. Species of the orders *Pleosporales* and *Glomerellales* were present in both collections, and *Botryosphaeriales* and *Diaporthales* only in the French one. The most prevalent species were *Medicopsis romeroi*, *Neocucurbitaria keratinophila*, *Neocucurbitaria unguis-hominis* and *Paraconiothyrium cyclothyrioides*, which had been recovered primarily from superficial tissues. The *Didymellaceae* was the most common family represented, with 27 isolates distributed into five genera. Most of the isolates tested were susceptible to antifungals, and only the geometric mean (GM) and minimal inhibitory concentration (MIC) values of itraconazole and caspofungin had higher values. This study provides a good picture of the great diversity of coelomycetous fungi in the European clinical context, and the basis for future studies on this interesting but neglected group of fungi.

1. Introduction

Human infections by coelomycetous fungi are rare and poorly characterized due to the difficulty in identifying these fungi using only phenotypic tools. The coelomycetous fungi are characterized by the production of conidia (asexual spores) into fruiting bodies (= conidiomata). The class Coelomycetes today lack scientific validity due to the demonstrated polyphyletic character of this sort of fungi (Wijayawardene et al., 2016). They cause superficial or subcutaneous infections, mostly following a traumatic inoculation of contaminated plant material or soil particles during agricultural work in tropical and subtropical areas (Stchigel and Sutton, 2013). The most common coelomycetous fungi involved in these infections are the etiologic agents of black-grain eumycetoma, such as *Biatriospora mackinnonii*; *Falciformispora* spp., *Medicopsis romeroi*, and *Pseudochaetosphaeronema larense* (Ahmed et al., 2014). Other common coelomycetous fungi include *Lasiodiplodia theobromae* and *Neoscytalidium dimidiatum*, which typically cause onychomycosis, subcutaneous phaeohyphomycosis, and rarely eumycetoma (Stchigel and Sutton, 2013; de Hoog et al., 2000). In addition, many species of *Phoma* and *Pyrenochaeta* have been reported as occasional agents of localized and systemic infections in humans (Punithalingam, 1979; Ferrer et al., 2009). The taxonomy of several coelomycetous genera mentioned before have been revised recently but they still constitute a group of highly polyphyletic taxa

* Corresponding author. Unitat de Micologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, 21 Sant Llorenç St., 43201, Reus, Spain. Fax: +34 977 759322.
E-mail address: jose.cano@urv.cat (J.F. Cano-Lira).
[1] D.G-H. and N.V-L contributed equally to this article.
[2] The full list of French Mycoses Study members is presented in the Acknowledgements section.

https://doi.org/10.1016/j.funbio.2019.02.001
1878-6146/

that are usually difficult to identify phenotypically (de Gruyter et al., 2013; Chen et al., 2015).

In a recent study on coelomycetes conducted in the USA, Valenzuela-Lopez et al. (2017) identified 230 fungal strains by sequencing the D1-D2 domains of the 28S rRNA gene (LSU), from which 152 (66.1 %) strains belonged to the order *Pleosporales*, the rest being distributed in several orders of the phylum *Ascomycota*. Most of these strains were recovered from superficial tissue. *N. dimidiatum*, *Paraconiothyrium cyclothyrioides* and members of the family *Didymellaceae* were the most prevalent taxa. In addition, those authors demonstrated the usefulness of the LSU as a good molecular marker for a preliminary identification of coelomycetous fungi at genus level. However, the nucleotide sequences of more phylogenetically informative genes need analysing in order to identify the fungi at species level. Genes such as the RNA polymerase II subunit 2 (*rpb2*), translation elongation factor 1-alpha (*tef1*), beta-tubulin (*tub2*) and the ribosomal internal transcribed spacer region (ITS), combined in a multi-locus analysis, have all been recommended for this purpose (Valenzuela-Lopez et al., 2018a).

Until now, the coelomycetous fungi involved in invasive fungal infections (IFIs) are poorly known in Europe, probably due to the infrequency of these fungi and the complexity of their identification in the absence of characteristic fruiting bodies when grown on culture media used in the clinical lab. In a recent French study, eighteen proven cases of cutaneous and subcutaneous primary infections by coelomycetous fungi were reported and analysed in patients from tropical and subtropical regions (Guégan et al., 2016).

For a better knowledge of the diversity of coelomycetous fungi involved in human infections, we studied a large set of clinical isolates that had been identified in two mycology reference centres in France and Spain, and determined their *in vitro* antifungal susceptibility pattern.

2. Material and methods

2.1. Fungal isolates

We studied 97 isolates of coelomycetous fungi recovered from clinical specimens, 51 isolates (CNRMA) of which were provided by the French National Reference Centre for Invasive Mycoses and Antifungals (NRCMA) at the *Institut Pasteur*, Paris. The Spanish National Centre of Microbiology at the *Instituto de Salud Carlos III*, Madrid provided 46 isolates (CNM-CM). The isolates were collected between 2005 and 2015. Table 1 gives information about the country of isolation and the location of the infection in the body.

2.2. Morphological and physiological characterization

For morphology studies, the isolates were cultured following the recipes and descriptions by Valenzuela-Lopez et al. (2018b).

2.3. Molecular identification and phylogenetic analysis

Total genomic DNA was extracted following the protocols by Valenzuela-Lopez et al. (2017). Preliminary molecular identification of the isolates was made using LSU nucleotide sequences in $BLAST_N$ searches. Twenty-eight LSU sequences of type or reference strains deposited in the GenBank database by the Westerdijk Fungal Biodiversity Institute (CBS) and the Mae Fah Luang University (MFLUCC) culture collections were used for identification and phylogenetic purposes. DNA sequences generated in this study were deposited in GenBank (accession numbers are given in Table 1).

For the phylogenetic study, sequences were aligned using the ClustalW application (Thompson et al., 1994) of the MEGA 6.06 (Tamura et al., 2013) computer program, and manually adjusted using the same software platform. Phylogenetic reconstructions were made by maximum-likelihood (ML) and Bayesian inference (BI) with MEGA 6.06 and MrBayes 3.2.4 (Huelsenbeck and Ronquist, 2001), respectively. The best substitution model for the gene matrix (TN93 + G) was estimated using MEGA 6.06. For ML analyses, nearest-neighbour interchange was used as the heuristic method for tree inference. Support for internal branches was assessed by 1000 ML bootstrapped pseudoreplicates. Bootstrap support (BS) of $\geq$70 was considered significant. For BI analyses, Markov chain Monte Carlo (MCMC) sampling was carried out with four million generations, with samples taken every 1000 generations. The 50 % majority rule consensus trees and posterior probability values (PP) were calculated after removing the first 25 % of the resulting trees for burn-in. A PP value of $\geq$0.95 was considered significant. Reference strains of *Colletotrichum gigasporum* (CBS 159.75), *Colletotrichum gloeosporioides* (CBS 122687) and *Colletotrichum hippeastri* (CBS 241.78) were used as outgroup.

2.4. Antifungal susceptibility testing

The *in vitro* susceptibility testing in both reference centres (n = 46 isolates) followed the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2008) procedure. The antifungals used were amphotericin B (Sigma–Aldrich Química, Madrid, Spain), itraconazole (Sigma–Aldrich Química, Madrid, Spain), posaconazole (Schering-Plough Research Institute, Kenilworth, N.J.), voriconazole (Pfizer S.A., Madrid, Spain), caspofungin (Merck & Co., Inc., Rahway, N.J.), micafungin (Astellas Pharma Inc, Tokyo, Japan) and terbinafine (Novartis, Basel, Switzerland). For the NCRMA, all antifungal drugs were obtained from ALSACHIM, Strasbourg, France.

The isolates were cultured on potato carrot agar (PCA; 20 g each of filtered potatoes and carrots, 20 g of agar, 1 L of distilled water) or oatmeal agar (OA; 30 g of filtered oat flakes, 15 g of agar–agar, 1 L tap water) for seven to 30 d at 25 °C and 30 °C to obtain sporulation. Conidia were then collected in sterile water containing 0.01 % (v/v) Tween 80 (Sigma–Aldrich, St. Louis, MO, USA), and the suspension was adjusted to 2–5 x 10^5 conidia/mL. The minimal effective concentration (MEC) was determined for each echinocandin and the minimal inhibitory concentration (MIC) for the other drugs (90 % inhibition for amphotericin B and 80 % for the azoles) after 24 h and 48 h of incubation at 35 °C. *Aspergillus flavus* ATCC 204304 and *Aspergillus fumigatus* ATCC 204305 were used as quality control strains in all tests carried out. Susceptibility profiles were determined for 46 isolates since non-sporulating isolates were excluded at the NRCMA.

3. Results

3.1. Locations of infections

The majority of the isolates were recovered from superficial tissue, mainly skin (44 %; 43/97), eyes (27 %; 26/97), nails/hairs (18 %; 17/97) and mouth/sinus (2 %; 2/97). A few were recovered from deeper sites: bones (4 %, 4/97), blood (2 %, 2/97), cerebrospinal fluid (n = 1), bone marrow (n = 1) and lung (n = 1) (Tables 1 and 2).

3.2. Phylogenetic analyses

The maximum-likelihood (ML) phylogenetic analysis of the LSU sequences (approximately 584 pb) demonstrated that the 97 isolates were distributed into four orders, but scattered into fourteen

Table 1
Taxonomical identification of the isolates studied, origin and GenBank accession numbers. New sequences generated are indicate in bold.

Order	Species	Strain no.[a]	Origin	Country	GenBank accesion no.[b]
Botryosphaeriales	*Diplodia seriata*	CBS 112555^T	*Vitis vinifera* dead plant	Portugal	KF766327
		CNRMA 6.1007	bone	France	**LT965964**
	Lasiodiplodia sp.	CNRMA 15.383	eye	France (West Indies, Guadeloupe)	**LT965965**
	Lasiodiplodia theobromae	CBS 164.96^T	fruit along coral reef coast	Papua New Guinea	NG_042460
		CNRMA 10.1369	skin	France (West Indies, Martinique)	**LT965966**
		CNRMA 10.813	eye	France (West Indies, Martinique)	**LT965967**
		CNRMA 11.360	eye	France (West Indies, Martinique)	**LT965968**
		CNRMA 13.891	skin	France	**LT965969**
		CNRMA 14.708	eye	France (West Indies, Guadeloupe)	**LT965970**
	Neofusicoccum luteum	CBS 110299	*Vitis vinifera* cane	Portugal	AY928043
		CNRMA 12.597	eye	France	**LT965971**
Diaporthales	*Diaporthe sclerotioides*	CBS 296.67^T	*Cucumis sativus* root	The Netherlands	AF439628
	Diaporthe sp.	CBS 477	*Cucumis sativus*	USA	AF439631
		CNRMA 8.522	eye	France	**LT965972**
		CNRMA 9.205	eye	France (West Indies, Guadeloupe)	**LT965973**
		CNRMA 11.385	eye	France (West Indies, Martinique)	**LT965974**
		CNRMA 12.311	blood	France	**LT965975**
		CNRMA 13.515	skin	France	**LT965976**
		CNRMA 14.198	skin	France	**LT965977**
Glomerellales	*Colletotrichum gigasporum*	CBS 159.75	air and stored grains	India	DQ286206
		CNRMA 16.553	skin	France (West Indies, Guadeloupe)	**LT965978**
	Colletotrichum gloeosporioides	CBS 122687	*Leucospermum* sp. leaf litter	South Africa	EU552111
		CNRMA 15.504	eye	France (West Indies, Martinique)	**LT965979**
	Colletotrichum hippeastri	CBS 241.78	*Hippeastrum* sp.	The Netherlands	DQ286167
	Colletotrichum sp.	CNM-CM4760	corneal swab	Spain	**LT965980**
		CNM-CM 6116	conjuntival	Spain	**LT965981**
		CNM-CM 7345	humour acuosus	Spain	**LT965982**
Pleosporales	*Didymella gardeniae*	CBS 626.68^T	*Gardenia jasminoides* leaf	India	GQ387595
		CNM-CM 3697	nail	Spain	**LT965983**
		CNM-CM 3895	nail	Spain	**LT965984**
		CNM-CM 5036	scales	Spain	**LT965985**
		CNM-CM 5814	conjunctival exudate	Spain	**LT965986**
		CNM-CM 7499	conjunctival exudate	Spain	**LT965987**
		CNRMA 11.794	skin	France	**LT965988**
	Didymella glomerata	CBS 528.66	*Chrysanthemum* sp. cutting	The Netherlands	EU754184
		CNM-CM 3356	toenail	Spain	**LT965989**
		CNM-CM 3546	nail	Spain	**LT965990**
		CNM-CM 4675	nail	Spain	**LT965991**
		CNM-CM 7099	cutaneous exudate	Spain	**LT965992**
		CNRMA 9.1046	skin	France	**LT965993**
		CNRMA 10.867	skin	France	**LT965994**
		CNRMA 15.6	mouth/sinus	France	**LT965995**
	Epicoccum nigrum	CBS 173.73^T	*Dactylis glomerata* seed	USA	GU237975
		CNM-CM 5281	skin	Spain	**LT965996**
		CNM-CM 5724	vitreous humour	Spain	**LT965997**
	Epicoccum sorghinum	CBS 179.80	*Sorghum vulgare*	Puerto Rico	GU237978
		CNRMA 7.167	bone	France (New Caledonia)	**LT965998**
		CNRMA 10.947	skin	France (New Caledonia)	**LT965999**
		CNRMA 10.948	skin	France (New Caledonia)	**LT966000**
	Medicopsis romeroi	CBS 252.60^T	maduromycosis	Venezuela	EU754207
		CNM-CM 3387	knee ulcer	Spain	**LT966001**
		CNM-CM 7645	cutaneous exudate	Spain	**LT966002**
		CNRMA 4.200	eye	France	**LT966003**
		CNRMA 5.321	skin	France	**LT966005**
		CNRMA 7.1225	skin	France	**LT966007**
		CNRMA 8.1363	skin	France	**LT966008**
		CNRMA 11.680	skin	France	**LT966010**
		CNRMA 11.949	bone	France	**LT966011**
		CNRMA 14.407	skin	France	**LT966013**
		CNRMA 15.461	bone	France	**LT966014**
		CNRMA 15.7	skin	France	**LT966015**
	Neoascochyta desmazieri	CBS 297.69^T	*Lolium perenne*	Germany	KT389726
		CNM-CM 6201	nail	Spain	**LT966016**
	Neocucurbitaria cava	CBS 257.68^T	wheat-field soil	Germany	EU754199
		CNRMA 15.708	mouth/sinus	France	**LT966017**
	Neocucurbitaria keratinophila	CBS 121759^T	corneal scrapings	Spain	LT623215
		CNM-CM 5882	cutaneous exudate	Spain	**LT966018**
		CNM-CM 6401	fingernail	Spain	**LT966019**
		CNM-CM 6455	cutaneous exudate	Spain	**LT966020**
		CNM-CM 7013	cutaneous exudate	Spain	**LT966021**
		CNM-CM 7457	cutaneous exudate	Spain	**LT966022**
		CNM-CM 7731	cutaneous exudate	Spain	**LT966023**
		CNM-CM 8010	conjunctival exudate	Spain	**LT966024**

(*continued on next page*)

Table 1 (*continued*)

Order	Species	Strain no.[a]	Origin	Country	GenBank accesion no.[b]
		CNM-CM 8674	toenail	Spain	**LT966025**
	Neocucurbitaria unguis-hominis	CBS 112.79	airborn	Wales	GQ387622
		CNM-CM 7037	nail	Spain	**LT966026**
		CNM-CM 7089	cutaneous lession	Spain	**LT966027**
		CNM-CM 8717	urine	Spain	**LT966028**
		CNM-CM 8743	toenail	Spain	**LT966029**
		CNRMA 4.1112	eye	France	**LT966030**
		CNRMA 6.243	eye	France	**LT966031**
		CNRMA 16.153	eye	France	**LT966032**
		CNRMA 16.19	lung	France	**LT966033**
	Neocucurbitaria sp.	CNM-CM 6489	wound exudate	Spain	**LT966034**
		CNM-CM 7025	hair	Spain	**LT966035**
		CNM-CM 7132	toenail	Spain	**LT966036**
	Paraconiothyrium cyclothyrioides	CBS 972.95^T	soil	Papua New Guinea	JX496232
		CNM-CM 6313	conjunctival exudate	Spain	**LT966037**
		CNM-CM 6513	nail	Spain	**LT966038**
		CNM-CM 4767	abscess	Spain	**LT966039**
		CNRMA 11.383	skin	France (West Indies, Martinique)	**LT966041**
		CNRMA 11.855	skin	France	**LT966042**
		CNRMA 13.245	skin	France	**LT966043**
		CNRMA 16.374	skin	France (West Indies, Guadeloupe)	**LT966044**
		CNRMA 16.556	skin	France (West Indies, Guadeloupe)	**LT966045**
	Paraconiothyrium fuckelii	CBS 797.95	*Rubus* sp. dead stem	Denmark	JX496226
		CNRMA 3.240	eye	France	**LT966046**
		CNRMA 4.493	eye	France	**LT966047**
	Paraphaeosphaeria michotii	MFLUCC 13-0349	Poaceae dead leaves	Italy	KJ939282
		CNM-CM 6000	skin	Spain	**LT966048**
	Paraphoma fimeti	CBS 170.70^T	*Apium graveolens* seeds	The Netherlands	GQ387584
		CNM-CM 8075	wound exudate	Spain	**LT966049**
	Paraphoma sp.	CNRMA 9.467	skin	France	**LT966050**
		CNRMA 15.665	skin	France	**LT966051**
	Phaeosphaeriopsis obtusispora	CBS 246.64	*Aloe arborescens* dead leaf	Portugal	JX681119
	Phoma herbarum	CBS 615.75	*Rosa multiflora* dead stem	The Netherlands	EU754186
		CNM-CM 2132	right toe	Spain	**LT966052**
		CNM-CM 3526	bone marrow	Spain	**LT966053**
		CNM-CM 3597	blood culture	Spain	**LT966054**
		CNM-CM 8031	nail	Spain	**LT966055**
		CNRMA 9.1095	skin	France	**LT966056**
		CNRMA 11.1097	eye	France	**LT966057**
		CNRMA 12.1227	eye	France	**LT966058**
	pleosporelean fungus	CNRMA 11.1115	skin	France	**LT966059**
	Preussia sp.	CNM-CM 7343	nail	Spain	**LT966060**
	Preussia terricola	CBS 317.65^T	*Musa sapientum* rhizosphere	Honduras	GQ203725
	Preussia typharum	CBS 107.69	Dung of deer	Japan	GQ203726
		CNM-CM 7335	nail	Spain	**LT966061**
	Pseudophaeosphaeria rubi	MFLUCC 14-0259	*Rubus idaeus* dead branch	Italy	KX765299
	Tintelnotia destructans	CBS 127737^T	anterior eye chamber cornea	Germany	KY090664
		CNM-CM 7430	Unknown	Spain	**LT966062**
	Tintelnotia sp.	CNM-CM 7080	nail	Spain	**LT966063**
		CNM-CM 7981	cutaneous exudate	Spain	**LT966064**
	Xenodidymella saxea	CBS 419.92^T	Corroded mediterranean marble	Unknown	GU238141
		CNRMA 16.76	Cerebrospinal fluid	France	**LT966065**

[a] **CBS**: Strains from Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; **CNM-CM**: Isolates from the National Centre for Microbiology, Instituto Carlos III, Madrid, Spain; **CNRMA**: Isolates from the National Reference Center for Invasive Mycoses and Antifungals; Institut Pasteur, Paris, France; **MFLUCC**: Strains from Mae Fah Luang University Culture Collection, Chiang Rai, Thailand. Type strains are indicated by a superscript^T.

[b] LSU, large subunit ribosomal DNA sequences. Sequences generated in this study are indicated in bold.

Table 2
Localization of infections due to coelomycetous fungi isolates.

Orders	no. of isolates obtained from:		
	Superficial infection	Deep infection	Total no. of isolates
Botryosphaeriales	7	1	8
Diaporthales	5	1	6
Glomerellales	5		5
Pleosporales	71	7	78
Total no. of isolates (%)	**88 (91)**	**9 (9)**	**97 (100)**

clades (Fig. 1). Most of the isolates (81 %; 78/97) belonged to the order *Pleosporales*, which were distributed into nine clades corresponding to 23 species of twelve genera, followed by those of the *Botryosphaeriales* (8 %; 8/97), the *Diaporthales* (6 %; 6/97) and the *Glomerellales* (5 %; 5/97).

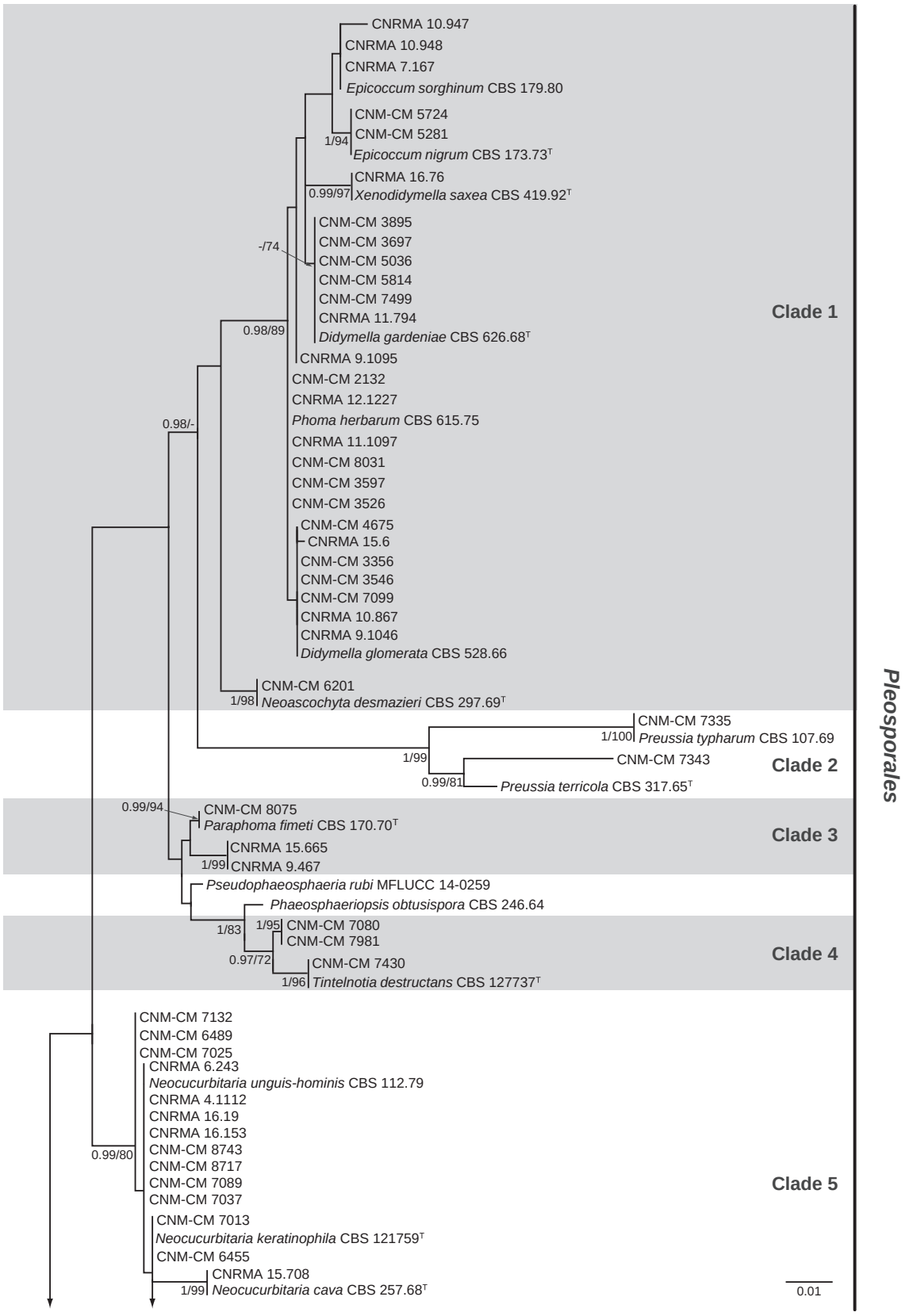


Fig. 1. Maximum likelihood tree obtained from the D1-D2 of LSU (584 bp) sequences of the 125 strains, where 28 belong to type or reference strains. The branch lengths are proportional to phylogenetic distance. Bayesian posterior probability scores ≥0.95 and Bootstrap support values ≥ 70 % are indicated on the nodes. Some branches were shortened to fit them to the page, these are indicated by two diagonal lines with the number of times a branch was shortened. The species of the genus *Colletotrichum* were used to root the tree. Superscript ^{T} indicated the type strains.

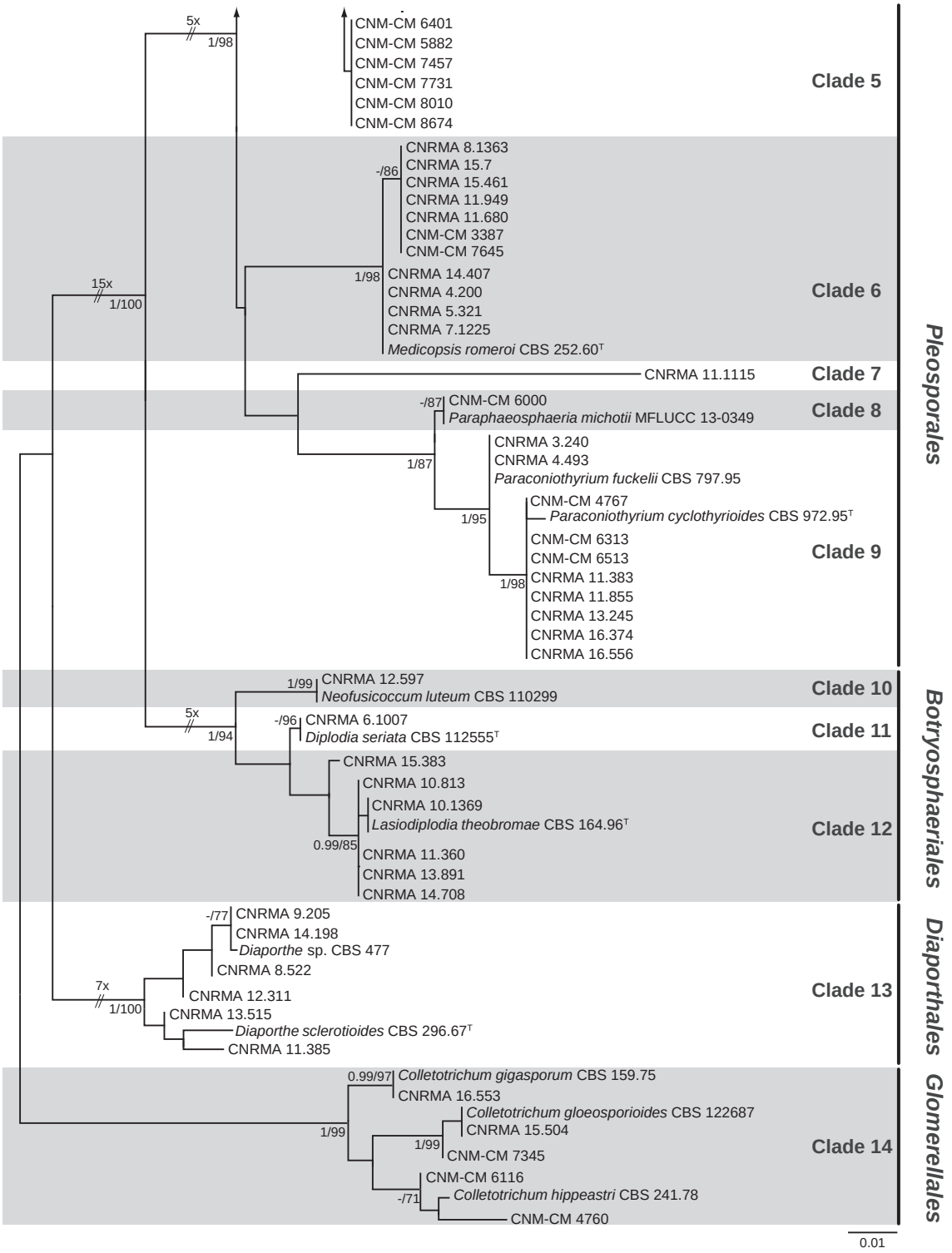


Fig. 1. (*continued*).

The most common species identified was *M. romeroi* (11 %; 11/97), followed by *P. cyclothyrioides*, *Neocucurbitaria keratinophila* and *Neocucurbitaria unguis-hominis* (8 % each; 8/97). These species were mostly isolated from cutaneous lesions (Table 2).

Clade 1 of the *Pleosporales* corresponded to the family *Didymellaceae*, which included 27 isolates distributed into five genera, morphologically characterized by their production of pycnidial conidiomata and hyaline, aseptate conidia. The five genera were *Didymella*, *Epicoccum*, *Neoascochyta*, *Phoma* and *Xenodidymella*. *Didymella* was represented by 13 isolates, six of them clustering with the type strain of *Didymella gardeniae* (CBS 626.68), and the other seven clustered with a reference strain of *Didymella glomerata* (CBS 528.66). The genus *Epicoccum* grouped five of the isolates, three of them clustering with a reference strain of *Epicoccum*

sorghinum (CBS 179.80) and the other two with the type strain of the type species of the genus, *Epicoccum nigrum* (CBS 173.73). The genus *Phoma* was represented by seven clinical isolates and a reference strain of *Phoma herbarum* (CBS 615.75). Two additional isolates included in this clade (CNRMA 16.76 and CNM-CM 6201) grouped with the type strains of *Xenodidymella saxea* (CBS 419.92) and *Neoascochyta desmazieri* (CBS 297.69), respectively.

Clade 2 had two species of *Preussia*: CNM-CM 7335 grouped with a reference strain of *Preussia typharum* (CBS 107.69), while CNM-CM 7343 represented an unknown species forming a sister clade with the type strain of *Preussia terricola* (CBS 317.65).

Clade 3 grouped three isolates of *Paraphoma*, one of them (CNM-CM 8075) clustered with the type strain of *Paraphoma fimeti* (CBS 170.70), and the remaining two (CNRMA 15.665 and CNRMA 9.467) representing unidentified phoma-like species.

Clade 4 had two sister clades of the genus *Tintelnotia*, which produced pycnidia and hyaline, aseptate conidia. The isolate CNM-CM 7430 was identified as *Tintelnotia destructans*. However, the other two isolates (CNM-CM 7080 and CNM-CM 7981) did not cluster with any known species of the genus and might represent new species.

Clade 5 had 20 isolates of *Neocucurbitaria*. *N. keratinophila* and *N. unguis-hominis* were the most common species, both with eight isolates each. *Neocucurbitaria cava*, with a single isolate (CNRMA 15.708), was also included in this clade. Three Spanish isolates, CNM-CM 6489, CNM-CM 7025 and CNM-CM 7132 were identified as *Neocucurbitaria* sp. due to being phylogenetically different from the other isolates and, again, might be a new species of the genus. *Neocucurbitaria* spp. produces pycnidia, ornamented or not, with bristle-like setose structures, and hyaline, aseptate conidia.

Clade 6 had eleven isolates of *M. romeroi* (syn. *Pyrenochaeta romeroi)*, which produces pycnidia and hyaline, aseptate conidia.

Clade 7 is represented by a single isolate (CNRMA 11.1115), phylogenetically distinct from the known pleosporalean fungi, possibly representing a novel taxon.

Clades 8 and 9 belonged to the family *Didymosphaeriaceae*. Clade 8 included a single isolate (CNM-CM 6000) phylogenetically related to a reference strain of *Paraphaeosphaeria michotii* (MFLUCC 13-0349). Clade 9 grouped ten isolates, two related to a reference strain of *Paraconiothyrium fuckelii* (CBS 797.95) and eight with the type strain of *P. cyclothyrioides* (CBS 972.95). Members of the *Didymosphaeriaceae* form pycnidia and pale brown, 0-1 septate conidia.

The order *Botryosphaeriales* are present in Clades 10 to 12. Clade 10 had only one isolate (CNRMA 12.597) which clustered with a reference strain of *Neofusicoccum luteum* (CBS 110299); Clade 11 also had a single isolate (CNRMA 6.1007) that clustered with the type strain of *Diplodia seriata* (CBS 112555), and Clade 12 grouped six isolates, five of them clustering with the type strain of *L. theobromae*, and CNRMA 15.383 identified as *Lasiodiplodia* sp. These fungi produce stromatic conidiomata and aseptate, hyaline to brown, thick-walled conidia.

Clade 13 included the type strain of *Diaporthe sclerotioides* (CBS 296.67) and six isolates corresponding to unidentified species of the genus *Diaporthe* (*Diaporthales*), none of them able to be morphologically distinguished since they produce pycnidia and small hyaline conidia.

Clade 14, corresponding to the *Glomerellales*, was used as outgroup. Five isolates nested in the *Colletotrichum* clade, two clustering with reference strains of *C. gigasporum* (CBS 159.75) and *C. gloeosporioides* (CBS 122687), respectively; and the other three, could not be identified. All the isolates showed the typical morphology of *Colletotrichum*, i.e., acervuli, conidia variable in shape, flattened with thickened tip branches (appressoria).

3.3. Antifungal susceptibility testing

The MIC was determined for 46 of the isolates included here (16 from Spain and 30 from France) (Table 3, Table S1). Globally, the geometric mean (GM) and MIC_{50} values of itraconazole and caspofungin were the highest (Table 3). The MIC of amphotericin B (0.06–1 mg/L) was generally low among the *Pleosporales* with the exception of one isolate of *M. romeroi* and one of *D. gardeniae*, with MICs of 8 and 32 mg/L, respectively. The azole MICs ranged between 0.03 and 1 mg/L for isolates belonging to the genera *Paraconiothyrium*, *Paraphoma*, *Tintelnotia* and *Neocucurbitaria*, with the exception of two isolates of *N. unguis-hominis*, which showed higher values (16 mg/L). The terbinafine MIC was low except for *Diaporthe* spp. and a few isolates of *Colletotrichum* spp. and *M. romeroi*.

4. Discussion

The present study is the largest on this taxonomically complex group of fungi from clinical origin, with almost a hundred isolates morphologically and molecularly characterized from two southern European countries (France and Spain). Most of these coelomycetous fungi belonged to the order *Pleosporales* and were most commonly recovered from superficial infections. Similar results were observed in a previous work that focused on coelomycetous fungi collected at a North American reference centre (Valenzuela-Lopez et al., 2017). However, the diversity of the fungi identified in that study was higher, i.e. eleven orders were represented against four here.

In the present study, *M. romeroi* was the most frequently isolated species whereas the most common taxon in the American study was *N. dimidiatum*. Interestingly, while *M. romeroi* is usually reported as an etiologic agent of black grain eumycetoma (Ahmed et al., 2014; van de Sande, 2013), our isolates were mainly recovered from eye and non-mycetoma subcutaneous infections.

The second most frequently isolated species were *P. cyclothyrioides*, *N. unguis-hominis* and *N. keratinophila*. *P. cyclothyrioides* is an emerging pathogen (Colombier et al., 2015; Guégan et al., 2016; Valenzuela-Lopez et al., 2017) and was represented by eight isolates recovered from skin or superficial locations and mainly from tropical regions. *N. unguis-hominis*, initially described as an agent of human onychomycosis (Punithalingam and English, 1975), was equally distributed across both centres (n = 8 isolates). Regarding *N. keratinophyla*, this species was reported for the first time from a corneal infection in Spain (Ferrer et al., 2009). Interestingly, as well as being the first case reported for this species, all the isolates of *N. keratinophyla* were recovered in Spain from superficial tissue.

Table 3
Overall *in vitro* antifungal activity against the 46 coelomycetous isolates as determined by EUCAST[a] methodology.

Antifungal agent	MIC/MEC values (mg/L)[b]				
	range	median	GM	MIC_{50}	MIC_{90}
Amphotericin B	0.03–16	0.5	0.41	0.25	1
Itraconazole	0.014–16	2	1.72	0.5	16
Voriconazole	0.03–16	0.5	0.70	0.6	4
Posaconazole	0.014–16	0.5	0.58	0.25	8
Caspofungin	0.125–16	2	2.17	1	8
Micafungin	0.015–16	0.5	0.53	0.125	8
Terbinafin	0.014–16	0.25	0.39	0.25	2

[a] EUCAST, European Committee on Antimicrobial Susceptibility Testing procedure.
[b] MIC, minimum inhibitory concentration; MEC, minimal effective concentration; MIC_{50} and MIC_{90}, MIC encompassing 50 and 90 % of isolates tested, respectively.

Other coelomycetous fungi we identified in the present work were *D. glomerata* and *Phoma herbarum*. Although *Phoma* spp. are commonly reported as a coelomycete involved in human infections (Punithalingam, 1979; Bennett et al., 2018), recent extensive changes in taxonomy and nomenclature have spread all but one of the species into different genera of the *Didymellaceae*, *Phoma herbarum* conserved as the type species of the genus (Chen et al., 2015; Valenzuela-Lopez et al., 2018b). Interestingly, *D. gardeniae* was commonly found in our study (five isolates from Spain and one from France).

Recently, Ahmed et al. (2017) proposed *T. destructans*, a new phoma-like fungus belonging to the *Phaeosphaeriaceae* able to cause eye and nail infections. They reported the successful use of terbinafine against a case of keratitis by this species. Two of the Spanish isolates recovered from superficial specimens (one cutaneous exudate and one nail sample) were molecularly related to the above-mentioned species but phylogenetically different and might represent a new taxon.

L. theobromae (order *Botryosphaeriales*) is the only species of this genus involved in human opportunistic infections (Saha et al., 2012). Valenzuela-Lopez et al. (2017) found a higher species diversity in the North American study than we report here, since five of the French isolates were identified as *L. theobromae*. The other three isolates of the *Botryosphaeriales* we found were related, one to a different species of *Lasiodiplodia* and the other two to other genera, specifically *Neofusicoccum* and *Diplodia*.

Four species of the genus *Diaporthe* (formerly *Phomopsis*; order *Diaporthales*), i.e. *D. bougainvilleicola*, *D. longicolla*, *Diaporthe phaseolorum* and *D. phoenicicola*, are considered opportunistic pathogens that cause mycoses that range from superficial to deep infections (Cariello et al., 2013; Gajjar et al., 2011; Garcia-Reyne et al., 2011; Iriart et al., 2011). Six isolates from France were phylogenetically placed into the latter genus. However, our results are only preliminary since only one phylogenetic marker was analysed. This fact was pointed out by other authors in this genus, which is a polyphyletic fungus (Gomes et al., 2013).

We also report the finding of five clinical isolates of *Colletotrichum*. Two of the isolates corresponded to *C. gigasporum* (formerly *Colletotrichum crassipes*) and *C. gloeosporioides*, taxa that have previously been reported as agents of keratitis, endophthalmitis and phaeohyphomicotic cyst; the other three isolates could not be identified at species level. This genus encompasses numerous plant pathogens that are found worldwide, although mainly in tropical and subtropical regions (Cannon et al., 2012). The taxonomy of *Colletotrichum* is complicated and the genus is organized in species-complexes (Cannon et al., 2012; Liu et al., 2014). Species such as *Colletotrichum coccodes*, *C. crassipes*, *Colletotrichum dematium*, *C. gloeosporioides*, *Colletotrichum graminicola* and *Colletotrichum truncatum* cause superficial and deep infections (endophthalmitis, keratitis, subcutaneous cyst or more rarely arthritis) (Cano et al., 2004). Further studies, including different phylogenetic markers, are needed to delimit the different species and clarify their pathogenic role.

The antifungal susceptibility of coelomycetous fungi involved in human infections is poorly known, mainly because they do not easily sporulate. In spite of the limited number of isolates tested here, amphotericin B seemed the most active drug *in vitro* together with terbinafine, in agreement with Valenzuela-Lopez et al. (2017). Until more *in vitro* data is available, the antifungal treatment of the infection by this sort of fungus remains purely empirical. In a recent study, Guégan et al. (2016) recommended extensive surgical resection of affected tissues as a first-line treatment for solitary subcutaneous lesions by coelomycetous fungi, followed by an antifungal therapy (posaconazole or voriconazole) in the case of relapse or amphotericin B in refractory cases.

Since our study is based on isolates from the two reference centres, we cannot comment on the incidence of infections due to coelomycetes nor compare their epidemiology between France and Spain. However, we still provide a good picture of the great diversity of coelomycetous fungi in the clinical context, and the basis for future studies on this interesting but neglected group of fungi.

Conflicts of interest

No conflict of interest declared.

Acknowledgments

We thank Cécile Gautier (National Reference Center for invasive Mycoses and Antifungals [NRCMA]) for technical assistance. This work was supported by the Spanish *Ministerio de Economía y Competitividad*, grant CGL2017-88094-P.

Olga Rivero-Menendez holds a fellowship from the *Fondo de Investigaciones Sanitarias* (grant FI14CIII/00025). Ana Alastruey-Izquierdo is supported by a research project from the *Fondo de Investigación Sanitaria* (FIS) (project PI16CIII/00035).

Members of the French Mycoses Study Group who contributed their clinical isolates to this study are as follows: Annecy (S. Bland), Bichat (C. Chochillon), Boulogne-Billancourt (N.Ait-Ammar), Caen (C. Duhamel), Clermont Ferrand (P.Poirier), Cochin (A. Paugam), Corbeil-Essones (D. Kubab), Guadeloupe (M. Nicolas), La Réunion (S. Picot), Lyon (A.L. Bienvenu), Martinique (N. Desbois), Nantes (F. Morio), Necker (M.E. Bougnoux), Nouvelle Calédonie (R. Goursaud), Pitié (A. Fekkar), Poitiers (C. Kauffmann), Quinze-Vingts (L. Merabet), Rouen (L. Favennec), Saint Etienne (H. Raberin), Saint-Louis (A. Alanio), Toulouse (S. Cassaing).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.02.001.

References

Ahmed, S.A., van de Sande, W.W., Stevens, D.A., Fahal, A., van Diepeningen, A.D., Menken, S.B., de Hoog, G.S., 2014. Revision of agents of black-grain eumycetoma in the order Pleosporales. Persoonia 33, 141–154.

Ahmed, S.A., Hofmüller, W., Seibold, M., de Hoog, G.S., Harak, H., Tammer, I., van Diepeningen, A.D., Behrens-Baumann, W., 2017. *Tintelnotia*, a new genus in *Phaeosphaeriaceae* harbouring agents of cornea and nail infections in humans. Mycoses 60, 244–253.

Bennett, A., Ponder, M.M., Garcia-Diaz, J., 2018. *Phoma* infections: classification, potential food Sources, and its clinical impact. Microorganisms 6, E58. https://doi.org/10.3390/microorganisms6030058.

Cannon, P.F., Damm, U., Johnston, P.R., Weir, B.S., 2012. *Colletotrichum* - current status and future directions. Stud. Mycol. 73, 181–213.

Cano, J., Guarro, J., Gené, J., 2004. Molecular and morphological identification of *Colletotrichum* species of clinical interest. J. Clin. Microbiol. 42, 2450–2454.

Cariello, P.F., Wickes, B.L., Sutton, D.A., Castlebury, L.A., Levitz, S.M., Finberg, R.W., Thompson, E.H., Daly, J.S., 2013. *Phomopsis bougainvilleicola* prepatellar bursitis in a renal transplant recipient. J. Clin. Microbiol. 51, 692–695.

Chen, Q., Jiang, J.R., Zhang, G.Z., Cai, L., Crous, P.W., 2015. Resolving the *phoma* enigma. Stud. Mycol. 82, 137–217.

Colombier, M.A., Alanio, A., Denis, B., Melica, G., Garcia-Hermoso, D., Levy, B., Peraldi, M.N., Glotz, D., Bretagne, S., Gallien, S., 2015. Dual invasive infection with *Phaeoacremonium parasiticum* and *Paraconiothyrium cyclothyrioides* in a renal transplant recipient: case report and comprehensive review of the literature of *Phaeoacremonium* phaeohyphomycosis. J. Clin. Microbiol. 53, 2084–2094.

de Gruyter, J., Woudenberg, J.H., Aveskamp, M.M., Verkley, G.J., Groenewald, J.Z., Crous, P.W., 2013. Redisposition of phoma-like anamorphs in Pleosporales. Stud. Mycol. 75, 1–36.

de Hoog, G.S., Guarro, J., Figueras, M.J., Gené, J., 2000. Atlas of Clinical Fungi, second ed. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Ferrer, C., Pérez-Santonja, J.J., Rodríguez, A.E., Colom, M.F., Gené, J., Alio, J.L., Verkley, G.J., Guarro, J., 2009. New *Pyrenochaeta* species causing keratitis. J. Clin. Microbiol. 47, 1596–1598.

Gajjar, D.U., Pal, A.K., Parmar, T.J., Arora, A.I., Ganatra, D.A., Kayastha, F.B., Ghodadra, B.K., Vasavada, A.R., 2011. Fungal scleral keratitis caused by *Phomopsis phoenicicola*. J. Clin. Microbiol. 49, 2365–2368.

Garcia-Reyne, A., López-Medrano, F., Morales, J.M., García Esteban, C., Martín, I., Eraña, I., Meije, Y., Lalueza, A., Alastruey-Izquierdo, A., Rodríguez-Tudela, J.L., Aguado, J.M., 2011. Cutaneous infection by *Phomopsis longicolla* in a renal transplant recipient from Guinea: first report of human infection by this fungus. Transpl. Infect. Dis. 13, 204–207.

Gomes, R.R., Glienke, C., Videira, S.I., Lombard, L., Groenewald, J.Z., Crous, P.W., 2013. *Diaporthe*: a genus of endophytic, saprobic and plant pathogenic fungi. Persoonia 31, 1–41.

Guégan, S., Garcia-Hermoso, D., Sitbon, K., Ahmed, S., Moguelet, P., Dromer, F., Lortholary, O., French Mycosis Study Group., 2016. Ten-Year experience of cutaneous and/or subcutaneous infections due to Coelomycetes in France. Open Forum Infect. Dis. https://doi.org/10.1093/ofid/ofw106.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Iriart, X., Binois, R., Fior, A., Blanchet, D., Berry, A., Cassaing, S., Amazan, E., Papot, E., Carme, B., Aznar, C., Couppié, P., 2011. Eumycetoma caused by *Diaporthe phaseolorum* (*Phomopsis phaseoli*): a case report and a mini-review of *Diaporthe/Phomopsis* spp invasive infections in humans. Clin. Microbiol. Infect. 17, 1492–1494.

Liu, F., Cai, L., Crous, P.W., Damm, U., 2014. The *Colletotrichum gigasporum* species complex. Persoonia 33, 83–97.

Punithalingam, E., English, M.P., 1975. *Pyrenochaeta unguis-hominis* sp. nov. on human toe-nails. Trans. Br. Mycol. Soc. 64, 539–541.

Punithalingam, E., 1979. Sphaeropsidales in culture from humans. Nova Hedwigia 31, 119–158.

Saha, S., Sengupta, J., Banerjee, D., Khetan, A., 2012. *Lasiodiplodia theobromae* keratitis: a case report and review of literature. Mycopathologia 174, 335–339.

Stchigel, A.M., Sutton, D.A., 2013. Coelomycete fungi in the clinical lab. Curr. Fungal. Infect. Rep. 7, 171–191.

Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST), 2008. EUCAST Technical Note on the method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia-forming moulds. Clin. Microbiol. Infect. 14, 982–984.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Valenzuela-Lopez, N., Sutton, D.A., Cano-Lira, J.F., Paredes, K., Wiederhold, N., Guarro, J., Stchigel, A.M., 2017. Coelomycetous fungi in the clinical setting: morphological convergence and cryptic diversity. J. Clin. Microbiol. 55, 552–567.

Valenzuela-Lopez, N., Cano-Lira, J.F., Stchigel, A.M., Guarro, J., 2018a. DNA sequencing to clarify the taxonomical conundrum of the clinical coelomycetes. Mycoses 61, 1–69.

Valenzuela-Lopez, N., Cano-Lira, J.F., Guarro, J., Sutton, D.A., Wiederhold, N., Crous, P.W., Stchigel, A.M., 2018b. Coelomycetous dothideomycetes with emphasis on the families *Cucurbitariaceae* and *Didymellaceae*. Stud. Mycol. 90, 1–69.

van de Sande, W.W., 2013. Global burden of human mycetoma: a systematic review and meta-analysis. PLoS Negl. Trop. Dis. 7, e2550. https://doi.org/10.1371/journal.pntd.0002550.

Wijayawardene, N.N., Hyde, K.D., Wanasinghe, D.N., Papizadeh, M., Goonasekara, I.D., Camporesi, E., Bhat, D.J., McKenzie, E.H.C., Phillips, A.J.L., Diederich, P., Tanaka, K., Li, W.J., Tangthirasunun, N., Phookamsak, R., Dai, D.Q., Dissanayake, A.J., Weerakoon, G., Maharachchikumbura, S.S.N., Hashimoto, A., Matsumura, M., Bahkali, A.H., Wang, Y., 2016. Taxonomy and phylogeny of dematiaceous coelomycetes. Fungal Divers. 77, 1–316.

ISHAM
INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2018, 0, 1–6
doi: 10.1093/mmy/myy132
Advance Access Publication Date: 0 2018
Original Article

Original Article

Neocucurbitaria keratinophila: An emerging opportunistic fungus causing superficial mycosis in Spain

Nicomedes Valenzuela-Lopez[1,2], José F. Cano-Lira[1,*], Alberto M. Stchigel[1], Olga Rivero-Menendez[3], Ana Alastruey-Izquierdo[3] and Josep Guarro[1]

[1]Mycology Unit, Medical School and IISPV, University Rovira i Virgili, C/ Sant Llorenç 21, 43201 Reus, Spain, [2]Unidad de Microbiología, Departamento de Tecnología Médica, Facultad de Ciencias de la Salud, Universidad de Antofagasta, Chile and [3]Mycology Reference Laboratory, Spanish National Center for Microbiology, Instituto de Salud Carlos III, Madrid, Spain

*To whom correspondence should be addressed. Prof. José F. Cano-Lira, Unitat de Micologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, 21 Sant Llorenç St., 43201, Reus, Spain. Tel: 34 977 759350; E-mail: jose.cano@urv.cat

Received 28 August 2018; Revised 30 October 2018; Accepted 7 November 2018; Editorial Decision 5 November 2018

Abstract

Although there have been few reports of opportunistic infections (superficial and systemic) caused by coelomycetous fungi, they are becoming more frequent. *Neocucurbitaria keratinophila* (formerly *Pyrenochaeta keratinophila*), characterized by producing pycnidial conidiomata and small hyaline conidia, seems to be an emergent opportunistic pathogen in Spain. Since this fungus was first reported from human keratitis, eight strains have been isolates from clinical cases in Spain. This is a retrospective study of these fungal strains, including phenotypic and molecular characterizations, and *in vitro* antifungal susceptibility assays. These clinical strains were identified by sequencing four phylogenetic markers such as the internal transcribed spacer region (ITS1-5.8S-ITS2) and fragments of the 28S nrRNA (LSU), beta-tubulin (*tub*2), and RNA polymerase II subunit 2 (*rpb*2) genes, and by morphological characterization. All the strains tested were susceptible to the majority of antifungals, being isavuconazole the only drug that showed a poor antifungal activity.

Key words: antifungal susceptibility, coelomycetes, superficial mycosis, *Neocucurbitaria*, *Pyrenochaeta*.

Introduction

Coelomycetous fungi are commonly found as saprobic or parasites of terrestrial vascular plants, although they can also be found in diverse environments such as soil, freshwater, salty water, sewage and even inorganic materials, and more rarely as human opportunistic pathogens.[1,2] Although the class Coelomycetes is now obsolete, it is still used in clinics to refer fungi that are morphologically characterized by producing asexual fruiting bodies called conidiomata, inside which numerous conidia are produced on conidiophores and/or from conidiogenous cells. Although human infections by coelomycetous fungi are relatively scarce in comparison with other fungi, they are becoming more frequently reported, ranging from superficial to deep infections.[1–5] Currently, identifying coelomycetous fungi is still complex because of the small number of discriminative morphological features and the high number of taxa involved.[1,6] For that reason, several studies performed a multilocus analysis to resolve their identification.[7–9] However, in the clinical lab it is more practical to use specific phylogenetic markers, for example, the internal transcribed spacer region (ITS), beta-tubulin (*tub*2), or RNA polymerase II subunit 2 (*rpb*2) allowing an easier identification without the need of phylogenetic reconstruction.[10]

In a recent study on coelomycetous fungi from clinical origin [D. Garcia-Hermoso, N. Valenzuela-Lopez, O. Rivero-Menendez, et al. Fungal Biology. Send manuscript to publisher][9bis], we found that a relatively high number of isolates of *Neocucurbitaria keratinophila* (formerly *Pyrenochaeta keratinophila*) had been deposited between 2005 and 2015 at the

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

SUSCEPTIBILITY

Molecular Identification and Susceptibility Testing of Molds Isolated in a Prospective Surveillance of Triazole Resistance in Spain (FILPOP2 Study)

Ana Alastruey-Izquierdo,[a,n] Laura Alcazar-Fuoli,[a,n] Olga Rivero-Menéndez,[a] Josefina Ayats,[b,n] Carmen Castro,[c] Julio García-Rodríguez,[d] Lidia Goterris-Bonet,[e] Elisa Ibáñez-Martínez,[f] María José Linares-Sicilia,[g] M. Teresa Martin-Gomez,[e] Estrella Martín-Mazuelos,[c] Teresa Pelaez,[h] Javier Peman,[f] Antonio Rezusta,[i] Susana Rojo,[j] Rocio Tejero,[g] Diego Vicente Anza,[k,l,m] Jesús Viñuelas,[i] Maria Soledad Zapico,[k] Manuel Cuenca-Estrella,[a] on behalf of the FILPOP2 Project from GEMICOMED (SEIMC) and REIPI

[a]Instituto Salud Carlos III, National Centre for Microbiology, Madrid, Spain
[b]Hospital Universitari de Bellvitge, Barcelona, Spain
[c]Hospital Universitario Virgen de Valme, Seville, Spain
[d]Hospital Universitario La Paz, Madrid, Spain
[e]Hospital Universitario Vall d'Hebron, Barcelona, Spain
[f]Hospital Universitario La Fe, Valencia, Spain
[g]Hospital Universitario Reina Sofía, Córdoba, Spain
[h]Hospital General Universitario Gregorio Marañón, Madrid, Spain
[i]Hospital Universitario Miguel Servet, Zaragoza, Spain
[j]Hospital Universitario Central de Asturias, Oviedo, Spain
[k]Hospital Universitario Donostia, San Sebastian, Spain
[l]Centro de Investigación Biomédica en Red de Enfermedades Respiratorias, Madrid, Spain
[m]Universidad del País Vasco UPV/EHU, Vizcaya, Spain
[n]Spanish Network for Research in Infectious Diseases, Instituto de Salud Carlos III, Madrid, Spain

ABSTRACT Antifungal resistance is increasing by the emergence of intrinsically resistant species and by the development of secondary resistance in susceptible species. A previous study performed in Spain revealed levels of azole resistance in molds of between 10 and 12.7%, but secondary resistance in *Aspergillus fumigatus* was not detected. We used itraconazole (ITZ)-supplemented medium to select resistant strains. A total of 500 plates supplemented with 2 mg/liter of ITZ were sent to 10 Spanish tertiary hospitals, and molecular identification and antifungal susceptibility testing were performed. In addition, the *cyp51A* gene in those *A. fumigatus* strains showing azole resistance was sequenced. A total of 493 isolates were included in the study. Sixteen strains were isolated from patients with an infection classified as proven, 104 were isolated from patients with an infection classified as probable, and 373 were isolated from patients with an infection classified as colonization. *Aspergillus* was the most frequent genus isolated, at 80.3%, followed by *Scedosporium-Lomentospora* (7.9%), *Penicillium-Talaromyces* (4.5%), *Fusarium* (2.6%), and the order *Mucorales* (1%). Antifungal resistance was detected in *Scedosporium-Lomentospora* species, *Fusarium*, *Talaromyces*, and *Mucorales*. Three strains of *A. fumigatus sensu stricto* were resistant to azoles; two of them harbored the TR_{34}+L98H mechanism of resistance, and the other one had no mutations in *cyp51A*. The level of azole resistance in *A. fumigatus* remains low, but cryptic species represent over 10% of the isolates and have a broader but overall higher range of antifungal resistance.

KEYWORDS antifungal resistance, *Aspergillus*, cryptic species, *Scedosporium*, *Fusarium*, *Mucorales*, azole resistance, *cyp51A*, TR_{34}/L98H, aspergillosis

Received 23 February 2018 **Returned for modification** 12 April 2018 **Accepted** 15 June 2018

Accepted manuscript posted online 25 June 2018

Citation Alastruey-Izquierdo A, Alcazar-Fuoli L, Rivero-Menéndez O, Ayats J, Castro C, García-Rodríguez J, Goterris-Bonet L, Ibáñez-Martínez E, Linares-Sicilia MJ, Martin-Gomez MT, Martín-Mazuelos E, Pelaez T, Peman J, Rezusta A, Rojo S, Tejero R, Anza DV, Viñuelas J, Zapico MS, Cuenca-Estrella M, on behalf of the FILPOP2 Project from GEMICOMED (SEIMC) and REIPI. 2018. Molecular identification and susceptibility testing of molds isolated in a prospective surveillance of triazole resistance in Spain (FILPOP2 study). Antimicrob Agents Chemother 62:e00358-18. https://doi.org/10.1128/AAC.00358-18.

Address correspondence to Ana Alastruey-Izquierdo, anaalastruey@isciii.es.

The incidence of fungal diseases causing fatal infections has risen due to an increase in populations at risk. Mortality rates range from 40% to 90% in high-risk patients, such as those with hematological malignancies (1, 2). Although *Aspergillus fumigatus* is the most common filamentous fungus involved in invasive diseases, emerging molds, such as *Mucorales*, *Scedosporium* spp., *Fusarium* spp., and other species of *Aspergillus*, are being increasingly reported (3, 4). The prevalence and relevance of these emerging fungal pathogens in the clinical setting are presently unknown. However, a prominent feature is that these emerging fungi show decreased susceptibility *in vitro* to most antifungal drugs (5), and disseminated mold infections are often very difficult to treat. A scarce number of epidemiological studies of mold infections involving multiple centers have been reported (6, 7). Epidemiological studies are essential to know the prevalence of fungal pathogens and are key to implementing control measures to decrease infection rates. In addition, they are essential to detect the emergence of resistance and to define rates of resistance in different geographical areas and groups of patients.

Antifungal resistance is increasing both by the emergence of more-resistant/less-susceptible species and by the development of secondary resistance (8). Particularly important are the high rates of azole-resistant *A. fumigatus* reported for clinical samples in the Netherlands and the United Kingdom (9–11). In the Netherlands, azole resistance has an overall prevalence of 5.3%, with a range from 1.8% to 12.8%, depending on the geographical area and hospital studied (11). In the United Kingdom, a clinical collection data set of 519 *A. fumigatus* isolates showed that the frequency of itraconazole (ITZ) resistance *in vitro* was 5%, with a significant increase since 2004 (10). Later, the rise of azole resistance continued in 2008 and 2009, with rates of 14% and 20%, respectively (12). Since then, triazole resistance has been described worldwide (13, 14).

In Spain, the multicenter epidemiological study FILPOP (6) described the epidemiology of mold infections in the country and the rates of resistance. The rates of triazole resistance ranged from 10 to 12.7%, depending on the species and the drug tested; however, secondary resistance in *A. fumigatus* was not detected. Taking into account the high levels of secondary resistance described in neighboring countries, we hypothesize that this resistance could be underestimated due to the sampling method that we used. In this work, we propose a strategy using a selective medium supplemented with an antifungal (ITZ) to detect specifically resistant isolates in respiratory samples and to avoid the overgrowth of azole-susceptible species belonging to the conventional flora. Plates with the selective medium were used to culture clinical samples directly to increase the rate and chance of isolation of azole-resistant strains.

RESULTS

Five hundred six isolates were obtained from 10 Spanish hospitals. One was a dermatophyte isolated from a nail, which was excluded from the study; seven were yeast; and five did not grow in the reference laboratory (RL) and were not further analyzed. Thus, a total of 493 isolates were included in the study. One hundred fifty-five isolates grew in ITZ-supplemented medium, and 338 grew in regular Sabouraud (SAB) medium without supplements. Each isolate was characterized independently since typing techniques were not applied at this stage.

Four hundred seventy-seven (96.8%) strains were isolated from respiratory samples (333 sputum samples, 79 BAS [bronchoaspirate] samples, 38 BAL [bronchoalveolar lavage] fluid samples, 14 tracheal aspirate samples, and 13 other respiratory samples), 10 were isolated from biopsy specimens, 4 were isolated from wound exudates, and 2 were isolated from otic exudates. Sixteen strains were isolated from patients with an infection classified as proven, 104 were isolated from patients with an infection classified as probable, and 373 were isolated from patients with an infection classified as colonization. Underlying diseases included hematological malignancy (7%), solid-organ transplant (9.7%), other cancer (11.6%), HIV (5%), chronic obstructive pulmonary disease (COPD) (16.6%), cystic fibrosis (9.3%), asthma (2.6%), other causes of immunosuppression (4.5%), other respiratory diseases (14.8%), other causes (6.1%), and un-

TABLE 1 Numbers of strains isolated per type of sample and type of infection

Sample type	No. of strains isolated from samples from infection categorized as:			Total no. of strains isolated
	Colonization	Probable	Proven	
Sputum	277	52	4	333
BAS	60	17	2	79
BAL fluid	21	17	0	38
Tracheal aspirate	7	5	2	14
Other respiratory sites[a]	8	5	0	13
Biopsy specimen	0	2	8	10
Wound exudate	0	4	0	4
Otic exudate	0	2	0	2
Total	373	104	16	493

[a]Other respiratory sites include sinuses, lung tissue, and oropharyngeal swabs.

known (12.6%). Table 1 summarizes the number of strains isolated from each type of sample by type of infection.

Identification of strains. *Aspergillus* was the most frequent genus isolated, at 80.3%, followed by *Scedosporium-Lomentospora* (7.9%), *Penicillium-Talaromyces* (4.5%), *Fusarium* (2.6%), and the order *Mucorales* (1%). Table 2 shows the identification to the species level of the strains analyzed; *A. fumigatus* was the most frequently isolated species, with 260 isolates (52.74%), followed by *Aspergillus niger* (5.27%), *Aspergillus flavus* (5.07%), *Aspergillus terreus* (4.67%), and *Scedosporium apiospermum* (4.26%). Cryptic species of *Aspergillus* accounted for 11.5% of the isolates. *A. fumigatus* was the most frequently isolated species in cases of both colonization (186 isolates) and infections (74 isolates); however, while *A. niger* and *A. flavus* ranked second (26 total isolates) and third (25 total isolates) in the total number of isolates, they were found less

TABLE 2 Numbers of strains isolated per species and type of infection

Group	No. of strains isolated from samples from infection categorized as:			Total no. of strains isolated
	Colonization	Probable	Proven	
A. fumigatus	186	68	6	260
A. niger	20	6	0	26
A. flavus	23	2	0	25
A. terreus	15	6	2	23
S. apiospermum	12	5	4	21
A. tubingensis	15	1	0	16
Scedosporium boydii	11	0	1	12
A. alliaceus	5	5	0	10
Fusarium proliferatum	7	2	0	9
A. lentulus	6	0	0	6
A. nidulans	5	1	0	6
A. calidoustus	4	1	0	5
Aspergillus quadrilineatus	5	0	0	5
Penicillium chrysogenum	5	0	0	5
Aspergillus sydowii	2	2	0	4
Paecilomyces lilacinus	3	1	0	4
Penicillium citrinum	4	0	0	4
Lomentospora prolificans	1	1	1	3
S. ellipsoideum	3	0	0	3
Other *Aspergillus* spp.	8	2	0	10
Other *Talaromyces* spp.	9	0	0	9
Mucorales	3	0	2	5
Basidiomycetes	4	1	0	5
Other *Fusarium* spp.	4	0	0	4
Other *Penicillium* spp.	4	0	0	4
Other	9	0	0	9
Total	373	104	16	493

frequently (6 and 2 isolates, respectively) than *S. apiospermum* (9 isolates) and *A. terreus* (8 isolates) in infections classified as probable or proven (Table 2). These differences were not statistically significant.

Susceptibility testing. Three strains did not grow in the medium and under the conditions used for susceptibility testing and were not analyzed. Table 3 shows geometric means (GMs), MIC_{50}s, MIC_{90}s, and MIC ranges for the species that had 10 or more isolates. *Scedosporium* species had elevated MICs for all antifungals, with voriconazole being the most active compound, with an MIC_{50} of 1 mg/liter, followed by echinocandins. Azoles and echinocandins showed no activity against any of the *Fusarium* isolates analyzed; only amphotericin B showed activity (MICs of ≤2 mg/liter for 9 out of 13 isolates). *Mucorales* species isolated showed elevated MICs of voriconazole and echinocandins and low MICs of amphotericin B and posaconazole. *Penicillium-Talaromyces* showed low MICs of most antifungals except for *Penicillium citrinum*, with high MICs (16 mg/liter) of voriconazole. Other species with high MICs were *Scopulariopsis brevicaulis* and *Alternaria* spp., with high MICs for all antifungals, and *Purpureocillium lilacinus* (syn., *Paecilomyces lilacinus*), with elevated MICs of amphotericin B, ITZ, and echinocandins. Table 4 shows the numbers of *Aspergillus* isolates with MICs of >2 mg/liter for amphotericin B, ITZ, and voriconazole and >0.25 mg/liter for posaconazole. There were no significant differences between MIC values for infectious strains and those for strains isolated from cases defined as colonization ($P > 0.01$).

Analysis of the impact of itraconazole-supplemented medium. Table 5 shows the numbers and percentages of strains growing in Sabouraud and ITZ-supplemented media. Sixty-nine percent of the isolates analyzed were isolated in SAB medium, while 31% were isolated in ITZ-supplemented medium (338 versus 155 isolates).

Resistant isolates (MICs of ITZ of >2 mg/liter) were preferably isolated in ITZ-supplemented medium (15.6% of the total isolates in ITZ-supplemented medium versus 13.1% of the total isolates in Sabouraud medium), although differences were not statistically significant ($P = 0.5$). *Scedosporium-Lomentospora* isolates were preferentially isolated in ITZ-supplemented medium (11% versus 6.5%), as were *Penicillium-Talaromyces* isolates (5.2 versus 4.1%), although differences were not statistically significant compared with the percentages of *Aspergillus* spp. ($P = 0.08$ and $P = 0.5$).

We compared the results obtained in this study with those obtained in the FILPOP1 study, where we analyzed the epidemiology of mold infections in 29 centers in Spain between 2010 and 2011. Although the results cannot be comparable because of important methodological differences, the percentages of *Scedosporium-Lomentospora* spp., *Penicillium/Talaromyces* spp., and *Fusarium* spp. increased in this study compared with the previous one, while the percentages of *Aspergillus* spp. and *Mucorales* decreased (Table 6).

Characterization of resistance mechanisms in *Aspergillus fumigatus*. Three strains of *A. fumigatus sensu stricto* were resistant to azoles: two of them harbored the TR_{34}+L98H mechanism of resistance, and the other one had no mutations in *cyp51A*.

DISCUSSION

The emergence of azole resistance in *A. fumigatus* has been described worldwide, with some European countries showing very high rates (13). These strains have been clinically associated with poorer outcomes (11). Another problem in antifungal resistance is the shift of the epidemiology toward the emergence of intrinsically resistant species, such as *Scedosporium* spp., *Fusarium* spp., or *Mucorales* (4, 15). In addition, in recent years, molecular studies have described new species of fungi that are indistinguishable by classical methods of identification and have been described as cryptic species (16, 17). These cryptic species are more resistant to some of the antifungals available and have been related with higher rates of mortality (18). Cryptic species have been found in clinical samples in percentages higher than those of other considered emerging pathogens, such as *Scedosporium* or *Fusarium* (6, 19).

In a previous study (FILPOP) performed in 30 Spanish hospitals between 2010 and 2011, we found no azole-resistant *A. fumigatus* isolates but a rate of cryptic species of

TABLE 3 Antifungal susceptibility profiles of the species isolated most frequently[a]

Group (no. of isolates) and parameter	MIC (mg/liter)					MEC (mg/liter)		
	AMB	ITZ	VCZ	PCZ	TRB	CPF	MCF	ANF
A. fumigatus (260)								
GM	0.36	0.20	0.47	0.06	2.94	0.35	0.01	0.02
MIC_{50}	0.5	0.12	0.5	0.06	4	0.25	0.015	0.015
MIC_{90}	0.5	0.5	1	0.12	8	1	0.03	0.03
Range	0.015–1	0.03–16	0.12–4	0.015–8	0.25–32	0.004–32	0.003–4	0.007–8
A. niger (26)								
GM	0.25	0.88	0.73	0.14	0.32	0.16	0.01	0.01
MIC_{50}	0.25	0.5	1	0.12	0.25	0.12	0.015	0.007
MIC_{90}	0.5	16	1	0.25	2	1	0.03	0.015
Range	0.12–0.5	0.25–16	0.5–2	0.06–1	0.12–4	0.03–2	0.004–0.06	0.007–0.03
A. flavus (25)								
GM	0.92	0.18	0.74	0.08	0.28	0.66	0.06	0.05
MIC_{50}	1	0.12	0.5	0.12	0.25	0.5	0.06	0.03
MIC_{90}	2	0.5	1	0.25	2	4	0.12	4
Range	0.5–4	0.06–0.5	0.5–2	0.03–0.25	0.03–2	0.12–32	0.015–4	0.007–8
A. terreus (23)								
GM	1.06	0.09	0.70	0.08	0.25	0.65	0.02	0.02
MIC_{50}	1	0.06	0.5	0.06	0.25	0.5	0.015	0.015
MIC_{90}	2	0.5	1	0.5	2	2	0.03	0.03
Range	0.25–4	0.015–16	0.25–8	0.015–16	0.06–2	0.06–32	0.004–4	0.007–8
S. apiospermum (21)								
GM	4.27	2.68	1.64	0.76	12.26	1.69	0.36	0.61
MIC_{50}	4	16	1	0.5	32	1	0.25	2
MIC_{90}	32	16	16	16	32	32	4	8
Range	0.25–32	0.12–16	0.25–16	0.015–16	0.06–32	0.06–32	0.006–4	0.008–8
A. tubingensis (16)								
GM	0.23	0.92	1.14	0.15	0.42	0.22	0.02	0.01
MIC_{50}	0.25	1	1	0.25	0.5	0.25	0.03	0.015
MIC_{90}	0.5	1	2	0.25	2	0.5	0.06	0.03
Range	0.12–1	0.25–16	0.5–2	0.015–0.5	0.06–2	0.06–0.5	0.004–0.12	0.007–0.03
S. boydii (12)								
GM	3.76	5.97	0.75	0.74	28.51	1.77	0.22	0.78
MIC_{50}	4	16	0.5	1	32	2	0.25	2
MIC_{90}	16	16	1	4	32	16	4	8
Range	0.12–32	0.015–16	0.25–16	0.015–16	8–32	0.06–16	0.007–4	0.007–8
A. alliaceus (10)								
GM	22.63	0.10	0.44	0.04	0.35	4.90	0.43	0.49
MIC_{50}	32	0.12	0.5	0.03	1	32	4	8
MIC_{90}	32	0.25	1	0.12	2	32	4	8
Range	8–32	0.03–0.25	0.25–1	0.015–0.12	0.03–2	0.12–32	0.015–4	0.015–8
Fusarium spp. (13)								
GM	2.35	16.00	8.00	10.44	5.22	28.76	4.00	8.00
MIC_{50}	2.00	16.00	8.00	16.00	4.00	32.00	4.00	8.00
MIC_{90}	32.00	16.00	16.00	16.00	32.00	32.00	4.00	8.00
Range	0.5–32	16–16	1–16	1–16	1–32	8–32	4–4	8–8
Mucorales (5)								
GM	0.11	0.33	4.00	0.16	1.49	2.98	1.12	2.28
MIC_{50}	0.12	0.25	4.00	0.12	0.50	32.00	4.00	8.00
MIC_{90}	0.25	1.00	8.00	0.50	32.00	32.00	4.00	8.00
Range	0.03–0.25	0.12–1	2–8	0.06–0.5	0.12–32	0.06–32	0.007–4	0.015–8
All (493)								
GM	0.62	0.35	0.71	0.11	1.87	0.58	0.03	0.04
MIC_{50}	0.5	0.25	0.5	0.06	2	0.5	0.015	0.015
MIC_{90}	4	16	4	1	16	8	4	8
Range	0.015–32	0.015–16	0.015–16	0.015–16	0.03–32	0.012–32	0.0015–4	0.007–8

[a]AMB, amphotericin B; TRB, terbinafine; VCZ, voriconazole; PCZ, posaconazole; CPF, caspofungin; MCF, micafungin; ANF, anidulafungin.

TABLE 4 Numbers of *Aspergillus* species strains resistant *in vitro* to amphotericin B, itraconazole, voriconazole, and posaconazole according to EUCAST breakpoints

Group	Total no. of isolates	No. of isolates resistant to:			
		AMB at >2 mg/liter	ITZ at >2 mg/liter	VCZ at >2 mg/liter	PCZ at >0.25 mg/liter
A. fumigatus	260	2	3	2	3
A. lentulus	6	3		4	
A. fumigatiaffinis	2	2			
Aspergillus felis	1			1	
A. niger	26		4		2
A. tubingensis	16		1		1
A. flavus	25	1			
A. alliaceus	10	10			
Aspergillus tamarii	1				
A. terreus	23	2	1	1	3
A. citrinoterreus	2				
A. nidulans	6				
A. quadrilineatus	5	1	1	1	1
Aspergillus delacroxii	1	1	1		
Aspergillus spinulosporus	1				
A. calidoustus	5	1	5	5	4
A. puniceus	1	1	1	1	1
A. sydowii	4				
Aspergillus chevalieri	1				
Total	396	24	17	15	15

15% (6). In this work, we have used ITZ-supplemented plates to screen for azole resistance. Thus, 3 out of 260 (1.2%) *A. fumigatus* isolates analyzed were resistant to azoles; 2 of these strains harbor the most frequent mechanism of azole resistance (TR_{34}+L98H), while the other strain showed no mutations in *cyp51A*. The TR_{34}+L98H mechanism of resistance has been linked to the use of azoles in agriculture and is the most frequent mechanism of azole resistance worldwide (20). Other mechanisms of resistance related to mutations in *cyp51A*, such as TR_{46}/Y121F/T289A and G448S, have been described in isolates from Spain (21, 22) but were not found in this work. One out of the three azole-resistant *A. fumigatus* isolates had no mutations in *cyp51A*. Azole-resistant isolates with no mutations in *cyp51A* were described previously (23, 24). Other mechanisms of azole resistance could be present and will be further analyzed in this isolate.

The main sources of resistance in our isolates were cryptic species of *Aspergillus* and emerging molds such as *Fusarium*, *Scedosporium*, and *Mucorales*. Breakpoints for these species have not been defined; however, infections of patients with these pathogens are associated with poorer outcomes (25).

Cryptic species of *Aspergillus* accounted for 11.5% of the total number of isolates. Among members of the *A. fumigatus* complex, *A. lentulus* and *A. fumigatiaffinis* showed high MICs of amphotericin B and voriconazole, as reported previously (26, 27). Within the *A. niger* complex, *A. niger* and *A. tubingensis* were isolated in this study. In accordance with

TABLE 5 Numbers and percentages of strains isolated in each medium

Group	No. (%) of strains isolated in:	
	SAB medium	ITZ-supplemented medium
Aspergillus spp.	277 (82.0)	119 (76.8)
Fusarium spp.	9 (2.7)	4 (2.6)
Scedosporium-Lomentospora spp.	22 (6.5)	17 (11.0)
Penicillium-Talaromyces spp.	14 (4.1)	8 (5.2)
Other genera	16 (4.7)	7 (4.5)
All	338 (100)	155 (100)
Resistant[a]	44 (13.1)	24 (15.6)
Susceptible	292 (86.9)	130 (84.4)

[a]Strains were classified as susceptible or resistant according to *A. fumigatus* breakpoints for itraconazole. Three isolates were not included in this group since they did not grow for antifungal susceptibility testing.

TABLE 6 Numbers and percentages of strains isolated in the FILPOP1 and FILPOP2 studies by group of species

Group	No. (%) of isolates	
	FILPOP1[a]	FILPOP2[b]
Aspergillus spp.	278 (86.3)	396 (80.32)
Scedosporium-Lomentospora spp.	15 (4.7)	39 (7.91)
Mucorales	12 (3.7)	5 (1.01)
Penicillium-Talaromyces spp.	7 (2.2)	22 (4.46)
Fusarium spp.	4 (1.2)	13 (2.64)
Others	6 (1.9)	18 (3.65)
Total	322 (100)	493 (100)

[a]See reference 6.
[b]This study.

the results obtained here, previous works reported that susceptibility within this group is variable and strain dependent (28, 29). *Aspergillus terreus* and *A. flavus* complex isolates have been associated with higher MICs of amphotericin B. In this work, the ranges of MICs of amphotericin B were 0.25 to 2 mg/liter for *A. flavus* and 0.25 to 4 for *A. terreus*. Two out of 23 isolates of *A. terreus* showed MICs of 4 mg/liter, while all isolates of *Aspergillus alliaceus* (*A. flavus* complex) showed MICs of >4 mg/liter for amphotericin B, in accordance with previous results (6, 27, 30). *Aspergillus citrinoterreus* (*A. terreus* complex) isolates have been reported to be more susceptible to itraconazole, voriconazole, and posaconazole than *A. terreus sensu stricto* isolates, both of which have high amphotericin B MICs (31). In this study, two isolates of *A. citrinoterreus* were found, with no differences among susceptibilities of *A. terreus* isolates. *Aspergillus ustus* complex isolates have been associated with high MICs of all antifungals (6, 17, 27, 32). Among the species of this complex, we isolated five *Aspergillus calidoustus* isolates and one *Aspergillus puniceus* isolate, with all of them being resistant to azoles and echinocandins and with amphotericin B being the only compound with some activity.

Species of *Scedosporium-Lomentospora* represented almost 8% of our total number of strains. *Scedosporium* species had elevated MICs of amphotericin B and ITZ, with the most active antifungals being voriconazole and echinocandins. *Lomentospora prolificans* (syn., *Scedosporium prolificans*) is panresistant, with no antifungal showing an *in vitro* effect. These results are in accordance with those of previous studies (33, 34).

Fusarium species accounted for 2.6% of the total number of isolates and showed elevated MICs of all antifungals. The echinocandins and azoles had no activity; the only antifungal compound with low MICs against some strains was amphotericin B. Other authors have reported different patterns according to the species complexes; thus, *Fusarium solani* isolates are usually resistant to azoles and show higher MICs of amphotericin B than other species, whereas *Fusarium oxysporum* and *Fusarium verticillioides* can be susceptible to voriconazole and posaconazole (35). In our study, most isolates (9 out of 13) were identified as *Fusarium proliferatum* (*Fusarium fujikuroi* complex); a study analyzing 81 strains of the *Fusarium fujikuroi* complex found that amphotericin B was the most active drug, followed by voriconazole, posaconazole, isavuconazole, and natamycin, while fluconazole, itraconazole, and micafungin showed poor activity (36). Our isolates showed high MICs (>2 mg/liter) of all antifungals but amphotericin B, with only one strain showing an MIC of >2 mg/liter, in accordance with results reported previously (37).

Unexpectedly, in this study, we could not find statistical differences in the detection of resistant isolates when using ITZ-supplemented plates. Only *Scedosporium-Lomentospora* isolates showed high percentages of isolation in ITZ-supplemented medium compared with the other species. However, when we compare the results obtained in the FILPOP1 study (6) with those obtained in this one (Table 6), we see that the percentages of *Scedosporium-Lomentospora* species, *Penicillium-Talaromyces* species, and *Fusarium* species isolates increased, while the percentage of *Aspergillus*

species and *Mucorales* isolates decreased. Although the results are not comparable because of important methodological differences (the numbers of participating centers and the numbers of strains analyzed, etc.), this could indicate that selective medium is favoring the isolation of rare species by decreasing the rate of recovery of fast-growing species such as *Aspergillus* spp. and *Mucorales*. This is in agreement with previous works where selective media have been recommended for the isolation of *Scedosporium* species (38–40).

In conclusion, this study shows that antifungal resistance is present in Spain. The level of azole resistance in *A. fumigatus* remains low, but cryptic species represent over 10% of the isolates and have different patterns of antifungal resistance. Apart from *Aspergillus*, other emerging molds, such as *Scedosporium-Lomentospora*, *Fusarium*, and *Mucorales*, showed high MICs of several antifungals. Taking into account these results and the impact of appropriate antifungal treatment on survival, we recommend screening for antifungal resistance and performing antifungal susceptibility testing for all isolates coming from sterile sites in order to determine the best treatment option for patients infected with these pathogens.

MATERIALS AND METHODS

Strains and isolates. A total of 500 Sabouraud (SAB; Oxoid SA, Madrid, Spain) plates supplemented with 2 mg/liter of ITZ (Sigma-Aldrich Química, Madrid, Spain) were sent to 10 Spanish tertiary hospitals from different regions in Spain: Gregorio Marañón (1,525 beds; Madrid, Madrid), La Paz (1,524 beds; Madrid, Madrid), Virgen de Valme (605 beds; Seville, Andalusia), Reina Sofía (1,233 beds; Córdoba, Andalusia), La Fe (1,050 beds; Valencia, Valencia), Donostia (1,054 beds; Guipúzcoa, Basque Country), Vall d'Hebron (1,251 beds; Barcelona, Catalonia), Bellvitge (1,022 beds; Barcelona, Catalonia), Central de Asturias (989 beds; Oviedo, Asturias), and Miguel Servet (1,234 beds; Zaragoza, Aragon). The number of beds in each hospital was determined according to a report from 2017 with current numbers at the end of 2016 (41). Samples from respiratory secretions, biopsy specimens, and other sterile sites were included in the study. The samples were cultured in classical media and in 2 mg/liter ITZ-supplemented medium.

All samples positive for filamentous fungi were sent to the Mycology Reference Laboratory (RL) of the Spanish National Center of Microbiology for identification and antifungal susceptibility testing.

Clinical data. Basic clinical data, such as source of isolation, underlying disease, antifungal treatment, and outcome of the patient, were gathered when possible. Study approval was obtained from the research ethics committee of the Instituto de Salud Carlos III, with reference number CEI PI56_2014.

The cases of invasive fungal diseases were classified as proven and probable infections according to European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) criteria (42); we included colonization as a third category in cases where infection could not be confirmed but a clinically relevant isolate was detected. Cases that could not be classified according to these criteria were defined as colonization.

Morphological identification. At the RL, the strains were subcultured in different media to ascertain their macroscopic and microscopic morphology. The media included malt extract agar (MEA) (2% malt extract; Oxoid SA, Madrid, Spain), potato dextrose agar (PDA; Oxoid SA), oatmeal agar (OMA; Oxoid SA), potassium chloride agar (Oxoid SA), and Czapek-Dox agar (Difco, Soria Melguizo SA, Madrid, Spain). Cultures were incubated at 30°C and 37°C. Fungal morphological features were examined macro- and microscopically by conventional methods (43).

Molecular identification. Molds were subcultured in glucose-yeast extract-peptone (GYEP) medium (0.3% yeast extract, 1% peptone; Difco, Soria Melguizo) with 2% glucose (Sigma-Aldrich Química, Madrid, Spain) for 24 to 48 h at 30°C. Genomic DNA was isolated by using an extraction procedure described previously (44). Molecular identification was performed by sequencing informative targets. DNA segments comprising the internal transcribed spacer 1 (ITS1) and ITS2 regions were amplified for all the strains with primer set ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (45). In the case of *Aspergillus* and *Scedosporium* isolates, a portion of the beta tubulin gene was sequenced with the following primers: βtub3 (5′-TTCACCTTCAGACCGGT-3′) and βtub2 (5′-AGTTGTCG GGACGGAATAG-3′) (16) for *Aspergillus* and TUB-F (5′-CTGTCCAACCCCTCTTACGGCGACCTGAAC-3′) and TUB-R (5′-ACCCTCACCAGTATACCAATGCAAGAAAGC-3′) (46) for *Scedosporium*. Also, DNA segments comprising the elongation factor alpha region were amplified for *Fusarium* isolates with primers EF1 (5′-ATGGGTAAGARGACAAGAC-3′) and EF2 (5′-GGARGTACCAGTSATCATGTT-3′) (47). All primers were synthesized by Sigma Genosys (Madrid, Spain). The reactions were performed in a GeneAmp 9700 PCR system (Applied Biosystems) under conditions described previously (6). Sequencing reactions were done with 2 μl of a sequencing kit reagent (BigDye Terminator cycle sequencing, ready reaction; Applied Biosystems), 1 μM primers (the same as for PCR, except that *Aspergillus* β-tubulin primers βtub1 [5′-AATTGGTGCCGCTTTCTGG-3′] and βtub4 [5′-AGCGTCCATGGTACCAGG-3′] were used), and 3 μl of the PCR product in a final volume of 10 μl.

Sequences were assembled and edited by using the SeqMan II and EditSeq software packages (Lasergene; DNAStar, Inc., Madison, WI, USA). All sequences were compared with reference sequences from the GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) and MycoBank (http://www.MycoBank.org/) databases with InfoQuest FP software, version 4.50 (Bio-Rad Laboratories, Madrid, Spain), as well as

with the database belonging to the Department of Mycology of the Spanish National Centre for Microbiology, which holds 13,000 sequences from strains belonging to 290 different fungal species. This database was designed by the Spanish National Centre for Microbiology and has restricted access (26, 34, 37, 48).

Antifungal susceptibility testing. Microdilution testing was performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard methodology (49). *A. fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains. The antifungal agents used in the study were amphotericin B (Sigma-Aldrich Química, Madrid, Spain), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer SA, Madrid, Spain), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ), terbinafine (Novartis, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, NJ), micafungin (Astellas Pharma, Inc., Tokyo, Japan), and anidulafungin (Pfizer SA, Madrid, Spain). The final concentrations tested ranged from 0.03 to 16 mg/liter for amphotericin B, terbinafine, and caspofungin; from 0.015 to 8 mg/liter for itraconazole, voriconazole, and posaconazole; from 0.007 to 4 mg/liter for anidulafungin; and from 0.004 to 2 mg/liter for micafungin. The plates were incubated at 35°C for 48 h in a humid atmosphere. Visual readings were performed at 24 and 48 h with the help of a mirror. The endpoint for amphotericin B, itraconazole, voriconazole, posaconazole, and terbinafine was the antifungal concentration that produced a complete inhibition of visual growth at 24 and 48 h (MIC). For the echinocandins, the endpoint was the antifungal concentration that produced a visible change in the morphology of the hyphae compared with the growth control well (minimum effective concentration [MEC]). EUCAST has set breakpoints to interpret antifungal susceptibility testing results for amphotericin B (resistant strain MIC value of >2 mg/liter), itraconazole (MIC > 2 mg/liter), voriconazole (MIC > 2 mg/liter), and posaconazole (MIC > 0.25 mg/liter) (50). These breakpoint values have been set for only some *Aspergillus* spp. but were used in this study to analyze rates of resistance *in vitro* for all *Aspergillus* species. Breakpoints of echinocandins and terbinafine have not been set yet, and rates of resistance were not calculated.

Analysis of the impact of itraconazole-supplemented medium. We investigated the impact of the supplemented medium by calculating the percentage of isolates growing in each medium overall and the most frequently isolated genera. In addition, isolates were classified as susceptible or resistant to ITZ by using the existing breakpoint for *A. fumigatus*; thus, isolates with an MIC of >2 mg/liter were classified as resistant, and the rest were classified as susceptible. One *Aspergillus nidulans* isolate showed an MIC of 2 mg/liter; although it should be classified as intermediate according to EUCAST breakpoints, we include it in susceptible group for practical purposes.

Characterization of resistance mechanisms in *Aspergillus fumigatus*. *A. fumigatus* isolates showing MICs over the breakpoint for resistance (>2 mg/liter for ITZ and voriconazole and >0.25 mg/liter for posaconazole) were studied for mutations in the *cyp51A* gene. The *cyp51A* gene, including its promoter region, was amplified and sequenced according to procedures described previously (51) for the detection of specific mutations associated with azole resistance.

Statistical analysis. Descriptive and comparative analyses were done. Differences in the proportions of fungal species were determined by Fisher's exact test or by chi-square analysis. The significance of the differences between MICs was determined by analysis of variance (with Bonferroni's *post hoc* test) or by nonparametric tests. A P value of <0.01 was considered statistically significant. Statistical analysis was performed with IBM SPSS Statistics 19.0 (SPSS Iberica, Madrid, Spain).

ACKNOWLEDGMENTS

This study was supported by an investigator-sponsored research grant from Gilead Sciences (IN-ES-131-1600). A.A.-I. is supported by a research project from the Fondo de Investigación Sanitaria (PI13/02145 and PI16CIII/00035); she has received research grants or honoraria as a speaker or advisor from Gilead Sciences, F2G, and Scynexis. O.R.-M. holds a predoctoral fellowship from the Fondo de Investigación Sanitaria (FI14CIII/00025). M.T.M.-G. has acted as invited speaker in meetings sponsored by Pfizer and MSD. T.P. is supported by a project from the Fondo de Investigación Sanitaria (PI13/02783). J.P. has received honoraria as a speaker for Pfizer, MSD, Astellas, and Gilead. J.V. has received travel grants from MSD, Becton Dickinson, and Beckman. M.C.-E. has received grant support from Astellas, bioMérieux, Gilead Sciences, Merck Sharp & Dohme, Pfizer, Schering Plough, Soria Melguizo, Ferrer International, CIDARA, F2G, Basilea, Amplyx, and Scynexis.

We thank Cristina Armentia and Teresa Merino for technical assistance.

REFERENCES

1. Pagano L, Akova M, Dimopoulos G, Herbrecht R, Drgona L, Blijlevens N. 2011. Risk assessment and prognostic factors for mould-related diseases in immunocompromised patients. J Antimicrob Chemother 66(Suppl 1):i5–i14. https://doi.org/10.1093/jac/dkq437.
2. Akan H, Antia VP, Kouba M, Sinko J, Tanase AD, Vrhovac R, Herbrecht R. 2013. Preventing invasive fungal disease in patients with haematological malignancies and the recipients of haematopoietic stem cell transplantation: practical aspects. J Antimicrob Chemother 68(Suppl 3):iii5–iii16. https://doi.org/10.1093/jac/dkt389.
3. Shoham S. 2013. Emerging fungal infections in solid organ transplant recipients. Infect Dis Clin North Am 27:305–316. https://doi.org/10.1016/j.idc.2013.02.004.

4. Malani AN, Kauffman CA. 2007. Changing epidemiology of rare mould infections: implications for therapy. Drugs 67:1803–1812. https://doi.org/10.2165/00003495-200767130-00001.
5. Kanafani ZA, Perfect JR. 2008. Antimicrobial resistance. Resistance to antifungal agents: mechanisms and clinical impact. Clin Infect Dis 46:120–128. https://doi.org/10.1086/524071.
6. Alastruey-Izquierdo A, Mellado E, Pelaez T, Peman J, Zapico S, Alvarez M, Rodriguez-Tudela JL, Cuenca-Estrella M. 2013. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP study). Antimicrob Agents Chemother 57:3380–3387. https://doi.org/10.1128/AAC.00383-13.
7. Pappas PG, Alexander BD, Andes DR, Hadley S, Kauffman CA, Freifeld A, Anaissie EJ, Brumble LM, Herwaldt L, Ito J, Kontoyiannis DP, Lyon GM, Marr KA, Morrison VA, Park BJ, Patterson TF, Perl TM, Oster RA, Schuster MG, Walker R, Walsh TJ, Wannemuehler KA, Chiller TM. 2010. Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Clin Infect Dis 50:1101–1111. https://doi.org/10.1086/651262.
8. Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. 31 July 2017. The global problem of antifungal resistance: prevalence, mechanisms, and management. Lancet Infect Dis https://doi.org/10.1016/S1473-3099(17)30316-X.
9. Verweij PE, Mellado E, Melchers WJ. 2007. Multiple-triazole-resistant aspergillosis. N Engl J Med 356:1481–1483. https://doi.org/10.1056/NEJMc061720.
10. Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, Laverdiere M, Arendrup MC, Perlin DS, Denning DW. 2009. Frequency and evolution of azole resistance in Aspergillus fumigatus associated with treatment failure. Emerg Infect Dis 15:1068–1076. https://doi.org/10.3201/eid1507.090043.
11. van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, Kuijper EJ, Van Tiel FH, Melchers WJ, Verweij PE. 2011. Clinical implications of azole resistance in Aspergillus fumigatus, The Netherlands, 2007-2009. Emerg Infect Dis 17:1846–1854. https://doi.org/10.3201/eid1710.110226.
12. Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, Denning DW. 2010. Azole antifungal resistance in Aspergillus fumigatus: 2008 and 2009. J Antimicrob Chemother 65:2116–2118. https://doi.org/10.1093/jac/dkq279.
13. Rivero-Menendez O, Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M. 2016. Triazole resistance in Aspergillus spp.: a worldwide problem? J Fungi 2:E21. https://doi.org/10.3390/jof2030021.
14. Verweij PE, Chowdhary A, Melchers WJ, Meis JF. 2016. Azole resistance in Aspergillus fumigatus: can we retain the clinical use of mold-active antifungal azoles? Clin Infect Dis 62:362–368. https://doi.org/10.1093/cid/civ885.
15. Richardson M, Lass-Florl C. 2008. Changing epidemiology of systemic fungal infections. Clin Microbiol Infect 14(Suppl 4):S5–S24. https://doi.org/10.1111/j.1469-0691.2008.01978.x.
16. Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA. 2005. Aspergillus lentulus sp. nov., a new sibling species of A. fumigatus. Eukaryot Cell 4:625–632. https://doi.org/10.1128/EC.4.3.625-632.2005.
17. Varga J, Houbraken J, Van Der Lee HA, Verweij PE, Samson RA. 2008. Aspergillus calidoustus sp. nov., causative agent of human infections previously assigned to Aspergillus ustus. Eukaryot Cell 7:630–638. https://doi.org/10.1128/EC.00425-07.
18. Zbinden A, Imhof A, Wilhelm MJ, Ruschitzka F, Wild P, Bloemberg GV, Mueller NJ. 2012. Fatal outcome after heart transplantation caused by Aspergillus lentulus. Transpl Infect Dis 14:E60–E63. https://doi.org/10.1111/j.1399-3062.2012.00779.x.
19. Balajee SA, Kano R, Baddley JW, Moser SA, Marr KA, Alexander BD, Andes D, Kontoyiannis DP, Perrone G, Peterson S, Brandt ME, Pappas PG, Chiller T. 2009. Molecular identification of Aspergillus species collected for the Transplant-Associated Infection Surveillance Network. J Clin Microbiol 47:3138–3141. https://doi.org/10.1128/JCM.01070-09.
20. Chowdhary A, Kathuria S, Xu J, Meis JF. 2013. Emergence of azole-resistant Aspergillus fumigatus strains due to agricultural azole use creates an increasing threat to human health. PLoS Pathog 9:e1003633. https://doi.org/10.1371/journal.ppat.1003633.
21. Pelaez T, Monteiro MC, Garcia-Rubio R, Bouza E, Gomez-Lopez A, Mellado E. 2015. First detection of Aspergillus fumigatus azole-resistant strain due to cyp51A TR46/Y121F/T289A in an azole-naive patient in Spain. New Microbes New Infect 6:33–34. https://doi.org/10.1016/j.nmni.2015.04.005.
22. Pelaez T, Gijon P, Bunsow E, Bouza E, Sanchez-Yebra W, Valerio M, Gama B, Cuenca-Estrella M, Mellado E. 2012. Resistance to voriconazole due to a G448S substitution in Aspergillus fumigatus in a patient with cerebral aspergillosis. J Clin Microbiol 50:2531–2534. https://doi.org/10.1128/JCM.00329-12.
23. Arendrup MC, Mavridou E, Mortensen KL, Snelders E, Frimodt-Moller N, Khan H, Melchers WJ, Verweij PE. 2010. Development of azole resistance in Aspergillus fumigatus during azole therapy associated with change in virulence. PLoS One 5:e10080. https://doi.org/10.1371/journal.pone.0010080.
24. Denning DW, Park S, Lass-Florl C, Fraczek MG, Kirwan M, Gore R, Smith J, Bueid A, Moore CB, Bowyer P, Perlin DS. 2011. High-frequency triazole resistance found in nonculturable Aspergillus fumigatus from lungs of patients with chronic fungal disease. Clin Infect Dis 52:1123–1129. https://doi.org/10.1093/cid/cir179.
25. Nucci M. 2003. Emerging moulds: Fusarium, Scedosporium and Zygomycetes in transplant recipients. Curr Opin Infect Dis 16:607–612. https://doi.org/10.1097/00001432-200312000-00015.
26. Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A, Cuenca-Estrella M, Rodriguez-Tudela JL. 2008. Aspergillus section Fumigati: antifungal susceptibility patterns and sequence-based identification. Antimicrob Agents Chemother 52:1244–1251. https://doi.org/10.1128/AAC.00942-07.
27. Alastruey-Izquierdo A, Alcazar-Fuoli L, Cuenca-Estrella M. 2014. Antifungal susceptibility profile of cryptic species of Aspergillus. Mycopathologia 178:427–433. https://doi.org/10.1007/s11046-014-9775-z.
28. Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A, Cuenca-Estrella M, Rodriguez-Tudela JL. 2009. Species identification and antifungal susceptibility patterns of species belonging to Aspergillus section Nigri. Antimicrob Agents Chemother 53:4514–4517. https://doi.org/10.1128/AAC.00585-09.
29. Howard SJ, Harrison E, Bowyer P, Varga J, Denning DW. 2011. Cryptic species and azole resistance in the Aspergillus niger complex. Antimicrob Agents Chemother 55:4802–4809. https://doi.org/10.1128/AAC.00304-11.
30. Ozhak-Baysan B, Alastruey-Izquierdo A, Saba R, Ogunc D, Ongut G, Timuragaoglu A, Arslan G, Cuenca-Estrella M, Rodriguez-Tudela JL. 2010. Aspergillus alliaceus and Aspergillus flavus co-infection in an acute myeloid leukemia patient. Med Mycol 48:995–999. https://doi.org/10.3109/13693781003749418.
31. Guinea J, Sandoval-Denis M, Escribano P, Pelaez T, Guarro J, Bouza E. 2015. Aspergillus citrinoterreus, a new species of section Terrei isolated from samples of patients with nonhematological predisposing conditions. J Clin Microbiol 53:611–617. https://doi.org/10.1128/JCM.03088-14.
32. Alastruey-Izquierdo A, Cuesta I, Houbraken J, Cuenca-Estrella M, Monzon A, Rodriguez-Tudela JL. 2010. In vitro activity of nine antifungal agents against clinical isolates of Aspergillus calidoustus. Med Mycol 48:97–102. https://doi.org/10.3109/13693780902803040.
33. Lackner M, de Hoog GS, Verweij PE, Najafzadeh MJ, Curfs-Breuker I, Klaassen CH, Meis JF. 2012. Species-specific antifungal susceptibility patterns of Scedosporium and Pseudallescheria species. Antimicrob Agents Chemother 56:2635–2642. https://doi.org/10.1128/AAC.05910-11.
34. Alastruey-Izquierdo A, Cuenca-Estrella M, Monzon A, Rodriguez-Tudela JL. 2007. Prevalence and susceptibility testing of new species of Pseudallescheria and Scedosporium in a collection of clinical mold isolates. Antimicrob Agents Chemother 51:748–751. https://doi.org/10.1128/AAC.01177-06.
35. Al-Hatmi A, Curfs-Breuker I, de Hoog G, Meis J, Verweij P. 2017. Antifungal susceptibility testing of Fusarium: a practical approach. J Fungi 3:E19. https://doi.org/10.3390/jof3020019.
36. Al-Hatmi AM, van Diepeningen AD, Curfs-Breuker I, de Hoog GS, Meis JF. 2015. Specific antifungal susceptibility profiles of opportunists in the Fusarium fujikuroi complex. J Antimicrob Chemother 70:1068–1071. https://doi.org/10.1093/jac/dku505.
37. Alastruey-Izquierdo A, Cuenca-Estrella M, Monzon A, Mellado E, Rodriguez-Tudela JL. 2008. Antifungal susceptibility profile of clinical Fusarium spp. isolates identified by molecular methods. J Antimicrob Chemother 61:805–809. https://doi.org/10.1093/jac/dkn022.
38. Tortorano AM, Richardson M, Roilides E, van Diepeningen A, Caira M, Munoz P, Johnson E, Meletiadis J, Pana ZD, Lackner M, Verweij P, Freiberger T, Cornely OA, Arikan-Akdagli S, Dannaoui E, Groll AH, Lagrou K, Chakrabarti A, Lanternier F, Pagano L, Skiada A, Akova M, Arendrup MC, Boekhout T, Chowdhary A, Cuenca-Estrella M, Guinea J, Guarro J, de Hoog S, Hope W, Kathuria S, Lortholary O, Meis JF, Ullmann AJ, Petrikkos

G, Lass-Florl C, European Society of Clinical Microbiology and Infectious Diseases Fungal Infection Study Group, European Confederation of Medical Mycology. 2014. ESCMID and ECMM joint guidelines on diagnosis and management of hyalohyphomycosis: Fusarium spp., Scedosporium spp. and others. Clin Microbiol Infect 20(Suppl 3):S27–S46. https://doi.org/10.1111/1469-0691.12465.

39. Pham T, Giraud S, Schuliar G, Rougeron A, Bouchara JP. 2015. Scedo-Select III: a new semi-selective culture medium for detection of the Scedosporium apiospermum species complex. Med Mycol 53:512–519. https://doi.org/10.1093/mmy/myv015.
40. Rainer J, Kaltseis J, de Hoog SG, Summerbell RC. 2008. Efficacy of a selective isolation procedure for members of the Pseudallescheria boydii complex. Antonie Van Leeuwenhoek 93:315–322. https://doi.org/10.1007/s10482-007-9206-y.
41. Ministerio de Sanidad, Servicios Sociales e Iguladid. 2017. Catálogo nacional de hospitales 2017. Ministerio de Sanidad, Servicios Sociales e Iguladid, Madrid, Spain. https://www.msssi.gob.es/ciudadanos/prestaciones/centrosServiciosSNS/hospitales/docs/CNH2015.pdf.
42. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Munoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE. 2008. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 46:1813–1821. https://doi.org/10.1086/588660.
43. de Hoog GS, Guarro J, Tran CS, Wintermans RGF, Gene J. 1995. Hyphomycetes, p 380–1007. *In* de Hoog GS, Guarro J (ed), Atlas of clinical fungi. John Wiley & Sons, Chichester, England.
44. Holden DW. 1994. DNA mini prep method for Aspergillus fumigatus (and other filamentous fungi), p 3–4. Telos Press, New York, NY.
45. White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p 315–322. *In* Innis MA, Gelfand DH (ed), PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA.
46. Cruse M, Telerant R, Gallagher T, Lee T, Taylor JW. 2002. Cryptic species in Stachybotrys chartarum. Mycologia 94:814–822. https://doi.org/10.1080/15572536.2003.11833175.
47. Kristensen R, Torp M, Kosiak B, Holst-Jensen A. 2005. Phylogeny and toxigenic potential is correlated in Fusarium species as revealed by partial translation elongation factor 1 alpha gene sequences. Mycol Res 109:173–186. https://doi.org/10.1017/S0953756204002114.
48. Alastruey-Izquierdo A, Castelli MV, Cuesta I, Monzon A, Cuenca-Estrella M, Rodriguez-Tudela JL. 2009. Activity of posaconazole and other antifungal agents against *Mucorales* strains identified by sequencing of internal transcribed spacers. Antimicrob Agents Chemother 53:1686–1689. https://doi.org/10.1128/AAC.01467-08.
49. Arendrup MC, Meletiadis J, Mouton JW, Lagrou K, Hamal P, Guinea J, Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing. 2017. EUCAST definitive document E.DEF 9.3.1: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9_3_1_Mould_testing__definitive.pdf. Accessed 25 February 2018.
50. EUCAST. 2018. Antifungal breakpoint tables for interpretation of MICs v 9.0. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/Antifungal_breakpoints_v_9.0_180212.pdf. Accessed 25 February 2018.
51. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Melchers WJ, Verweij PE, Cuenca-Estrella M, Rodriguez-Tudela JL. 2007. A new Aspergillus fumigatus resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations. Antimicrob Agents Chemother 51:1897–1904. https://doi.org/10.1128/AAC.01092-06.

Journal of *Fungi*

MDPI

Review

Triazole Resistance in *Aspergillus* spp.: A Worldwide Problem?

Olga Rivero-Menendez [1,2], Ana Alastruey-Izquierdo [1,2,*], Emilia Mellado [1,2] and Manuel Cuenca-Estrella [1,2]

1 Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III., Carretera de Majadahonda a Pozuelo Km. 2, Majadahonda, 28220 Madrid, Spain; orivero@isciii.es (O.R.-M.); emellado@isciii.es (E.M.); mcuenca-estrella@isciii.es (M.-C.E.)

2 Spanish Network for Research in Infectious Diseases (REIPI RD12/0015)—co-financed by European Development Regional Fund "A way to achieve Europe" ERDF, Madrid, Spain

* Correspondence: anaalastruey@isciii.es; Tel.: +34-918223784

Academic Editor: William J. Steinbach
Received: 1 May 2016; Accepted: 24 June 2016; Published: 4 July 2016

Abstract: Since the first description of an azole-resistant *A. fumigatus* strain in 1997, there has been an increasing number of papers describing the emergence of azole resistance. Firstly reported in the USA and soon after in Europe, it has now been described worldwide, challenging the management of human aspergillosis. The main mechanism of resistance is the modification of the azole target enzyme: 14-α sterol demethylase, encoded by the *cyp*51A gene; although recently, other resistance mechanisms have also been implicated. In addition, a shift in the epidemiology has been noted with other *Aspergillus* species (mostly azole resistant) increasingly being reported as causative agents of human disease. This paper reviews the current situation of *Aspergillus* azole resistance and its implications in the clinical setting.

Keywords: *Aspergillus fumigatus*; aspergillosis; azole drug resistance; *cyp*51A; mutations

1. Introduction

Invasive aspergillosis (IA) is a life-threatening infection caused by ubiquitous saprophytic *Aspergillus* species, which are the most common cause of invasive mold infections worldwide, especially in immunocompromised patients [1]. *Aspergillus fumigatus* is the leading agent of IA [2] but also of all other forms of aspergillosis, including allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) and aspergilloma [3]. This fungus produces billions of airborne conidia due to an abundant asexual reproduction cycle and has the ability of surviving in very different environments, such as those with temperatures up to 60 °C [4].

Despite the mortality and morbidity of IA remaining high due mainly to difficulties in early diagnosis, the survival rates of these patients have improved due to advances in diagnostics and treatment. The triazoles, itraconazole (ITC), voriconazole (VRC) and posaconazole (POS), are the mainstay of treatment for aspergillosis. Isavuconazole is a new extended-spectrum triazole, and its activity against *Aspergillus* has been proven [5]. Triazoles are the only anti-*Aspergillus* agents that are orally available, making them essential for long-term therapy [6]. Although VRC is recommended as first-line therapy for IA [7,8], ITC is still commonly used for chronic and allergic non-invasive forms of aspergillosis [8,9], and POS was shown to reduce the number of invasive fungal infections in neutropenic patients [10]. Additionally, there are some alternative therapies to triazoles that can function as rescue treatments, such as echinocandins or amphotericin B [8].

2. Antifungal Susceptibility Testing and Azole Resistance within *Aspergillus fumigatus*

The Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have developed reference methods to test antifungal susceptibility, which allow the detection of in vitro resistance nowadays. Both committees defined wild-type (WT) MIC (minimum inhibitory concentration) distributions in order to establish epidemiologic cutoff values (ECVs) for *A. fumigatus* and azoles [11,12]. Based on these data and taking into account the clinical outcome, pharmacokinetics and pharmacodynamics, EUCAST defined breakpoints for *A. fumigatus* and azoles (ITC > 2 μg/mL, VRC > 2 μg/mL, POS > 0.25 μg/mL and ISA > 1 μg/mL), which are used to categorize *A. fumigatus* strains as susceptible or resistant [13]. CLSI has also defined ECVs for *A. fumigatus* and azoles: ITC > 1 μg/mL, VRC > 1 μg/mL, POS > 0.5 μg/mL [11]. There are other commercial methods for in vitro susceptibility testing, such as Etest (BioMerieux, Marcy l'Etoile, France) or Sensititre YeastOne (SYO) (Trek Diagnostic Systems Ltd., East Grinstead, UK), that are complementary to EUCAST and CLSI and are easy to perform for routine use.

Since the first reported case in 1997 in clinical *A. fumigatus* isolates collected in the 1980s in the U.S. [14], an ever-growing number of triazole resistance strains have been published [1,6]. The increased description of azole-resistant *A. fumigatus* strains in the last few years may pose a threat to public health because of the lack of alternative treatment [15,16]. In addition, as in vitro antifungal susceptibility testing in *Aspergillus* is not routinely done in non-invasive settings, the prevalence of triazole resistance strains is likely to be underestimated [6].

3. Azole Resistance Development in *Aspergillus fumigatus*

Aspergillus fumigatus is usually susceptible to azoles, but as stated before, secondary resistance is increasingly reported. Since the first azole-resistant isolate detected in 1997 in the U.S., azole resistance has been increasingly reported from many other countries. Particularly in the past few years, there has been an increase in clinical resistant isolates described from the Netherlands [6]. This is well studied at the molecular level and will be further discussed in this review. The development of secondary resistance is thought to be acquired in two possible ways. In patients that suffer chronic aspergillosis and are under long-term azole treatment, resistance can develop through this exposure [17]. These patients are initially infected by a susceptible *A. fumigatus* strain that evolves to a resistant phenotype under azole treatment pressure. These resistant isolates are isogenic to the initial one that caused the infection. Camps et al. reviewed seven cases of acquired resistance during treatment showing an average delay of four months between the latest susceptible and the first resistant isolate [18]. The first reported case of this resistance route was described in 2001 in four isogenic *A. fumigatus* isolates recovered from a patient treated with ITC for a pulmonary *A. fumigatus* infection. Two of them were obtained before treatment with ITC, and two were isolated after treatment finished. The results suggested that the strain acquired resistance to this antifungal during treatment [19]. Alternatively, the use of azole fungicides in the environment that induce cross-resistance to medical triazoles in environmental *A. fumigatus* isolates has been suggested as another source of resistance development [20]. This environmental route was described in a study were a single mechanism of azole resistance was found in 94% of clinical isolates from several hospitals in The Netherlands [20], not being able to relate it to previous antifungal treatment. Finally, intrinsic azole resistance has also been described in other *Aspergillus* spp.

4. Mechanism of Azole Resistance

Since the first report of the *A. fumigatus* azole resistance strain, several studies have been published investigating the underlying molecular mechanisms. In *A. fumigatus*, the main targets of the azoles are Cyp51 proteins, encoded by two different, but related genes sharing 63% sequence identity, *cyp*51A and *cyp*51B [21]. The most frequent resistance mechanism is related to modifications in the azole

target (Cyp51A, a 14α sterol demethylase), although other mechanisms within *A. fumigatus* have been investigated.

5. Cyp51A Mutations

Up to now, most of the *A. fumigatus* azole resistant strains have been associated with point mutations or overexpression of *cyp*51A. The *cyp*51A encodes a 14α-sterol-demethylase, a key enzyme in the ergosterol biosynthesis pathway [22]. Ergosterol is the main component of fungal cell membranes. Triazoles bind with one of the nitrogen atoms of the triazole ring to the iron atom in the heme group located at the active site of Cyp51A [22]. This way, demethylation of C-14 of lanosterol is blocked, and ergosterol is not synthesized. Lack of ergosterol alters membrane fluidity and leads to fungal cell death [1]. Several single-nucleotide polymorphisms (SNPs), responsible for *cyp*51A amino acid substitutions, with or without tandem repeats in the promoter region of the gene, have been described. Both mechanisms affect the binding of azoles to the enzyme and lead to the development of resistance.

There are a few point mutations located at hot spot codons, whose link to azole resistance has been corroborated: (i) those associated with glycine 54 (G54), linked to cross-resistance to ITC and POS [23,24]; and (ii) amino acid substitutions at methionine 220 (M220), associated with different patterns of reduced susceptibility for triazoles [25]. Mutations in glycine 138 (G138), causing simultaneous resistance to itraconazole and voriconazole [26], and glycine 448 (G448S), resulting in VRC resistance, with some reduction in ITC and POS susceptibility, have also being reported in several studies [27–29]. Other point mutations, such as P216L, F219C, F219I, A284T, Y431C, G432S and G434C, have been occasionally described related to azole resistance, but further research is needed in order to confirm its role in the development of resistance [17,18,30–36]. In addition, a group of polymorphisms resulting in amino acid changes (F46Y, M172V, N248T, D255E and E427K) is frequently reported, alone or in combination, related to different patterns of susceptibility (they have been detected in azole susceptible and resistant strains), with consistently higher MICs than the wild type strains, although not always exceeding the breakpoint for resistance. More research is needed in order to determine the implication of each amino acid substitution (if any) in the azole profile shown by these strains (Table 1). All of these point mutations are generally described in strains isolated from patients that have been undergoing azole treatment.

A second group of *cyp*51A alterations with different resistance mechanisms has been reported, being normally described as panazole resistant. In *A. fumigatus*, this type of azole cross-resistance depends on specific mutations in *cyp*51A in combination with alterations in the promoter region, leading to multiazole-resistant strains [12,37,38]. These mechanisms are generated by combinations of *cyp*51A modifications: (i) the integration of a 34-bp tandem repeat (TR_{34}) in the promoter region of the gene, leading to an overexpression of *cyp*51A along with a substitution of leucine 98 to histidine (TR_{34}/L98H) [37]; this alteration is the most frequently identified resistance mechanism found in environmental *A. fumigatus* strains [39]; (ii) a 46-bp tandem repeat insertion in the promoter region and substitutions of tyrosine 121 to phenylalanine and threonine 289 to alanine (TR_{46}/Y121F/T289A) [40], which is related to VRC resistance; and (iii) a 53-bp tandem repeat in the promoter region without any *cyp*51A amino acid substitution [41,42].

One of the first studies on azole cross-resistance in *A. fumigatus* was performed in 17 clinical *A. fumigatus* isolates that were ITC resistant. These strains showed cross-resistance between ITC and POS, which have a similar molecule structure, but not with VRC [43,44]. Cross-resistance between azoles was studied by Howard et al. showing that 74% of the ITC resistant isolates studied were cross-resistant to POS and 65% to VRC [17]. The newest triazole isavuconazole has shown higher MICs in strains with reduced susceptibilities to other triazoles and presented a high degree of correlation with VRC susceptibility results [45]. In addition, other azole fungicides are widely used for crop protection (DMIs), which exhibit a related molecule structure to medical triazoles, leading to development of cross-resistance with azole in clinical use [46].

Table 1. Described *Aspergillus fumigatus cyp*51A mutations.

*cyp*51A Amino Acid No./Change	Continents	References
Described in resistant strains with a known mechanism		
G54/W/R/E/V/A	Europe Asia America Oceania	[12,17,18,23,32,47–53] [3,54–57] [58] [59]
M220/T/V/I/K/R/L	Europe Asia America	[12,17,20,25,32,33,35,47,48,50,52,60,61] [54,57] [58,62]
G448S	Europe Asia America Oceania	[17,27,29,63] [64] [58] [59]
Promoter tandem insertion + *cyp*51A amino acid No./change		
TR_{34}/L98H with or without S297T/F497I	Europe Asia America Africa Oceania	[12,17,20,32,35–38,40,47,48,50–53,60,65–76] [3,77–87] [58,88,89] [90] [59]
TR_{46}/Y121F/T289A with or without S297T/F497I	Europe Asia America Africa	[40,47,51,52,60,66,67,75,76,91–94] [82,95,96] [58,88,89] [90]
TR53	Europe America	[41] [88]
Described in resistant strains with an unknown mechanism		
G138/C/S	Europe America	[17,26,31] [58]
Described both in resistant and susceptible strains		
F46Y/M172V/N248T/D255E/E427K or some other combinations	Europe Asia Oceania	[17,33,34,36,53,61,65,71] [3] [59]
F46Y/M172V/E427K	Europe	[12,17,33,34,74,75]
Occasionally described in susceptible or resistant strains		
P216L		[17,18,53,61,75,97]
F219/S/C/I		[18,32,53,58]
I242V		[12,62]
N248K		[12,34,83]
Y431/S/C		[17,31,35,59]
G432/S/A		[30,83]
G434C		[17,31]

6. Azole Resistance Mechanisms are *cyp*51A Independent

Although triazole resistance in *A. fumigatus* is mainly attributed to *cyp*51A target mutations, a recent survey of resistant isolates in Manchester showed that >50% of resistant isolates had no mutation in *cyp*51A or its promoter [98]. There is also a reported case of a Dutch patient with chronic granulomatous disease treated with azole-echinocandin combination therapy, whose resistant isolate revealed a four-to-five-fold increased expression of *cyp*51A without having any *cyp*51A alterations [2]. Therefore, other mechanisms of resistance in clinical azole-resistant isolates without *cyp*51A mutations need to be explored.

Overexpression of *cyp51B*. In *A. fumigatus*, Cyp51 proteins are encoded by two different, but related genes sharing 63% sequence identity, *cyp*51A and *cyp*51B [21]. As described before, most of the azole-resistant strains have alterations in *cyp*51A; however, the role of *cyp*51B in *A. fumigatus* azole resistance remains unclear. Several *cyp*51B polymorphisms/mutations have been observed, but have never been linked to resistance. Only one study with a clinical azole-resistant isolate without *cyp*51A mutation or over-expression showed an over-expression of *cyp*51B [99]. Further studies are required to clearly define the relationship between this mechanism and azole resistance.

Overexpression of efflux pumps. Fungi have to beat intracellular toxin accumulation in order to successfully colonize human hosts [1]. This is achieved by efflux pumps, of which there are two main categories: ATP-binding cassette (ABC) proteins, primary transporters that take advantage of ATP hydrolysis, and major facilitator superfamily (MFS) pumps, secondary transporters that use the proton-motive force across the plasma membrane [100]. In *A. fumigatus*, at least 49 ABC family transporters and 278 MFS genes have been described, which is more than four-times the number identified in yeasts like *Saccharomyces cerevisiae* [101]. However, in *A. fumigatus*, despite the great number of existing genes encoding transporters, little is known about the connection between ABC or MFS efflux pumps and triazole resistance. To date, only five transporter genes are known to be related to azole resistance: *AfuMDR1*, *AfuMDR2*, *AfuMDR3*, *AfuMDR4* and *AtrF*.

AfuMDR1 and *AfuMDR2* ATP-binding cassette transporters were the first described, raising the possibility that these two genes could be directly involved in drug efflux in *A. fumigatus* [102]. Another ABC transporter, *atrF*, was cloned from a clinical isolate of *A. fumigatus* resistant to ITC, and five-fold higher levels of *atrF* mRNA compared to those in susceptible strains were revealed [103]. *AfuMDR3* and *AfuMDR4* were identified to be connected with triazole resistance in a study where resistant *A. fumigatus* mutants showed either constitutive high-level expression of both transporters or induction of expression when exposed to ITC. Two out of 23 mutants seemed to be ITC resistant due to overexpression of these genes, although evidence of a direct relationship between them and an ITC resistant phenotype is lacking. *AfuMDR3* has great similarity to MFS, and *AfuMDR4* is a member of the ABC proteins family [24]. Additionally, *AfuMDR4* has been shown to be induced with VRC in complex *A. fumigatus* biofilm populations and that this contributes to azole resistance [104]. Furthermore, exposure of a clinical azole-susceptible *A. fumigatus* isolate to VRC showed upregulation of five transporters of the ABC superfamily (*abcA-E*) and three of the MFS (*mfsA-C*) [105]. Lastly, a demonstrated link between transporters and azole resistance was the azole-induced expression of *cdr1B*. A *cdr*1B deleted mutant resulted in a four-fold susceptibility reduction in ITC MICs in an *A. fumigatus* clinical resistant isolate [106]. However, further studies are warranted in order to properly understand the relationship between the overexpression of pump efflux and azole resistance mechanisms in *A. fumigatus*.

Cholesterol import. The import of exogenous cholesterol under aerobic conditions, as a substitute for ergosterol after azole treatment, has also been described as a mechanism of resistance. The activity of ITC against *A. fumigatus* is compromised when cholesterol serum in RPMI medium is present [107]. In *A. fumigatus*, a sterol-regulatory element binding protein (*SrbA*) that plays a role in the azole resistance by *erg11* (*cyp*51A) regulation has been characterized [108]. The *srb*A null mutant (Δ*srb*A) was highly susceptible to FLC and VRC, which was explained by a reduction in *erg*11A transcript in response to both azoles. However, further studies on the genetic regulatory network mediated by *Srb*A in *A. fumigatus* and its role in triazole drug interactions need to be carried out [109,110].

Role of Hsp90. Heat shock protein 90 (Hsp90) is a eukaryotic molecular chaperone that helps crucial regulatory proteins in their folding, transport and maturation steps under environmental stress. Its involvement in the resistance of *Candida albicans* to azole and echinocandin antifungals is well established, but the function of Hsp90 in *A. fumigatus* remains unclear [111]. Using *S. cerevisiae* mutants expressing different levels of this chaperone, it was revealed that Hsp90 potentiates the acquisition of azole resistance and plays a key role in its continuance once it has been acquired. In *C. albicans* and

Aspergillus terreus, Hsp90 inhibitors can beat azole and echinocandin resistance in vivo [112]. However, the mechanisms by which Hsp90 controls these functions remain to be fully investigated.

HapE mutation. Another described mechanism is caused by a mutation in *HapE*, a CCAAT-binding transcription factor complex subunit. Two isogenic isolates with the wild-type *cyp*51A genotype, one azole susceptible isolated before treatment and the second with a resistant phenotype isolated post-treatment, were whole-genome sequenced in order to detect the resistance conferring mutation. Six out of a sixty-nine of identified point mutations in protein-coding regions were confirmed, and sexual crossing experiments revealed that a P88L substitution in HapE was the only one leading to resistance in progeny. This mutation in HapE can lead to a resistant phenotype by itself, as it was proven by cloning the mutated *hapE* gene into an azole-susceptible reference strain. This increase in resistance has been suggested to be due to a gain of function mutation if the mutated Hap-complex binds to a CCAAT-box in the promoter region of *cyp*51A and induces its expression [113].

7. Prevalence of Azole Resistance in *Aspergillus fumigatus* throughout the World

To date, Europe is the continent with the highest reported azole resistance in *A. fumigatus* (Table 2). Two reports in the late 2000s in the Netherlands and UK raised the alarm about an increase of azole resistance cases. The first one, in 2007, involved a series of Dutch patients suffering IA caused by panazole resistant strains, even those who had not been under azole treatment. One new resistance mechanism was found in these strains, TR_{34}/L98H [37,38]. The second study, in 2009, described a wide range of *cyp*51A mutations found in patients in the U.K., becoming clear that a dramatic increase in azole resistance in *A. fumigatus* was occurring [17]. Since then, azole resistant cases in clinical samples have been reported in almost every European country, including Austria [70], Belgium [68,76,92,94], Denmark [35,61,66,70], France [19,27,30,48,50,73,91,114], Germany [32,47,51,60,72], Greece [115], Italy [36], The Netherlands [18,20,37,38,40,41,53,65,67,74–76], Poland [69,116], Portugal [117], Romania [118], Spain [12,23,25,29,34,37,49,63,70,93,119], Sweden [120], Turkey [71] and the UK [17,26,31,33,65]. Even though G54 and M220 point mutations have been occasionally reported in Europe since they were described [12,17,18,20,23,25,32,35,48,50,51,60,71], the TR_{34}/L98H is by far the most common mutation found, both in environmental and clinical samples. Since its first report in 2007 in Spanish and Dutch isolates [37], TR_{34}/L98H has been detected across Europe (Figure 1) [12,32,35,38,41,48,50,51,53,60,67,69,71,75]. In 2009 a new resistance mechanism, TR_{46}/Y121F/T289A, was identified in The Netherlands [40]. Since then, it has also been reported in several countries [39,51,60,66,67,75,76,91–93]. Azole resistance in environmental strains in Europe has been commonly detected, with TR_{34}/L98H and TR_{46}/Y121F/T289A being the most often described mechanisms (Figure 1), and therefore, their emergence has been related with the extensive use of agricultural fungicides. Van der Linden et al. found that out of 140 environmental resistant strains, 14 had the TR_{46}/Y121F/T289A mechanism, while 126 had TR_{34}/L98H [40]. In Germany, an analysis of 455 environmental isolates revealed 45 that harbored the TR_{34}/L98H mutation and six TR_{46}/Y121F/T289A [47]. Another analysis reported 16% resistance (to ITC and POS) in environmental *A. fumigatus* isolates in Italy [36]. Other, less frequent point mutations have been described as related to the azole-resistant phenotype, but further research is needed in order to confirm it.

Table 2. Azole resistance prevalence in *A. fumigatus* by continent and/or country. Only significant publications with more than 50 isolates tested are reported.

Continent/Country	% Resistance	Source of the Isolates	References
	Europe		
Belgium	5.7	C	[76]
France	0.85–10.6	C	[30,48,50]
Germany	1.1–12	C and E	[32,47,60]
Netherlands	2.1–20	C and E	[20,53,67,74]
Poland	2.25	C	[69]
Spain	1.8	C	[63]
Turkey	10.2	C	[71]
United Kingdom	6.6–28	C	[17,33]
	Other continents		
Asia *	1.9–11.1	C and E	[55,77,78,80–86,121]
Africa (Tanzania)	13.9	E	[90]
America (USA)	0.6–11.8	C	[58,122]
Oceania (Australia)	2.6	C	[59]
	International surveillance studies		
America-Asia-Australia-Europe	1.4–5.8	C and E	[52,70,123,124]

C = clinical strains, E = environmental strains; * including China, India, Iran, Japan, Kuwait and Pakistan.

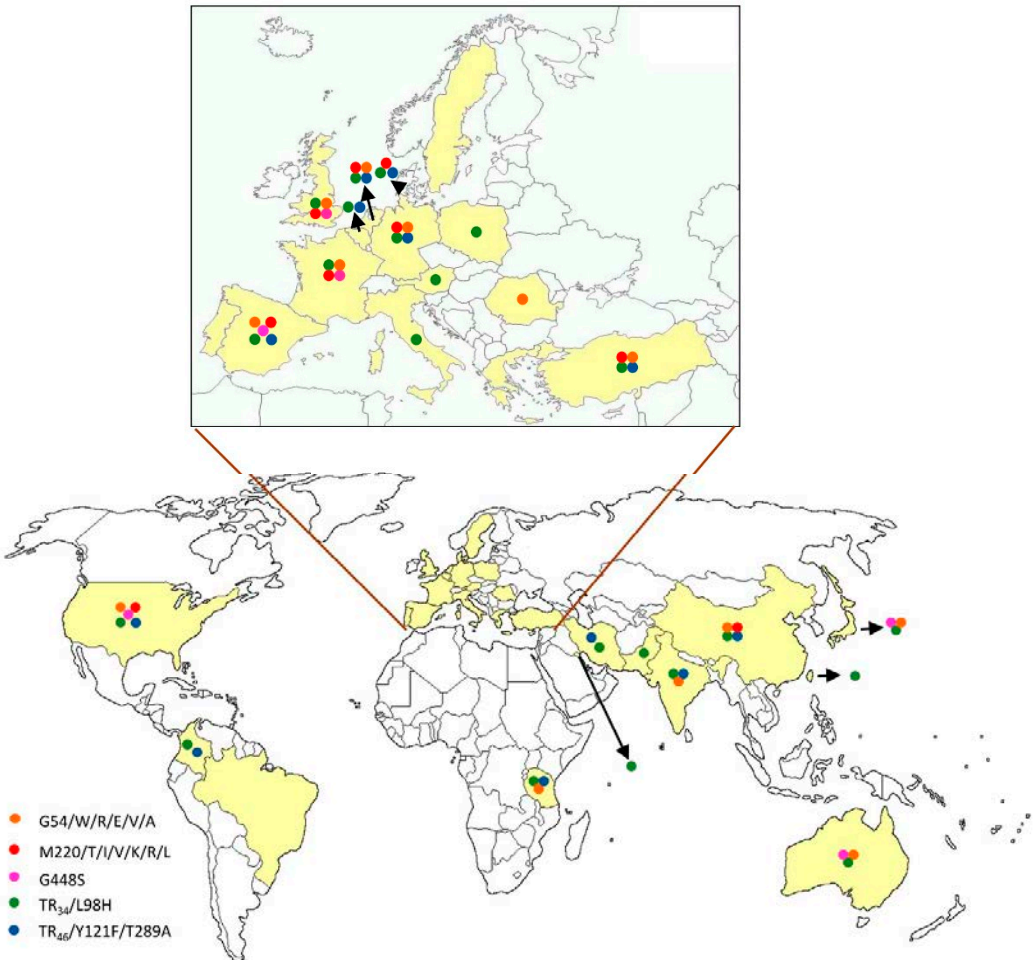


Figure 1. Worldwide distribution of azole resistance in *A. fumigatus* by mechanisms.

Reports from Asiatic countries suggest that triazole resistance rates in Asia are lower than in Europe (Table 2). The first two reports describing azole resistance in *A. fumigatus* in this area were

published in 2005. One was from clinical strains from Taiwan, where two out of 40 isolates showed azole resistance, but mutations in *cyp*51A were not investigated [125]; and the second one was based on six isogenic isolates obtained from a Chinese patient treated with azoles and suffering from lung aspergilloma. ITC resistance was found in four post-treatment isolates, one of them with a M220I mutation and the rest with G54R [54]. Several other cases have been reported since then. The ARTEMIS global antifungal susceptibility program included more than 100 medical centers worldwide and detected several clinical isolates from China that had a TR_{34}/L98H resistance mechanism [123]. This alteration has also been reported in 7.9% of the multi-azole resistant strains isolated from azole-naïve patients in Taiwan [87] and in three out of fourteen resistant clinical isolates in Pakistan [85]. In contrast, TR_{34}/L98H has not been described in Japan, with reports showing a low azole resistant strains rate. Kikuchi et al. found three resistant isolates out of 171 *A. fumigatus* clinical strains isolated between 1987 and 2008 [121]. Some novel mutations have been reported in this country, such as the P216L [97] or F332K [126], and the G448S and TR_{46}/Y121F/T289A mechanisms were recently identified in Japan for the first time [64,95]. Azole resistance prevalence in *A. fumigatus* is also low in India, where three studies revealed the presence of TR_{34}/L98H as the resistance mechanism in clinical isolates: 44 out of 630 (6.9%), two out of 103 (1.9%) and 10 out of 685 (1.5%) [3,80,81]. Similar findings have been observed in Middle East countries, like Iran (3.5% of clinical samples) [84] or Kuwait (two out of 16 clinical isolates and one out of 50 environmental isolates) [77]. Azole resistance in environmental strains in Asia is also lower than in Europe (Table 2). In fact, a recent report on the use of azole fungicides on a pumpkin farm revealed no azole resistance in 50 *A. fumigatus* isolates [127]. Several environmental studies have been performed in India, describing the TR_{46}/Y121F/T289A mechanism for the first time in Asia in isolates from agricultural fields [82] and showing that 44 out of 630 *A. fumigatus* sampled from the soil of paddy fields, tea gardens, cotton trees, flower pots and indoor air of hospitals were resistant and harbored the TR_{34}/L98H resistance mechanism [81]. A report from Iran described 12.2% of environmental resistant strains [79], and in Kuwait, 7% of environmental samples were also resistant [78], all of them carrying TR_{34}/L98H. This difference in environmental azole resistance rates between Asia and Europe could be due to the lower use of azole fungicides in Asian countries [128].

The first study involving a large number of isolates in the U.S. included 181 *A. fumigatus* isolates from transplant patients with proven IA from 2001–2006 (multicenter prospective study). Only one of these isolates was triazole resistant [122] and indicates a low azole resistance prevalence in this country. Similarly, 1096 *A. fumigatus* clinical strains from all over the U.S. collected between 2011 and 2013 were studied; 51 of them were sequenced for *cyp*51A mutations. One isolate possessed the M220I mutation in *cyp*51A, and 13 isolates had another mutation, I242V; TR_{34}/L98H was not identified [62]. A recent comprehensive study in the U.S. included 220 clinical *A. fumigatus* isolates obtained from 2001–2014, with the description of two isolates harboring TR_{34}/L89H mutations and the other two with TR_{46}/Y121F/T289A. This was the first report of both resistance mechanisms in *A. fumigatus* isolates in the United States. Other point mutations detected in the 26 azole resistant strains were G54R/W/E, M220I/K/V, G138S/C, G448S and F219S [58]. To our knowledge, no environmental sample studies have been reported in this country yet, but there is also lower use of fungicides in the U.S. as compared to Europe [128].

Some investigations have been carried out in South American countries, such as Brazil, where six out of 170 clinical *A. fumigatus* collected between 2000 and 2012 showed azole resistance, but neither the TR_{34}/L98H nor the TR_{46}/Y121F/T289A mechanisms were found [129]. An environmental study has been carried out in Colombia, known to be the fourth country in the world for pesticide use, 30% of which are fungicides. Sixty soil samples from flower beds and flower fields were analyzed, describing one TR_{34}/L98H, 17 TR_{46}/Y121F/T289A and one TR53 isolates [88]. Colombia is the second biggest flower exporter after The Netherlands, which could explain the high environmental azole resistance rate in *A. fumigatus* [129].

Azole resistance has also been reported in Africa; 15 out of 108 environmental samples taken in Tanzania were azole resistant, 11 of them with the TR_{34}/L98H mutation and four with

TR_{46}/Y121F/T289A [90]. Another study in the same country describes G54E as responsible for 46.4% of resistant environmental *A. fumigatus* isolates from this country [118]. To our knowledge, no reports from clinical samples have been published in Africa yet.

In Australia, 418 *A. fumigatus* clinical strains were collected from 2000–2013, revealing nine isolates with reduced susceptibility to ITC, VRC and POS. All of them had between two and five amino acid substitutions, including G54R, F46Y, Y431S, G448S, M172V, N248T, D255E, E427K and TR_{34}/L98H, the latter being identified in two isolates. The first TR_{34}/L98H *A. fumigatus* was recovered in 2004, and it is believed to be Australian-acquired in a patient on long-term ITC therapy, while the second isolate was suspected to have been acquired in Europe while the patient was travelling in 2012 [59].

8. Azole Resistance in Other *Aspergillus* Species

A shift in epidemiology of fungal infections towards a greater number of species able to cause disease in humans has occurred [130]. The leading cause of IA is *A. fumigatus* (85%), followed by *A. flavus* (5%–10%), *A. terreus* (2%–10%) and *A. niger* (2%–3%) [100]. However, the use of molecular tools has led to the description of new species within the genus *Aspergillus*. Some of these species are considered cryptic or sibling because they are difficult to differentiate by classical methods, and they have been frequently misidentified. Their prevalence in the clinical setting has been reported to be between 10% and 15% in two studies. The TRANSNET (Transplant-Associated Infection Surveillance Network) study included 218 *Aspergillus* isolates from transplant recipients with proven or probable IA from 2001–2006 from the U.S. and documented an 11% cryptic species [131]. The FILPOP study (population-based survey of filamentous fungi) from Spain described 15% cryptic species among 323 isolates analyzed [119]. The importance of these cryptic species in the clinical setting is based on their different susceptibility profile, as they are frequently more resistant to the antifungals available [132]. As these cryptic species are difficult to differentiate, it has been recommended that when using classical identification methods in the clinical setting, an *Aspergillus* isolate should be classified to the "species complex" level, thereby accounting for gathering all closely-related cryptic species.

The *Aspergillus fumigatus* complex includes several species that have been reported in human infections: *Aspergillus lentulus*, *A. udagawae* (syn. *Neosartorya udagawae*), *A. pseudofischeri* (syn. *Neosartorya pseudofischeri*), *A. viridinutans*, *A. fumigatiaffinis*, *A. fumisynnematus* and *A. hiratsukae* (syn. *Neosartorya hiratsukae*) [119,131,133]. Antifungal susceptibility testing of these species revealed heterogeneous patterns. *Aspergillus lentulus*, *A. fumigatiaffinis* and *A. udagawae* show high MICs for AmB, with the first two of these also having high MICs for azoles, but *A. udagawae* has intermediate values for VRC and low MICs for ITC or PCZ. *Aspergillus viridinutans* and *A. pseudofischeri* have reduced susceptibility for azoles, but not for AmB, and *A. hiratsukae* and *A. fumisynnematus* are susceptible to all drugs [132–137].

The *A. niger* includes *A. tubingensis*, the second most frequent species of the complex in clinical isolates, and has been found with similar prevalence as *A. niger* in some studies [76,119]. *Aspergillus awamori* and *A. foetidus* have also been described in clinical samples, although there is debate about their classification as new species or subspecies of *A. niger* [138]. The susceptibility profile of these species is isolate dependent, and three patterns have been described regarding ITC: low MICs, high MICs and isolates that show a paradoxical effect (which are able to grow in the presence of high antifungal concentrations, but remain fully susceptible at intermediate-to-low concentrations [139]) for this antifungal [140]. *Aspergillus niger* and *A. awamori* have been reported to have higher MICs to azoles than *A. tubingensis* [141].

Aspergillus flavus is the second most common *Aspergillus* causing IA, and it is reported as the most prevalent in countries with arid climates, such as those in the Middle East, Africa and Southeast Asia, as it is capable of surviving in extreme conditions [142]. *Aspergillus alliaceus* is part of the *A. flavus* complex. This species has elevated MICs to AmB and echinocandins, but is variable regarding azoles. The first report describing *A. alliaceus* stated that ITC was the most active antifungal in vitro against this mold [143], but the first study reporting IA caused by *A. alliaceus* (together with *A. flavus*) defined

VRC as the best option for treatment, as the isolate tested was resistant to ITC and POS [144]. VRC resistance has also been reported in clinical strains of *A. flavus*, and T788G and Y319H mutations in the *cyp*51C gene have been found to be associated with these high MICs to VRC [145,146].

Aspergillus terreus shows high MICs to AmB both in vitro [147,148] and in vivo [149], and reduced susceptibility to azoles has also been described. A study from 2012 reports a *cyp*51A mutation, M217I, in some clinical *A. terreus* isogenic isolates causing ITC resistance [150]. The *A. terreus* complex includes *Aspergillus alabamensis*, *A. floccosus*, *A. neoafricanus*, *A. aureoterreus A. hortai*, *A. pseudoterreus* [151] and *Aspergillus citrinoterreus*. They all have high MICs to AmB, but *A. hortai* and *A. citrinoterreus* are more susceptible to azoles than *A. terreus* [152,153].

The *Aspergillus ustus* complex is known for its elevated MICs to most drugs. *Aspergillus calidoustus* was described in 2008 as being able to grow at 37 °C, in contrast to *A. ustus*, and has been isolated from human infections [154]. Triazoles have been reported to be inactive in vitro against *A. calidoustus* [122], and the same has been reported of other antifungal classes, so it is considered a multiresistant species. Other cryptic species with high MICs to all antifungals in this complex are *A. keveii* and *A. insuetus*, also isolated from clinical samples [155].

9. Treatment Options

Mortality rates in patients infected with azole-resistant strains (ITC > 2 μg/mL, VRC > 2 μg/mL, POS > 0.5 μg/mL, determined by the CLSI reference method) are higher than those affected with azole-susceptible ones (88% vs. 30%–50%) [53]. As mentioned above, VRC is the primary treatment for IA, but liposomal amphotericin B (L-AMB) is recommended as an alternative therapy [156]. L-AMB was demonstrated to develop no cross-resistance in a murine model of disseminated azole-resistant aspergillosis, being either active against azole-susceptible or azole-resistant strains [157]. However, this drug is not recommended to treat infections caused by *A. terreus* or other AMB-resistant species. Another approach to consider is an antifungal combination therapy that leads to a synergistic response. A great number of in vitro, in vivo and clinical studies have tested various antifungal combinations and found some of them effective against *A. fumigatus* [158]. Recent studies have focused on the combination of an azole, normally VRC, with an echinocandin, both for azole-susceptible and azole-resistant *A. fumigatus* strains. The efficacy of this combined therapy mainly relies on anidulafungin (AND) [159], which is currently not licensed for the treatment of IA. In one clinical study, mortality rates were 27.5% for monotherapy and 19.3% for combined therapy of VRC and AND [160]. In a murine model, AND was successful against 45% of VRC-resistant strains when used as monotherapy [161]. Further studies for combined therapy are warranted in order to find alternative treatment options, given the limitations of current monotherapy. Although azole-resistant strains have been present in clinical samples for more than two decades, it has been suggested that first-line therapy should remain as azoles whilst local azole resistance prevalence remains below 10% [162]. Still, therapeutic options for IA should be revised taking this issue into account.

10. Conclusions and Recommendations for Clinical Practice

Clinical and environmental triazole resistance in *Aspergillus* species is a growing public health concern that has become a worldwide problem. Even though the highest rates of triazole resistance have been described in Europe, several cases have been reported in every continent, and new resistance mechanisms are being described. Despite *A. fumigatus* being the most common *Aspergillus* species, triazole resistance has also been identified in many cryptic species of *Aspergillus*. Therefore, the morphological identification of an isolate cannot always drive the treatment strategy. We recommend performing antifungal susceptibility testing on every *Aspergillus* isolate associated with IA in order to select the best antifungal treatment. In addition, the prevalence of resistant strains should be investigated in every country to understand the prevalence of resistance and to adjust therapeutic options where high rates of resistant isolates are present. Moreover, the development of

molecular methods to detect azole resistance in culture-negative infections could be very useful in laboratory practice.

It is important to investigate more extensively the origin of environmental samples that are resistant to triazoles, since measures to reduce the use of agricultural azoles could be an important step in reducing resistance rates in the clinical setting, as stated in the technical report published by the European Centre for Disease prevention and Control (ECDC) [163].

Acknowledgments: The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Olga Rivero-Menendez holds a predoctoral fellowship from the Fondo de Investigaciones Sanitarias (FI14CIII/00025).

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Chowdhary, A.; Sharma, C.; Hagen, F.; Meis, J.F. Exploring azole antifungal drug resistance in *Aspergillus fumigatus* with special reference to resistance mechanisms. *Future. Microbiol.* **2014**, *9*, 697–711. [CrossRef] [PubMed]
2. Arendrup, M.C.; Mavridou, E.; Mortensen, K.L.; Snelders, E.; Frimodt-Moller, N.; Khan, H.; Melchers, W.J.; Verweij, P.E. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. *PLoS ONE* **2010**, *5*, e10080. [CrossRef] [PubMed]
3. Chowdhary, A.; Sharma, C.; Kathuria, S.; Hagen, F.; Meis, J.F. Prevalence and mechanism of triazole resistance in *Aspergillus fumigatus* in a referral chest hospital in Delhi, India and an update of the situation in Asia. *Front. Microbiol.* **2015**, *6*, 428. [CrossRef] [PubMed]
4. Kwon-Chung, K.J.; Sugui, J.A. *Aspergillus fumigatus*—what makes the species a ubiquitous human fungal pathogen? *PLoS Pathog.* **2013**, *9*, e1003743. [CrossRef] [PubMed]
5. Miceli, M.H.; Kauffman, C.A. Isavuconazole: A new broad-spectrum triazole antifungal agent. *Clin. Infect. Dis.* **2015**, *61*, 1558–1565. [CrossRef] [PubMed]
6. Verweij, P.E.; Chowdhary, A.; Melchers, W.J.; Meis, J.F. Azole resistance in *Aspergillus fumigatus*: Can we retain the clinical use of mold-active antifungal azoles? *Clin. Infect. Dis.* **2016**, *62*, 362–368. [CrossRef] [PubMed]
7. Maschmeyer, G.; Haas, A.; Cornely, O.A. Invasive aspergillosis: Epidemiology, diagnosis and management in immunocompromised patients. *Drugs* **2007**, *67*, 1567–1601. [CrossRef] [PubMed]
8. Walsh, T.J.; Anaissie, E.J.; Denning, D.W.; Herbrecht, R.; Kontoyiannis, D.P.; Marr, K.A.; Morrison, V.A.; Segal, B.H.; Steinbach, W.J.; Stevens, D.A.; et al. Treatment of aspergillosis: Clinical practice guidelines of the infectious diseases society of America. *Clin. Infect. Dis.* **2008**, *46*, 327–360. [CrossRef] [PubMed]
9. Howard, S.J.; Pasqualotto, A.C.; Denning, D.W. Azole resistance in allergic bronchopulmonary aspergillosis and *Aspergillus* bronchitis. *Clin. Microbiol. Infect.* **2010**, *16*, 683–688. [CrossRef] [PubMed]
10. Cornely, O.A.; Maertens, J.; Winston, D.J.; Perfect, J.; Ullmann, A.J.; Walsh, T.J.; Helfgott, D.; Holowiecki, J.; Stockelberg, D.; Goh, Y.T.; et al. Posaconazole vs. Fluconazole or itraconazole prophylaxis in patients with neutropenia. *N. Engl. J. Med.* **2007**, *356*, 348–359. [CrossRef] [PubMed]
11. Espinel-Ingroff, A.; Diekema, D.J.; Fothergill, A.; Johnson, E.; Pelaez, T.; Pfaller, M.A.; Rinaldi, M.G.; Canton, E.; Turnidge, J. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. For the CLSI broth microdilution method (M38-A2 document). *J. Clin. Microbiol.* **2010**, *48*, 3251–3257. [CrossRef] [PubMed]
12. Rodriguez-Tudela, J.L.; Alcazar-Fuoli, L.; Mellado, E.; Alastruey-Izquierdo, A.; Monzon, A.; Cuenca-Estrella, M. Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2008**, *52*, 2468–2472. [CrossRef] [PubMed]
13. EUCAST European committee on antimicrobial susceptibility testing. Antifungal breakpoint tables for interpretation of MICs v 8.0. Available online: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/Antifungal_breakpoints_v_8.0_November_2015.pdf (accessed on 25 April 2016).

14. Denning, D.W.; Venkateswarlu, K.; Oakley, K.L.; Anderson, M.J.; Manning, N.J.; Stevens, D.A.; Warnock, D.W.; Kelly, S.L. Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **1997**, *41*, 1364–1368. [PubMed]
15. Chowdhary, A.; Kathuria, S.; Xu, J.; Meis, J.F. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. *PLoS Pathog.* **2013**, *9*, e1003633. [CrossRef]
16. Verweij, P.E.; Snelders, E.; Kema, G.H.; Mellado, E.; Melchers, W.J. Azole resistance in *Aspergillus fumigatus*: A side-effect of environmental fungicide use? *Lancet Infect. Dis.* **2009**, *9*, 789–795. [CrossRef]
17. Howard, S.J.; Cerar, D.; Anderson, M.J.; Albarrag, A.; Fisher, M.C.; Pasqualotto, A.C.; Laverdiere, M.; Arendrup, M.C.; Perlin, D.S.; Denning, D.W. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg. Infect. Dis.* **2009**, *15*, 1068–1076. [CrossRef] [PubMed]
18. Camps, S.M.; van der Linden, J.W.; Li, Y.; Kuijper, E.J.; van Dissel, J.T.; Verweij, P.E.; Melchers, W.J. Rapid induction of multiple resistance mechanisms in *Aspergillus fumigatus* during azole therapy: A case study and review of the literature. *Antimicrob. Agents Chemother.* **2012**, *56*, 10–16. [CrossRef] [PubMed]
19. Dannaoui, E.; Borel, E.; Monier, M.F.; Piens, M.A.; Picot, S.; Persat, F. Acquired itraconazole resistance in *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* **2001**, *47*, 333–340. [CrossRef] [PubMed]
20. Snelders, E.; van der Lee, H.A.; Kuijpers, J.; Rijs, A.J.; Varga, J.; Samson, R.A.; Mellado, E.; Donders, A.R.; Melchers, W.J.; Verweij, P.E. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med.* **2008**, *5*, e219. [CrossRef] [PubMed]
21. Mellado, E.; Diaz-Guerra, T.M.; Cuenca-Estrella, M.; Rodriguez-Tudela, J.L. Identification of two different 14-alpha sterol demethylase-related genes (*cyp*51A and *cyp*51B) in *Aspergillus fumigatus* and other *Aspergillus* species. *J. Clin. Microbiol.* **2001**, *39*, 2431–2438. [CrossRef] [PubMed]
22. Snelders, E.; Karawajczyk, A.; Schaftenaar, G.; Verweij, P.E.; Melchers, W.J. Azole resistance profile of amino acid changes in *Aspergillus fumigatus cyp*51A based on protein homology modeling. *Antimicrob. Agents Chemother.* **2010**, *54*, 2425–2430. [CrossRef] [PubMed]
23. Diaz-Guerra, T.M.; Mellado, E.; Cuenca-Estrella, M.; Rodriguez-Tudela, J.L. A point mutation in the 14alpha-sterol demethylase gene *cyp*51A contributes to itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2003**, *47*, 1120–1124. [CrossRef] [PubMed]
24. Nascimento, A.M.; Goldman, G.H.; Park, S.; Marras, S.A.; Delmas, G.; Oza, U.; Lolans, K.; Dudley, M.N.; Mann, P.A.; Perlin, D.S. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrob. Agents Chemother.* **2003**, *47*, 1719–1726. [CrossRef] [PubMed]
25. Mellado, E.; Garcia-Effron, G.; Alcazar-Fuoli, L.; Cuenca-Estrella, M.; Rodriguez-Tudela, J.L. Substitutions at methionine 220 in the 14alpha-sterol demethylase (*cyp*51A) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. *Antimicrob. Agents Chemother.* **2004**, *48*, 2747–2750. [CrossRef] [PubMed]
26. Howard, S.J.; Webster, I.; Moore, C.B.; Gardiner, R.E.; Park, S.; Perlin, D.S.; Denning, D.W. Multi-azole resistance in *Aspergillus fumigatus*. *Int. J. Antimicrob. Agents* **2006**, *28*, 450–453. [CrossRef] [PubMed]
27. Bellete, B.; Raberin, H.; Morel, J.; Flori, P.; Hafid, J.; Manhsung, R.T. Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma. *Med. Mycol.* **2010**, *48*, 197–200. [CrossRef] [PubMed]
28. Manavathu, E.; Espinel-Ingroff, A.; Alangaden, G.; Chandrasekar, P. Molecular studies on voriconazole resistance in a clinical isolate of *Aspergillus fumigatus*. In Proceedings of 43rd Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, USA, 14–17 September 2003.
29. Pelaez, T.; Gijon, P.; Bunsow, E.; Bouza, E.; Sanchez-Yebra, W.; Valerio, M.; Gama, B.; Cuenca-Estrella, M.; Mellado, E. Resistance to voriconazole due to a G448S substitution in *Aspergillus fumigatus* in a patient with cerebral aspergillosis. *J. Clin. Microbiol.* **2012**, *50*, 2531–2534. [CrossRef] [PubMed]
30. Alanio, A.; Sitterle, E.; Liance, M.; Farrugia, C.; Foulet, F.; Botterel, F.; Hicheri, Y.; Cordonnier, C.; Costa, J.M.; Bretagne, S. Low prevalence of resistance to azoles in *Aspergillus fumigatus* in a French cohort of patients treated for haematological malignancies. *J. Antimicrob. Chemother.* **2011**, *66*, 371–374. [CrossRef] [PubMed]

31. Albarrag, A.M.; Anderson, M.J.; Howard, S.J.; Robson, G.D.; Warn, P.A.; Sanglard, D.; Denning, D.W. Interrogation of related clinical pan-azole-resistant *Aspergillus fumigatus* strains: G138C, Y431C, and G434C single nucleotide polymorphisms in *cyp*51A, upregulation of *cyp*51A, and integration and activation of transposon *Atf1* in the *cyp*51a promoter. *Antimicrob. Agents Chemother.* **2011**, *55*, 5113–5121. [CrossRef] [PubMed]
32. Bader, O.; Weig, M.; Reichard, U.; Lugert, R.; Kuhns, M.; Christner, M.; Held, J.; Peter, S.; Schumacher, U.; Buchheidt, D.; et al. *cyp*51A-based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. *Antimicrob. Agents Chemother.* **2013**, *57*, 3513–3517. [PubMed]
33. Bueid, A.; Howard, S.J.; Moore, C.B.; Richardson, M.D.; Harrison, E.; Bowyer, P.; Denning, D.W. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. *J. Antimicrob. Chemother.* **2010**, *65*, 2116–2118. [CrossRef] [PubMed]
34. Escribano, P.; Recio, S.; Pelaez, T.; Bouza, E.; Guinea, J. *Aspergillus fumigatus* strains with mutations in the *cyp*51A gene do not always show phenotypic resistance to itraconazole, voriconazole, or posaconazole. *Antimicrob. Agents Chemother.* **2011**, *55*, 2460–2462. [CrossRef] [PubMed]
35. Mortensen, K.L.; Jensen, R.H.; Johansen, H.K.; Skov, M.; Pressler, T.; Howard, S.J.; Leatherbarrow, H.; Mellado, E.; Arendrup, M.C. *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: A laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *J. Clin. Microbiol.* **2011**, *49*, 2243–2251. [CrossRef] [PubMed]
36. Prigitano, A.; Venier, V.; Cogliati, M.; De, L.G.; Esposto, M.C.; Tortorano, A.M. Azole-resistant *Aspergillus fumigatus* in the environment of Northern Italy, May 2011 to June 2012. *Euro. Surveill* **2014**, *19*, 20747. [CrossRef] [PubMed]
37. Mellado, E.; Garcia-Effron, G.; Alcazar-Fuoli, L.; Melchers, W.J.; Verweij, P.E.; Cuenca-Estrella, M.; Rodriguez-Tudela, J.L. A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp*51A alterations. *Antimicrob. Agents Chemother.* **2007**, *51*, 1897–1904. [CrossRef] [PubMed]
38. Verweij, P.E.; Mellado, E.; Melchers, W.J. Multiple-triazole-resistant aspergillosis. *N. Engl. J. Med.* **2007**, *356*, 1481–1483. [CrossRef] [PubMed]
39. Vermeulen, E.; Lagrou, K.; Verweij, P.E. Azole resistance in *Aspergillus fumigatus*: A growing public health concern. *Curr. Opin. Infect. Dis.* **2013**, *26*, 493–500. [CrossRef] [PubMed]
40. van der Linden, J.W.; Camps, S.M.; Kampinga, G.A.; Arends, J.P.; Debets-Ossenkopp, Y.J.; Haas, P.J.; Rijnders, B.J.; Kuijper, E.J.; van Tiel, F.H.; Varga, J.; et al. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clin. Infect. Dis.* **2013**, *57*, 513–520. [CrossRef] [PubMed]
41. Hodiamont, C.J.; Dolman, K.M.; Ten Berge, I.J.; Melchers, W.J.; Verweij, P.E.; Pajkrt, D. Multiple-azole-resistant *Aspergillus fumigatus* osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. *Med. Mycol.* **2009**, *47*, 217–220. [CrossRef] [PubMed]
42. Mellado, E.; Alcazar-Fuoli, L.; Pajkrt, D.; Verweij, P.E.; Melchers, W.J.; Cuenca-Estrella, M.; Rodriguez-Tudela, J.L. Alterations of the *cyp*51A gene promoter contribute to *Aspergillus fumigatus* multiple triazole resistance. In Proceedings of 47th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, USA, 17–20 September 2007.
43. Mosquera, J.; Denning, D.W. Azole cross-resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2002**, *46*, 556–557. [CrossRef] [PubMed]
44. Xiao, L.; Madison, V.; Chau, A.S.; Loebenberg, D.; Palermo, R.E.; McNicholas, P.M. Three-dimensional models of wild-type and mutated forms of cytochrome P450 14α-sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding. *Antimicrob. Agents Chemother.* **2004**, *48*, 568–574. [CrossRef] [PubMed]
45. Gregson, L.; Goodwin, J.; Johnson, A.; McEntee, L.; Moore, C.B.; Richardson, M.; Hope, W.W.; Howard, S.J. In vitro susceptibility of *Aspergillus fumigatus* to isavuconazole: Correlation with itraconazole, voriconazole, and posaconazole. *Antimicrob. Agents Chemother.* **2013**, *57*, 5778–5780. [CrossRef] [PubMed]
46. Snelders, E.; Camps, S.M.; Karawajczyk, A.; Schaftenaar, G.; Kema, G.H.; van der Lee, H.A.; Klaassen, C.H.; Melchers, W.J.; Verweij, P.E. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS ONE* **2012**, *7*, e31801. [CrossRef] [PubMed]

47. Bader, O.; Tunnermann, J.; Dudakova, A.; Tangwattanachuleeporn, M.; Weig, M.; Gross, U. Environmental isolates of azole-resistant *Aspergillus fumigatus* in Germany. *Antimicrob. Agents Chemother.* **2015**, *59*, 4356–4359. [CrossRef] [PubMed]
48. Burgel, P.R.; Baixench, M.T.; Amsellem, M.; Audureau, E.; Chapron, J.; Kanaan, R.; Honore, I.; Dupouy-Camet, J.; Dusser, D.; Klaassen, C.H.; et al. High prevalence of azole-resistant *Aspergillus fumigatus* in adults with cystic fibrosis exposed to itraconazole. *Antimicrob. Agents Chemother.* **2012**, *56*, 869–874. [CrossRef] [PubMed]
49. Escribano, P.; Recio, S.; Pelaez, T.; Gonzalez-Rivera, M.; Bouza, E.; Guinea, J. In vitro acquisition of secondary azole resistance in *Aspergillus fumigatus* isolates after prolonged exposure to itraconazole: Presence of heteroresistant populations. *Antimicrob. Agents Chemother.* **2012**, *56*, 174–178. [CrossRef] [PubMed]
50. Morio, F.; Aubin, G.G.; Danner-Boucher, I.; Haloun, A.; Sacchetto, E.; Garcia-Hermoso, D.; Bretagne, S.; Miegeville, M.; Le, P.P. High prevalence of triazole resistance in *Aspergillus fumigatus*, especially mediated by TR34/L98H, in a French cohort of patients with cystic fibrosis. *J. Antimicrob. Chemother.* **2012**, *67*, 1870–1873. [CrossRef] [PubMed]
51. Steinmann, J.; Hamprecht, A.; Vehreschild, M.J.; Cornely, O.A.; Buchheidt, D.; Spiess, B.; Koldehoff, M.; Buer, J.; Meis, J.F.; Rath, P.M. Emergence of azole-resistant invasive aspergillosis in HSCT recipients in Germany. *J. Antimicrob. Chemother.* **2015**, *70*, 1522–1526. [CrossRef] [PubMed]
52. van der Linden, J.W.; Arendrup, M.C.; Warris, A.; Lagrou, K.; Pelloux, H.; Hauser, P.M.; Chryssanthou, E.; Mellado, E.; Kidd, S.E.; Tortorano, A.M.; et al. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. *Emerg. Infect. Dis.* **2015**, *21*, 1041–1044. [CrossRef] [PubMed]
53. van der Linden, J.W.; Snelders, E.; Kampinga, G.A.; Rijnders, B.J.; Mattsson, E.; Debets-Ossenkopp, Y.J.; Kuijper, E.J.; van Tiel, F.H.; Melchers, W.J.; Verweij, P.E. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007–2009. *Emerg. Infect. Dis.* **2011**, *17*, 1846–1854. [CrossRef] [PubMed]
54. Chen, J.; Li, H.; Li, R.; Bu, D.; Wan, Z. Mutations in the *cyp*51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *J. Antimicrob. Chemother.* **2005**, *55*, 31–37. [CrossRef] [PubMed]
55. Tashiro, M.; Izumikawa, K.; Hirano, K.; Ide, S.; Mihara, T.; Hosogaya, N.; Takazono, T.; Morinaga, Y.; Nakamura, S.; Kurihara, S.; et al. Correlation between triazole treatment history and susceptibility in clinically isolated *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2012**, *56*, 4870–4875. [CrossRef] [PubMed]
56. Tashiro, M.; Izumikawa, K.; Minematsu, A.; Hirano, K.; Iwanaga, N.; Ide, S.; Mihara, T.; Hosogaya, N.; Takazono, T.; Morinaga, Y.; et al. Antifungal susceptibilities of *Aspergillus fumigatus* clinical isolates obtained in Nagasaki, Japan. *Antimicrob. Agents Chemother.* **2012**, *56*, 584–587. [CrossRef] [PubMed]
57. Xu, H.; Chen, W.; Li, L.; Wan, Z.; Li, R.; Liu, W. Clinical itraconazole-resistant strains of *Aspergillus fumigatus*, isolated serially from a lung aspergilloma patient with pulmonary tuberculosis, can be detected with real-time PCR method. *Mycopathologia* **2010**, *169*, 193–199. [CrossRef] [PubMed]
58. Wiederhold, N.P.; Gil, V.G.; Gutierrez, F.; Lindner, J.R.; Albataineh, M.T.; McCarthy, D.I.; Sanders, C.; Fan, H.; Fothergill, A.W.; Sutton, D.A. First detection of TR34/L98H and TR46/Y121F/T289A *cyp*51 mutations in *Aspergillus fumigatus* isolates in the United States. *J. Clin. Microbiol.* **2016**, *54*, 168–171. [CrossRef] [PubMed]
59. Kidd, S.E.; Goeman, E.; Meis, J.F.; Slavin, M.A.; Verweij, P.E. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses* **2015**, *58*, 350–355. [CrossRef] [PubMed]
60. Fischer, J.; van Koningsbruggen-Rietschel, S.; Rietschel, E.; Vehreschild, M.J.; Wisplinghoff, H.; Kronke, M.; Hamprecht, A. Prevalence and molecular characterization of azole resistance in *Aspergillus* spp. Isolates from German cystic fibrosis patients. *J. Antimicrob. Chemother.* **2014**, *69*, 1533–1536. [CrossRef] [PubMed]
61. Zhao, Y.; Stensvold, C.R.; Perlin, D.S.; Arendrup, M.C. Azole resistance in *Aspergillus fumigatus* from bronchoalveolar lavage fluid samples of patients with chronic diseases. *J. Antimicrob. Chemother.* **2013**, *68*, 1497–1504. [CrossRef] [PubMed]
62. Pham, C.D.; Reiss, E.; Hagen, F.; Meis, J.F.; Lockhart, S.R. Passive surveillance for azole-resistant *Aspergillus fumigatus*, United States, 2011–2013. *Emerg. Infect. Dis.* **2014**, *20*, 1498–1503. [CrossRef] [PubMed]
63. Escribano, P.; Pelaez, T.; Munoz, P.; Bouza, E.; Guinea, J. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? *Antimicrob. Agents Chemother.* **2013**, *57*, 2815–2820. [CrossRef] [PubMed]

64. Toyotome, T.; Fujiwara, T.; Kida, H.; Matsumoto, M.; Wada, T.; Komatsu, R. Susceptibility to azoles in clinical isolates of *Aspergillus fumigatus* and *A. tubingensis* fron Obihiro, Japan. In Proceedings of 7th Advances Against Aspergillosis, Manchester, UK, 3–5 March 2016.
65. Abdolrasouli, A.; Rhodes, J.; Beale, M.A.; Hagen, F.; Rogers, T.R.; Chowdhary, A.; Meis, J.F.; Armstrong-James, D.; Fisher, M.C. Genomic context of azole resistance mutations in *Aspergillus fumigatus* determined using whole-genome sequencing. *MBio.* **2015**, *6*, e00536. [PubMed]
66. Astvad, K.M.; Jensen, R.H.; Hassan, T.M.; Mathiasen, E.G.; Thomsen, G.M.; Pedersen, U.G.; Christensen, M.; Hilberg, O.; Arendrup, M.C. First detection of TR46/Y121F/T289A and TR34/L98H alterations in *Aspergillus fumigatus* isolates from azole-naive patients in Denmark despite negative findings in the environment. *Antimicrob. Agents Chemother.* **2014**, *58*, 5096–5101. [CrossRef] [PubMed]
67. Fuhren, J.; Voskuil, W.S.; Boel, C.H.; Haas, P.J.; Hagen, F.; Meis, J.F.; Kusters, J.G. High prevalence of azole resistance in *Aspergillus fumigatus* isolates from high-risk patients. *J. Antimicrob. Chemother.* **2015**, *70*, 2894–2898. [CrossRef] [PubMed]
68. Jeurissen, A.; Cooreman, S.; Van, K.W.; Van, L.J.; Vanhove, P.; Lagrou, K.; Heytens, L. Invasive pulmonary aspergillosis due to a multi-azole resistant *Aspergillus fumigatus*. *Acta Clin. Belg.* **2012**, *67*, 46–48. [PubMed]
69. Kurzyk, E.M.; Nawrot, U.; Mroczynska, M.; Wlodarczyk, K.; Ussowicz, M.; Zdziarski, P.; Arendrup, M.C.; Brillowska-Dabrowska, A. Detection of clinical *Aspergillus fumigatus* isolates resistant to triazoles. In Proceedings of the 7th Trends in Medical Mycology, Lisbon, Portugal, 9–12 October 2015; Volume 58, pp. 53–54.
70. Mortensen, K.L.; Mellado, E.; Lass-Florl, C.; Rodriguez-Tudela, J.L.; Johansen, H.K.; Arendrup, M.C. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob. Agents Chemother.* **2010**, *54*, 4545–4549. [CrossRef] [PubMed]
71. Ozmerdiven, G.E.; Ak, S.; Ener, B.; Agca, H.; Cilo, B.D.; Tunca, B.; Akalin, H. First determination of azole resistance in *Aspergillus fumigatus* strains carrying the TR34/L98H mutations in Turkey. *J. Infect. Chemother.* **2015**, *21*, 581–586. [CrossRef] [PubMed]
72. Rath, P.M.; Buchheidt, D.; Spiess, B.; Arfanis, E.; Buer, J.; Steinmann, J. First reported case of azole-resistant *Aspergillus fumigatus* due to the TR34/L98H mutation in Germany. *Antimicrob. Agents Chemother.* **2012**, *56*, 6060–6061. [CrossRef] [PubMed]
73. Rocchi, S.; Daguindau, E.; Grenouillet, F.; Deconinck, E.; Bellanger, A.P.; Garcia-Hermoso, D.; Bretagne, S.; Reboux, G.; Millon, L. Azole-resistant *Aspergillus fumigatus* isolate with the TR34/L98H mutation in both a fungicide-sprayed field and the lung of a hematopoietic stem cell transplant recipient with invasive aspergillosis. *J. Clin. Microbiol.* **2014**, *52*, 1724–1726. [CrossRef] [PubMed]
74. Snelders, E.; Huis In 't Veld, R.A.; Rijs, A.J.; Kema, G.H.; Melchers, W.J.; Verweij, P.E. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl. Environ. Microbiol.* **2009**, *75*, 4053–4057. [CrossRef] [PubMed]
75. van Ingen, J.; van der Lee, H.A.; Rijs, T.A.; Zoll, J.; Leenstra, T.; Melchers, W.J.; Verweij, P.E. Azole, polyene and echinocandin MIC distributions for wild-type, TR34/L98H and TR46/Y121F/T289A *Aspergillus fumigatus* isolates in The Netherlands. *J. Antimicrob. Chemother.* **2015**, *70*, 178–181. [CrossRef] [PubMed]
76. Vermeulen, E.; Maertens, J.; De, B.A.; Nulens, E.; Boelens, J.; Surmont, I.; Mertens, A.; Boel, A.; Lagrou, K. Nationwide surveillance of azole resistance in *Aspergillus* diseases. *Antimicrob. Agents Chemother.* **2015**, *59*, 4569–4576. [CrossRef] [PubMed]
77. Ahmad, S.; Joseph, L.; Hagen, F.; Meis, J.F.; Khan, Z. Concomitant occurrence of itraconazole-resistant and -susceptible strains of *Aspergillus fumigatus* in routine cultures. *J. Antimicrob. Chemother.* **2015**, *70*, 412–415. [CrossRef] [PubMed]
78. Ahmad, S.; Khan, Z.; Hagen, F.; Meis, J.F. Occurrence of triazole-resistant *Aspergillus fumigatus* with TR34/L98H mutations in outdoor and hospital environment in Kuwait. *Environ. Res.* **2014**, *133*, 20–26. [CrossRef] [PubMed]
79. Badali, H.; Vaezi, A.; Haghani, I.; Yazdanparast, S.A.; Hedayati, M.T.; Mousavi, B.; Ansari, S.; Hagen, F.; Meis, J.F.; Chowdhary, A. Environmental study of azole-resistant *Aspergillus fumigatus* with TR34/L98H mutations in the *cyp*51A gene in Iran. *Mycoses* **2013**, *56*, 659–663. [CrossRef] [PubMed]
80. Chowdhary, A.; Kathuria, S.; Randhawa, H.S.; Gaur, S.N.; Klaassen, C.H.; Meis, J.F. Isolation of multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR34/L98H mutations in the *cyp*51A gene in India. *J. Antimicrob. Chemother.* **2012**, *67*, 362–366. [CrossRef] [PubMed]

81. Chowdhary, A.; Kathuria, S.; Xu, J.; Sharma, C.; Sundar, G.; Singh, P.K.; Gaur, S.N.; Hagen, F.; Klaassen, C.H.; Meis, J.F. Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR34/L98H mutations in the *cyp*51A gene in India. *PLoS ONE* **2012**, *7*, e52871. [CrossRef] [PubMed]
82. Chowdhary, A.; Sharma, C.; Kathuria, S.; Hagen, F.; Meis, J.F. Azole-resistant *Aspergillus fumigatus* with the environmental TR46/Y121F/T289A mutation in India. *J. Antimicrob. Chemother.* **2014**, *69*, 555–557. [CrossRef] [PubMed]
83. Liu, M.; Zeng, R.; Zhang, L.; Li, D.; Lv, G.; Shen, Y.; Zheng, H.; Zhang, Q.; Zhao, J.; Zheng, N.; et al. Multiple *cyp*51A-based mechanisms identified in azole-resistant isolates of *Aspergillus fumigatus* from China. *Antimicrob. Agents Chemother.* **2015**, *59*, 4321–4325. [CrossRef] [PubMed]
84. Mohammadi, F.; Hashemi, S.J.; Zoll, J.; Melchers, W.J.; Rafati, H.; Dehghan, P.; Rezaie, S.; Tolooe, A.; Tamadon, Y.; van der Lee, H.A.; et al. Quantitative analysis of single-nucleotide polymorphism for rapid detection of TR_{34}. *Antimicrob. Agents Chemother.* **2015**, *60*, 387–392. [CrossRef] [PubMed]
85. Perveen, I.; Sehar, S.; Naz, I.; Ahmed, S. Prospective evaluation of azole resistance in *Aspergillus fumigatus* clinical isolates in Pakistan. In Proceedings of 7th Advances Against Aspergillosis, Manchester, UK, 3–5 March 2016.
86. Seyedmousavi, S.; Hashemi, S.J.; Zibafar, E.; Zoll, J.; Hedayati, M.T.; Mouton, J.W.; Melchers, W.J.; Verweij, P.E. Azole-resistant *Aspergillus fumigatus*, Iran. *Emerg. Infect. Dis.* **2013**, *19*, 832–834. [CrossRef] [PubMed]
87. Wu, C.J.; Wang, H.C.; Lee, J.C.; Lo, H.J.; Dai, C.T.; Chou, P.H.; Ko, W.C.; Chen, Y.C. Azole-resistant *Aspergillus fumigatus* isolates carrying TR34/L98H mutations in Taiwan. *Mycoses* **2015**, *58*, 544–549. [CrossRef] [PubMed]
88. Le Pape, P.; Lavergne, R.A.; Morio, F.; Alvarez-Moreno, C. Multiple fungicide-driven alterations in azole-resistant *Aspergillus fumigatus*, Colombia, 2015. *Emerg. Infect. Dis.* **2016**, *22*, 156–157. [CrossRef] [PubMed]
89. Wiederhold, N.P.; Garcia-Gil, V.; Lindner, J.R.; Sanders, C.; Fan, H.; Sutton, D.A.; Fothergill, A.W. Evaluation of *cyp*5A mechanisms of azole resistance in *Aspergillus fumigatus* isolates from the United States. In Proceedings of the 7th Trends in Medical Mycology, Lisbon, Portugal, 9–12 October 2015; Volume 58, p. 55.
90. Chowdhary, A.; Sharma, C.; van den Boom, M.; Yntema, J.B.; Hagen, F.; Verweij, P.E.; Meis, J.F. Multi-azole-resistant *Aspergillus fumigatus* in the environment in Tanzania. *J. Antimicrob. Chemother.* **2014**, *69*, 2979–2983. [CrossRef] [PubMed]
91. Lavergne, R.A.; Morio, F.; Favennec, L.; Dominique, S.; Meis, J.F.; Gargala, G.; Verweij, P.E.; Le Pape, P. First description of azole-resistant *Aspergillus fumigatus* due to TR46/Y121F/T289A mutation in France. *Antimicrob. Agents Chemother.* **2015**, *59*, 4331–4335. [CrossRef] [PubMed]
92. Montesinos, I.; Dodemont, M.; Lagrou, K.; Jacobs, F.; Etienne, I.; Denis, O. New case of azole-resistant *Aspergillus fumigatus* due to TR46/Y121F/T289A mutation in Belgium. *J. Antimicrob. Chemother.* **2014**, *69*, 3439–3440. [CrossRef] [PubMed]
93. Pelaez, T.; Monteiro, M.C.; Garcia-Rubio, R.; Bouza, E.; Gomez-Lopez, A.; Mellado, E. First detection of *Aspergillus fumigatus* azole-resistant strain due to *cyp*51A TR46/Y121F/T289A in an azole-naive patient in Spain. *New Microbes. New Infect.* **2015**, *6*, 33–34. [CrossRef] [PubMed]
94. Vermeulen, E.; Maertens, J.; Schoemans, H.; Lagrou, K. Azole-resistant *Aspergillus fumigatus* due to TR46/Y121F/T289A mutation emerging in Belgium, July 2012. *Euro. Surveill* **2012**, *17*, pii20326.
95. Hagiwara, D.; Takahashi, H.; Fujimoto, M.; Sugahara, M.; Misawa, Y.; Gonoi, T.; Itoyama, S.; Watanabe, A.; Kamei, K. Multi-azole resistant *Aspergillus fumigatus* harboring *cyp*51A TR46/Y121F/T289A isolated in Japan. *J. Infect. Chemother.* **2016**. [CrossRef] [PubMed]
96. Chen, Y.; Wang, H.; Lu, Z.; Li, P.; Zhang, Q.; Jia, T.; Zhao, J.; Tian, S.; Han, X.; Chen, F.; et al. Emergence of TR46/Y121F/T289A in an *Aspergillus fumigatus* isolate from a Chinese patient. *Antimicrob Agents Chemother* **2015**, *59*, 7148–7150. [CrossRef] [PubMed]
97. Hagiwara, D.; Takahashi, H.; Watanabe, A.; Takahashi-Nakaguchi, A.; Kawamoto, S.; Kamei, K.; Gonoi, T. Whole-genome comparison of *Aspergillus fumigatus* strains serially isolated from patients with aspergillosis. *J. Clin. Microbiol.* **2014**, *52*, 4202–4209. [CrossRef] [PubMed]
98. Denning, D.W.; Park, S.; Lass-Florl, C.; Fraczek, M.G.; Kirwan, M.; Gore, R.; Smith, J.; Bueid, A.; Moore, C.B.; Bowyer, P.; et al. High-frequency triazole resistance found in nonculturable *Aspergillus fumigatus* from lungs of patients with chronic fungal disease. *Clin. Infect. Dis.* **2011**, *52*, 1123–1129. [CrossRef] [PubMed]

99. Bueid, A.; Moore, C.B.; Denning, D.W.; Bowyer, P. High-level expression of *cyp*51B in azole-resistant clinical *Aspergillus fumigatus* isolates. *J. Antimicrob. Chemother.* **2013**. [CrossRef] [PubMed]
100. Cannon, R.D.; Lamping, E.; Holmes, A.R.; Niimi, K.; Baret, P.V.; Keniya, M.V.; Tanabe, K.; Niimi, M.; Goffeau, A.; Monk, B.C. Efflux-mediated antifungal drug resistance. *Clin. Microbiol. Rev.* **2009**, *22*, 291–321. [CrossRef] [PubMed]
101. Chamilos, G.; Kontoyiannis, D.P. Update on antifungal drug resistance mechanisms of *Aspergillus fumigatus*. *Drug Resist. Updat.* **2005**, *8*, 344–358. [CrossRef] [PubMed]
102. Tobin, M.B.; Peery, R.B.; Skatrud, P.L. Genes encoding multiple drug resistance-like proteins in *Aspergillus fumigatus* and *Aspergillus flavus*. *Gene* **1997**, *200*, 11–23. [CrossRef]
103. Slaven, J.W.; Anderson, M.J.; Sanglard, D.; Dixon, G.K.; Bille, J.; Roberts, I.S.; Denning, D.W. Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, *atrF*, in the presence of itraconazole in an itraconazole resistant clinical isolate. *Fungal. Genet. Biol.* **2002**, *36*, 199–206. [CrossRef]
104. Rajendran, R.; Mowat, E.; McCulloch, E.; Lappin, D.F.; Jones, B.; Lang, S.; Majithiya, J.B.; Warn, P.; Williams, C.; Ramage, G. Azole resistance of *Aspergillus fumigatus* biofilms is partly associated with efflux pump activity. *Antimicrob. Agents Chemother.* **2011**, *55*, 2092–2097. [CrossRef] [PubMed]
105. da Silva Ferreira, M.E.; Malavazi, I.; Savoldi, M.; Brakhage, A.A.; Goldman, M.H.; Kim, H.S.; Nierman, W.C.; Goldman, G.H. Transcriptome analysis of *Aspergillus fumigatus* exposed to voriconazole. *Curr. Genet.* **2006**, *50*, 32–44. [CrossRef] [PubMed]
106. Fraczek, M.G.; Bromley, M.; Buied, A.; Moore, C.B.; Rajendran, R.; Rautemaa, R.; Ramage, G.; Denning, D.W.; Bowyer, P. The *cdr*1B efflux transporter is associated with non-*cyp*51A-mediated itraconazole resistance in *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* **2013**, *68*, 1486–1496. [CrossRef] [PubMed]
107. Xiong, Q.; Hassan, S.A.; Wilson, W.K.; Han, X.Y.; May, G.S.; Tarrand, J.J.; Matsuda, S.P. Cholesterol import by *Aspergillus fumigatus* and its influence on antifungal potency of sterol biosynthesis inhibitors. *Antimicrob. Agents Chemother.* **2005**, *49*, 518–524. [CrossRef] [PubMed]
108. Willger, S.D.; Puttikamonkul, S.; Kim, K.H.; Burritt, J.B.; Grahl, N.; Metzler, L.J.; Barbuch, R.; Bard, M.; Lawrence, C.B.; Cramer, R.A., Jr. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. *PLoS Pathog.* **2008**, *4*, e1000200. [CrossRef] [PubMed]
109. Blatzer, M.; Barker, B.M.; Willger, S.D.; Beckmann, N.; Blosser, S.J.; Cornish, E.J.; Mazurie, A.; Grahl, N.; Haas, H.; Cramer, R.A. SREBP coordinates iron and ergosterol homeostasis to mediate triazole drug and hypoxia responses in the human fungal pathogen *Aspergillus fumigatus*. *PLoS Genet.* **2011**, *7*, e1002374. [CrossRef] [PubMed]
110. Blosser, S.J.; Cramer, R.A. SREBP-dependent triazole susceptibility in *Aspergillus fumigatus* is mediated through direct transcriptional regulation of *erg*11A (*cyp*51A). *Antimicrob. Agents Chemother.* **2012**, *56*, 248–257. [CrossRef] [PubMed]
111. Lamoth, F.; Juvvadi, P.R.; Fortwendel, J.R.; Steinbach, W.J. Heat shock protein 90 is required for conidiation and cell wall integrity in *Aspergillus fumigatus*. *Eukaryot. Cell* **2012**, *11*, 1324–1332. [CrossRef] [PubMed]
112. Cowen, L.E.; Lindquist, S. Hsp90 potentiates the rapid evolution of new traits: Drug resistance in diverse fungi. *Science* **2005**, *309*, 2185–2189. [CrossRef] [PubMed]
113. Camps, S.M.; Dutilh, B.E.; Arendrup, M.C.; Rijs, A.J.; Snelders, E.; Huynen, M.A.; Verweij, P.E.; Melchers, W.J. Discovery of a *hapE* mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing. *PLoS ONE* **2012**, *7*, e50034. [CrossRef] [PubMed]
114. Lescar, J.; Meyer, I.; Akshita, K.; Srinivasaraghavan, K.; Verma, C.; Palous, M.; Mazier, D.; Datry, A.; Fekkar, A. *Aspergillus fumigatus* harbouring the sole Y121F mutation shows decreased susceptibility to voriconazole but maintained susceptibility to itraconazole and posaconazole. *J. Antimicrob. Chemother.* **2014**, *69*, 3244–3247. [CrossRef] [PubMed]
115. Arabatzis, M.; Kambouris, M.; Kyprianou, M.; Chrysaki, A.; Foustoukou, M.; Kanellopoulou, M.; Kondyli, L.; Kouppari, G.; Koutsia-Karouzou, C.; Lebessi, E.; et al. Polyphasic identification and susceptibility to seven antifungals of 102 *Aspergillus* isolates recovered from immunocompromised hosts in Greece. *Antimicrob. Agents Chemother.* **2011**, *55*, 3025–3030. [CrossRef] [PubMed]
116. Ziolkowska, G.; Tokarzewski, S.; Nowakiewicz, A. Drug resistance of *Aspergillus fumigatus* strains isolated from flocks of domestic geese in Poland. *Poult. Sci.* **2014**, *93*, 1106–1112. [CrossRef] [PubMed]

117. Araujo, R.; Pina-Vaz, C.; Rodrigues, A.G. Susceptibility of environmental versus clinical strains of pathogenic *Aspergillus*. *Int. J. Antimicrob. Agents* **2007**, *29*, 108–111. [CrossRef] [PubMed]
118. Sharma, C.; Hagen, F.; Moroti, R.; Meis, J.F.; Chowdhary, A. Triazole-resistant *Aspergillus fumigatus* harbouring G54 mutation: Is it de novo or environmentally acquired? *J. Glob. Antimicrob. Resist.* **2015**. [CrossRef]
119. Alastruey-Izquierdo, A.; Mellado, E.; Pelaez, T.; Peman, J.; Zapico, S.; Alvarez, M.; Rodriguez-Tudela, J.L.; Cuenca-Estrella, M. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP study). *Antimicrob. Agents Chemother.* **2013**, *57*, 3380–3387. [CrossRef] [PubMed]
120. Chryssanthou, E. In vitro susceptibility of respiratory isolates of *Aspergillus* species to itraconazole and amphotericin B. Acquired resistance to itraconazole. *Scand. J. Infect. Dis.* **1997**, *29*, 509–512. [CrossRef] [PubMed]
121. Kikuchi, K.; Watanabe, A.; Ito, J.; Oku, Y.; Wuren, T.; Taguchi, H.; Yarita, K.; Muraosa, Y.; Yahiro, M.; Yaguchi, T.; et al. Antifungal susceptibility of *Aspergillus fumigatus* clinical isolates collected from various areas in Japan. *J. Infect. Chemother.* **2014**, *20*, 336–338. [CrossRef] [PubMed]
122. Baddley, J.W.; Marr, K.A.; Andes, D.R.; Walsh, T.J.; Kauffman, C.A.; Kontoyiannis, D.P.; Ito, J.I.; Balajee, S.A.; Pappas, P.G.; Moser, S.A. Patterns of susceptibility of *Aspergillus* isolates recovered from patients enrolled in the transplant-associated infection surveillance network. *J. Clin. Microbiol.* **2009**, *47*, 3271–3275. [CrossRef] [PubMed]
123. Lockhart, S.R.; Frade, J.P.; Etienne, K.A.; Pfaller, M.A.; Diekema, D.J.; Balajee, S.A. Azole resistance in *Aspergillus fumigatus* isolates from the ARTEMIS global surveillance study is primarily due to the TR34/L98H mutation in the *cyp*51A gene. *Antimicrob. Agents Chemother.* **2011**, *55*, 4465–4468. [CrossRef] [PubMed]
124. Castanheira, M.; Messer, S.A.; Rhomberg, P.R.; Pfaller, M.A. Antifungal susceptibility patterns of a global collection of fungal isolates: Results of the sentry antifungal surveillance program (2013). *Diagn Microbiol Infect. Dis* **2016**, *85*, 200–204. [CrossRef] [PubMed]
125. Hsueh, P.R.; Lau, Y.J.; Chuang, Y.C.; Wan, J.H.; Huang, W.K.; Shyr, J.M.; Yan, J.J.; Yu, K.W.; Wu, J.J.; Ko, W.C.; et al. Antifungal susceptibilities of clinical isolates of *Candida* species, *Cryptococcus neoformans*, and *Aspergillus* species from Taiwan: Surveillance of multicenter antimicrobial resistance in Taiwan program data from 2003. *Antimicrob. Agents Chemother.* **2005**, *49*, 512–517. [CrossRef] [PubMed]
126. Asano, M.; Kano, R.; Makimura, K.; Hasegawa, A.; Kamata, H. Molecular typing and in vitro activity of azoles against clinical isolates of *Aspergillus fumigatus* and *A. niger* in Japan. *J. Infect. Chemother.* **2011**, *17*, 483–486. [CrossRef] [PubMed]
127. Kano, R.; Kohata, E.; Tateishi, A.; Murayama, S.Y.; Hirose, D.; Shibata, Y.; Kosuge, Y.; Inoue, H.; Kamata, H.; Hasegawa, A. Does farm fungicide use induce azole resistance in *Aspergillus fumigatus*? *Med. Mycol.* **2015**, *53*, 174–177. [CrossRef] [PubMed]
128. European Commision Health & Consumer Protection Directorate-General. Opinion on azole antimycotic resistance. Available online: http://ec.europa.eu/food/fs/sc/ssc/out278_en.pdf (accessed on 25 April 2016).
129. Lockhart, S.R. Azole resistance in the Americas: Not catching up with europe (yet). In Proceedings of the 7th Trends in Medical Mycology, Lisbon, Portugal, 9–12 October 2015; Volume 58, p. 20.
130. Richardson, M.; Lass-Florl, C. Changing epidemiology of systemic fungal infections. *Clin. Microbiol. Infect.* **2008**, *14*, 5–24. [CrossRef] [PubMed]
131. Balajee, S.A.; Kano, R.; Baddley, J.W.; Moser, S.A.; Marr, K.A.; Alexander, B.D.; Andes, D.; Kontoyiannis, D.P.; Perrone, G.; Peterson, S.; et al. Molecular identification of *Aspergillus* species collected for the transplant-associated infection surveillance network. *J. Clin. Microbiol.* **2009**, *47*, 3138–3141. [CrossRef] [PubMed]
132. Alastruey-Izquierdo, A.; Alcazar-Fuoli, L.; Cuenca-Estrella, M. Antifungal susceptibility profile of cryptic species of *Aspergillus*. *Mycopathologia* **2014**, *178*, 427–433. [CrossRef] [PubMed]
133. Alcazar-Fuoli, L.; Mellado, E.; Alastruey-Izquierdo, A.; Cuenca-Estrella, M.; Rodriguez-Tudela, J.L. *Aspergillus* section *fumigati*: Antifungal susceptibility patterns and sequence-based identification. *Antimicrob. Agents Chemother.* **2008**, *52*, 1244–1251. [CrossRef] [PubMed]
134. Balajee, S.A.; Gribskov, J.; Brandt, M.; Ito, J.; Fothergill, A.; Marr, K.A. Mistaken identity: *Neosartorya pseudofischeri* and its anamorph masquerading as *Aspergillus fumigatus*. *J. Clin. Microbiol.* **2005**, *43*, 5996–5999. [CrossRef] [PubMed]

135. Montenegro, G.; Sanchez, P.S.; Jewtuchowicz, V.M.; Pinoni, M.V.; Relloso, S.; Temporitti, E.; Iovannitti, C.A.; Mujica, M.T. Phenotypic and genotypic characterization of *Aspergillus lentulus* and *Aspergillus fumigatus* isolates in a patient with probable invasive aspergillosis. *J. Med. Microbiol.* **2009**, *58*, 391–395. [CrossRef] [PubMed]
136. Sugui, J.A.; Vinh, D.C.; Nardone, G.; Shea, Y.R.; Chang, Y.C.; Zelazny, A.M.; Marr, K.A.; Holland, S.M.; Kwon-Chung, K.J. *Neosartorya udagawae* (*Aspergillus udagawae*), an emerging agent of aspergillosis: How different is it from *Aspergillus fumigatus*? *J. Clin. Microbiol.* **2010**, *48*, 220–228. [CrossRef] [PubMed]
137. Vinh, D.C.; Shea, Y.R.; Jones, P.A.; Freeman, A.F.; Zelazny, A.; Holland, S.M. Chronic invasive aspergillosis caused by *Aspergillus viridinutans*. *Emerg. Infect. Dis.* **2009**, *15*, 1292–1294. [CrossRef] [PubMed]
138. Hendrickx, M.; Beguin, H.; Detandt, M. Genetic re-identification and antifungal susceptibility testing of *Aspergillus* section *nigri* strains of the BCCM/IHEM collection. *Mycoses* **2012**, *55*, 148–155. [CrossRef] [PubMed]
139. Stevens, D.A.; Espiritu, M.; Parmar, R. Paradoxical effect of caspofungin: Reduced activity against *Candida albicans* at high drug concentrations. *Antimicrob Agents Chemother* **2004**, *48*, 3407–3411. [CrossRef] [PubMed]
140. Alcazar-Fuoli, L.; Mellado, E.; Alastruey-Izquierdo, A.; Cuenca-Estrella, M.; Rodriguez-Tudela, J.L. Species identification and antifungal susceptibility patterns of species belonging to *Aspergillus* section *nigri*. *Antimicrob. Agents Chemother.* **2009**, *53*, 4514–4517. [CrossRef] [PubMed]
141. Szigeti, G.; Kocsube, S.; Doczi, I.; Bereczki, L.; Vagvolgyi, C.; Varga, J. Molecular identification and antifungal susceptibilities of black *Aspergillus* isolates from otomycosis cases in Hungary. *Mycopathologia* **2012**, *174*, 143–147. [CrossRef] [PubMed]
142. Krishnan, S.; Manavathu, E.K.; Chandrasekar, P.H. *Aspergillus flavus*: An emerging non-*fumigatus Aspergillus* species of significance. *Mycoses* **2009**, *52*, 206–222. [CrossRef] [PubMed]
143. Balajee, S.A.; Lindsley, M.D.; Iqbal, N.; Ito, J.; Pappas, P.G.; Brandt, M.E. Nonsporulating clinical isolate identified as *Petromyces alliaceus* (anamorph *Aspergillus alliaceus*) by morphological and sequence-based methods. *J. Clin. Microbiol.* **2007**, *45*, 2701–2703. [CrossRef] [PubMed]
144. Ozhak-Baysan, B.; Alastruey-Izquierdo, A.; Saba, R.; Ogunc, D.; Ongut, G.; Timuragaoglu, A.; Arslan, G.; Cuenca-Estrella, M.; Rodriguez-Tudela, J.L. *Aspergillus alliaceus* and *Aspergillus flavus* co-infection in an acute myeloid leukemia patient. *Med. Mycol.* **2010**, *48*, 995–999. [CrossRef] [PubMed]
145. Liu, W.; Sun, Y.; Chen, W.; Liu, W.; Wan, Z.; Bu, D.; Li, R. The T788G mutation in the *cyp*51C gene confers voriconazole resistance in *Aspergillus flavus* causing aspergillosis. *Antimicrob. Agents Chemother.* **2012**, *56*, 2598–2603. [CrossRef] [PubMed]
146. Paul, R.A.; Rudramurthy, S.M.; Meis, J.F.; Mouton, J.W.; Chakrabarti, A. A novel Y319H substitution in *cyp*51C associated with azole resistance in *Aspergillus flavus*. *Antimicrob Agents Chemother* **2015**, *59*, 6615–6619. [CrossRef] [PubMed]
147. Lass-Florl, C.; Griff, K.; Mayr, A.; Petzer, A.; Gastl, G.; Bonatti, H.; Freund, M.; Kropshofer, G.; Dierich, M.P.; Nachbaur, D. Epidemiology and outcome of infections due to *Aspergillus terreus*: 10-year single centre experience. *Br. J. Haematol.* **2005**, *131*, 201–207. [CrossRef] [PubMed]
148. Steinbach, W.J.; Perfect, J.R.; Schell, W.A.; Walsh, T.J.; Benjamin, D.K., Jr. In vitro analyses, animal models, and 60 clinical cases of invasive *Aspergillus terreus* infection. *Antimicrob. Agents Chemother.* **2004**, *48*, 3217–3225. [CrossRef] [PubMed]
149. Graybill, J.R.; Hernandez, S.; Bocanegra, R.; Najvar, L.K. Antifungal therapy of murine *Aspergillus terreus* infection. *Antimicrob. Agents Chemother.* **2004**, *48*, 3715–3719. [CrossRef] [PubMed]
150. Arendrup, M.C.; Jensen, R.H.; Grif, K.; Skov, M.; Pressler, T.; Johansen, H.K.; Lass-Florl, C. In vivo emergence of *Aspergillus terreus* with reduced azole susceptibility and a *cyp*51A M217I alteration. *J. Infect. Dis.* **2012**, *206*, 981–985. [CrossRef] [PubMed]
151. Samson, R.A.; Peterson, S.W.; Frisvad, J.C.; Varga, J. New species in *Aspergillus* section *terrei*. *Stud. Mycol.* **2011**, *69*, 39–55. [CrossRef] [PubMed]
152. Guinea, J.; Sandoval-Denis, M.; Escribano, P.; Pelaez, T.; Guarro, J.; Bouza, E. *Aspergillus citrinoterreus*, a new species of section *terrei* isolated from samples of patients with nonhematological predisposing conditions. *J. Clin. Microbiol.* **2015**, *53*, 611–617. [CrossRef] [PubMed]

153. Kathuria, S.; Sharma, C.; Singh, P.K.; Agarwal, P.; Agarwal, K.; Hagen, F.; Meis, J.F.; Chowdhary, A. Molecular epidemiology and in vitro antifungal susceptibility of *Aspergillus terreus* species complex isolates in Delhi, India: Evidence of genetic diversity by amplified fragment length polymorphism and microsatellite typing. *PLoS ONE* **2015**, *10*, e0118997. [CrossRef] [PubMed]
154. Varga, J.; Houbraken, J.; van der Lee, H.A.; Verweij, P.E.; Samson, R.A. *Aspergillus calidoustus* sp. nov., causative agent of human infections previously assigned to *Aspergillus ustus*. *Eukaryot. Cell* **2008**, *7*, 630–638. [CrossRef] [PubMed]
155. Alastruey-Izquierdo, A.; Cuesta, I.; Houbraken, J.; Cuenca-Estrella, M.; Monzon, A.; Rodriguez-Tudela, J.L. In vitro activity of nine antifungal agents against clinical isolates of *Aspergillus calidoustus*. *Med. Mycol.* **2010**, *48*, 97–102. [CrossRef] [PubMed]
156. Verweij, P.E.; Ananda-Rajah, M.; Andes, D.; Arendrup, M.C.; Bruggemann, R.J.; Chowdhary, A.; Cornely, O.A.; Denning, D.W.; Groll, A.H.; Izumikawa, K.; et al. International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. *Drug Resist. Updat.* **2015**, *21*, 30–40. [CrossRef] [PubMed]
157. Seyedmousavi, S.; Melchers, W.J.; Mouton, J.W.; Verweij, P.E. Pharmacodynamics and dose-response relationships of liposomal amphotericin B against different azole-resistant *Aspergillus fumigatus* isolates in a murine model of disseminated aspergillosis. *Antimicrob. Agents Chemother.* **2013**, *57*, 1866–1871. [CrossRef] [PubMed]
158. Mukherjee, P.K.; Sheehan, D.J.; Hitchcock, C.A.; Ghannoum, M.A. Combination treatment of invasive fungal infections. *Clin. Microbiol. Rev.* **2005**, *18*, 163–194. [CrossRef] [PubMed]
159. Krishnan-Natesan, S.; Wu, W.; Chandrasekar, P.H. In vitro efficacy of the combination of voriconazole and anidulafungin against voriconazole-resistant *cyp*51A mutants of *Aspergillus fumigatus*. *Diagn. Microbiol. Infect. Dis.* **2012**, *73*, 135–137. [CrossRef] [PubMed]
160. Marr, K.A.; Schlamm, H.T.; Herbrecht, R.; Rottinghaus, S.T.; Bow, E.J.; Cornely, O.A.; Heinz, W.J.; Jagannatha, S.; Koh, L.P.; Kontoyiannis, D.P.; et al. Combination antifungal therapy for invasive aspergillosis: A randomized trial. *Ann. Intern. Med.* **2015**, *162*, 81–89. [CrossRef] [PubMed]
161. Seyedmousavi, S.; Bruggemann, R.J.; Melchers, W.J.; Verweij, P.E.; Mouton, J.W. Pharmacodynamics of anidulafungin against clinical *Aspergillus fumigatus* isolates in a nonneutropenic murine model of disseminated aspergillosis. *Antimicrob. Agents Chemother* **2013**, *57*, 303–308. [CrossRef] [PubMed]
162. Denning, D.W.; Bowyer, P. Voriconazole resistance in *Aspergillus fumigatus*: Should we be concerned? *Clin. Infect. Dis.* **2013**, *57*, 521–523. [CrossRef] [PubMed]
163. European Centre for Disease Prevention and Control. Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species. Available online: http://ecdc.europa.eu/en/publications/Publications/risk-assessment-impact-environmental-usage-of-triazoles-on-Aspergillus-spp-resistance-to-medical-triazoles.pdf (accessed on 1 May 2016).

AAC Journals.ASM.org

Rapid Development of *Candida krusei* Echinocandin Resistance during Caspofungin Therapy

A. Forastiero,[a*] V. Garcia-Gil,[a*] O. Rivero-Menendez,[a] R. Garcia-Rubio,[a] M. C. Monteiro,[a] A. Alastruey-Izquierdo,[a] R. Jordan,[c] I. Agorio,[b] E. Mellado[a]

Mycology Reference Laboratory, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain[a]; Mycology Laboratory, Hospital Británico de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina[b]; Infectious Diseases Department, Hospital Británico de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina[c]

In invasive candidiasis, there has been an epidemiological shift from *Candida albicans* to non-*albicans* species infections, including infections with *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*. Although the prevalence of *C. krusei* remains low among yeast infections, its intrinsic resistance to fluconazole raises epidemiological and therapeutic concerns. Echinocandins have *in vitro* activity against most *Candida* spp. and are the first-line agents in the treatment of candidemia. Although resistance to echinocandin drugs is still rare, individual cases of *C. krusei* resistance have been reported in recent years, especially with strains that have been under selective pressure. A total of 15 *C. krusei* strains, isolated from the blood, urine, and soft tissue of an acute lymphocytic leukemia patient, were analyzed. Strains developed echinocandin resistance during 10 days of caspofungin therapy. The molecular epidemiology of the isolates was investigated using two different typing methods: PCR-based amplification of the species-specific repetitive polymorphic CKRS-1 sequence and multilocus sequence typing. All isolates were genetically related, and the mechanism involved in decreased echinocandin susceptibility was characterized. Clinical resistance was associated with an increase in echinocandin MICs *in vitro* and was related to three different mutations in hot spot 1 of the target enzyme Fks1p. Molecular evidence of the rapid acquisition of resistance by different mutations in *FKS1* highlights the need to monitor the development of resistance in *C. krusei* infections treated with echinocandin drugs.

The incidence of opportunistic fungal infections has increased in recent decades due to the growing number of immunocompromised patients. Concomitantly, the number of fungal species identified as pathogenic for humans has also increased. *Candida albicans* is the most common cause of candidemia, but there have been increased numbers of isolations of non-*albicans* species of *Candida* in recent years, with the most prominent being *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (1).

Candida krusei has been described as a causative agent of disseminated fungal infections in susceptible patients, resulting in the lowest 90-day survival rates (53.6%) among common *Candida* spp. (2). Although the prevalence of *C. krusei* remains low (2%) among yeast infections (3), its intrinsic resistance to fluconazole raises epidemiological and therapeutic concerns (4). While antifungal drug resistance in *C. albicans* isolates continues to be low (5), *C. glabrata* and *C. krusei* have emerged as important and potentially antifungal (azole)-resistant causes of candidemia (6). Therefore, echinocandins are a good therapeutic option for the treatment of invasive candidiasis, despite their cost and the absence of an oral form. They (i) possess fungicidal activity against most species of *Candida*, including those resistant to polyenes and to azoles (7); (ii) lack major adverse effects; and (iii) show no interaction with other drugs (8). Furthermore, echinocandins have been approved for the treatment of several fungal infections, including invasive candidiasis and candidemia, and are also indicated for empirical therapy in select high-risk patients (those with persistent fever and neutropenia) (9–11).

Echinocandins interfere with fungal cell wall synthesis through noncompetitive inhibition of the β-1,3-glucan synthase (GS) complex, the enzyme required for the synthesis of glucan. Inhibition occurs by targeting the catalytic subunit, which is encoded by three related genes: *FKS1*, *FKS2*, and *FKS3* (12). This results in a weakening of the fungal cell wall, a breakdown of cellular integrity, and cell lysis (13). Reports of clinical isolates of *Candida* spp. with decreased *in vitro* echinocandin susceptibility are still sporadic (<1.7 to 2%), including among the vast majority of *C. krusei* isolates (14, 15). However, individual cases of *C. krusei* resistance to the echinocandins have been reported (16–21). Acquired resistance to echinocandin therapy has been associated with amino acid substitutions caused by mutations in specific hot spot regions (HS1, HS2, and the recently described HS3 region) of the conserved *FKS1* target gene in different *Candida* species, such as *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. lusitaniae*, and *C. kefyr* (19, 22–27), as well as in the *FKS2* gene of *C. glabrata* (25, 28). These mutations have been related to prior echinocandin exposure and therapeutic failure in candidemic patients (18, 29–32). The most prominent changes occurred at the serine 645 position in Fks1p, with mutations to proline, tyrosine, and phenylalanine,

Received 28 April 2015 **Returned for modification** 6 June 2015
Accepted 25 August 2015

Accepted manuscript posted online 31 August 2015

Citation Forastiero A, Garcia-Gil V, Rivero-Menendez O, Garcia-Rubio R, Monteiro MC, Alastruey-Izquierdo A, Jordan R, Agorio I, Mellado E. 2015. Rapid development of *Candida krusei* echinocandin resistance during caspofungin therapy. Antimicrob Agents Chemother 59:6975–6982. doi:10.1128/AAC.01005-15.

Address correspondence to E. Mellado, emellado@isciii.es.

* Present address: A. Forastiero, Laboratorio de Micología, Hospital Británico de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina; V. Garcia-Gil, Servicio de Microbiología y Parasitología, Hospital Universitario La Paz, Madrid, Spain.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.01005-15.

TABLE 1 Sources, *FKS1*-HS1 amino acid substitutions, and susceptibilities of *Candida krusei* isolates[a]

Isolate	*FKS1*-HS1 substitution	MIC (mg/liter)					
		AmB	FLC	VRC	CSF	MCF	ANF
BC1_31/05/13	wt	0.5	64	0.5	0.5	0.06	0.03
BC2_11/06/13	P663Q	1.0	32	0.25	4	0.5	0.125
CC1_11/06/13	P663Q	0.5	32	0.25	4	0.5	0.125
BC3_14/06/13	F655L	0.5	32	0.5	4	1	0.5
CC2_14/06/13	P663Q	0.5	64	0.5	4	1	0.125
STC_15/06/13	P663Q	0.5	32	0.25	4	1	0.125
UC_15/06/13	F655L	0.5	32	0.5	2	1	0.5
CTC1_16/06/13	P663Q	0.5	64	0.5	1	1	0.125
BC4_16/06/13	S659P/S	0.25	32	0.5	2	0.5	0.06
BC5_19/06/13	P663Q	0.25	32	0.5	4	1	0.125
CTC2_23/06/13	S659P/S	0.25	32	0.5	2	0.5	0.06
BC6_23/06/13	F655L	0.25	32	0.5	4	1	1
BC7_26/06/13	P663Q	0.25	32	0.5	2	1	0.125
CC3_26/06/13	P663Q	0.25	32	0.5	4	1	0.125
BC8_29/06/13	P663Q	0.5	64	0.5	4	1	0.25

[a] MICs were determined by following the recommendations proposed by AFST-EUCAST. BC, blood culture; CC, catheter culture; STC, soft tissue culture; UC, urine culture; CTC, catheter tip culture; wt, wild type; P, proline; Q, glutamine; F, phenylalanine; L, leucine; S, serine; AmB, amphotericin B; FLC, fluconazole; VRC, voriconazole; CSF, caspofungin; MCF, micafungin; ANF, anidulafungin.

expressed either homozygously or heterozygously for the mutant allele, indicating that expression of the resistance phenotype is dominant (33).

In this work, we analyzed 15 isolates of *C. krusei* from a patient with acute lymphocytic leukemia (ALL) who suffered from a candidemia that rapidly developed echinocandin resistance during 10 days of caspofungin therapy. Molecular typing of isolates and characterization of the resistance mechanism that confers decreased *C. krusei* echinocandin susceptibility are both described.

MATERIALS AND METHODS

Strains. Fifteen *Candida krusei* isolates were serially recovered from a hematologic patient with catheter-related candidemia secondary to neutropenia due to chemotherapy treatment. Isolates were obtained before and after caspofungin therapy, as follows: (i) before, blood culture 1 (BC1); and (ii) after, BC2 to BC8, soft tissue culture (STC), urine culture (UC), catheter tip cultures (CTC1 and CTC2), and catheter cultures (CC1 to CC3) (Table 1). These isolates were first identified as *C. krusei* by morphological features (BBL CHROMagar *Candida* medium and corn meal agar with Tween 80; Sigma-Aldrich, Madrid, Spain) and then confirmed by DNA internal transcribed spacer (ITS) sequencing. *Candida krusei* ATCC 6258 (A) and five unrelated *C. krusei* strains (B, CNM-CL5642; C, CNM-CL5724; D, CNM-CL6695; E, CNM-CL6709; and F, CNM-CL7057) were used as control strains where required.

Antifungal susceptibility testing. Antifungal susceptibility was determined by the broth microdilution (BMD) and Etest methods. MICs were determined by following the recommendations proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) (34). Etest was carried out according to the manufacturer's instructions (TecLaim S.A.). For amphotericin B (AmB), the breakpoints were defined based on the wild-type distribution of MICs determined by the EUCAST method (≤1.0 mg/liter) (35). In the case of anidulafungin, the breakpoints proposed by AFST-EUCAST were used to interpret susceptibility results (susceptible [S], ≤0.06 mg/liter; and resistant [R], >0.06 mg/liter) (36). Due to significant interlaboratory variation in MIC ranges for caspofungin, EUCAST breakpoints have not yet been established. Caspofungin susceptibility testing has been associated with significant variation due (at least in part) to variability in the potency of different lots of pure caspofungin and to a lower stability in water, which was originally recommended as a solvent (37–39). Therefore, the anidulafungin MIC was used to determine the *in vitro* susceptibility to the echinocandin class, as recommended by EUCAST (36). Because EUCAST breakpoints for voriconazole have not yet been established, these breakpoints were defined following the recommendations of CLSI document M27-S4 (S, ≤0.5 μg/ml; susceptible dose dependent [SDD], 1 μg/ml; and R, ≥2 μg/ml) (37). However, since breakpoints are method specific, using CLSI breakpoints to interpret the results obtained by using the EUCAST methodology might not be an optimal approach, so this interpretation was made only for a presumptive evaluation.

Amplification and sequencing of the *FKS1* gene. Genomic DNAs of all *C. krusei* isolates were extracted using DNeasy plant minikits (Qiagen Inc., Valencia, CA) and used as templates for PCR amplification. PCR amplification and full sequencing of the three hot spots of the *FKS1* gene (HS1, nucleotides 1963 to 1989; HS2, nucleotides 4090 to 4113; and newly described HS3, nucleotides 2116 to 2148) for all isolates were achieved using a panel of oligonucleotide primers (synthesized by Sigma Genosys, Madrid, Spain) (see Table S1 in the supplemental material). Amplification conditions consisted of 2 min of denaturation at 95°C, followed by 40 cycles of 30 s at 95°C for denaturation, 30 s at 55°C for annealing, and 60 s at 72°C for elongation, with a final elongation step of 2 min at 72°C. PCR products were purified with High Pure PCR product purification kits (Roche Diagnostics, Madrid, Spain) according to the manufacturer's recommendations. PCR products were semiquantified by agarose gel electrophoresis and used as templates for sequencing, which was performed with a dye terminator cycle sequencing quick-start kit (Beckman Coulter Inc., Fullerton, CA). The sequencing PCR products were then purified through G50 Sepharose columns (Amersham Biosciences) and, finally, sequenced on a CEQ8000 DNA analysis system (Beckman Coulter) with the forward and reverse primers used for PCR amplification. Sequences from all 15 *C. krusei* isolates were compared with that of the caspofungin-susceptible *C. krusei* (ATCC 6258) control strain.

The amino acid sequences of all three hot spot regions of the catalytic subunit of the 1,3-β-D-glucan synthase complex (Fks1p) were deduced from the nucleotide sequences and analyzed using the MegAlign software package (DNAstar, Inc., Lasergene, Madison, WI). The amino acid sequence alignments were derived by CLUSTAL analysis (40).

Molecular typing of *Candida krusei*. A PCR method was used to fingerprint *C. krusei* strains. The polymorphic species-specific repetitive sequence CKRS-1 (*C. krusei* repeated sequence 1), located in the nontranscribed intergenic regions (IGRs) of rRNA genes, was amplified using the

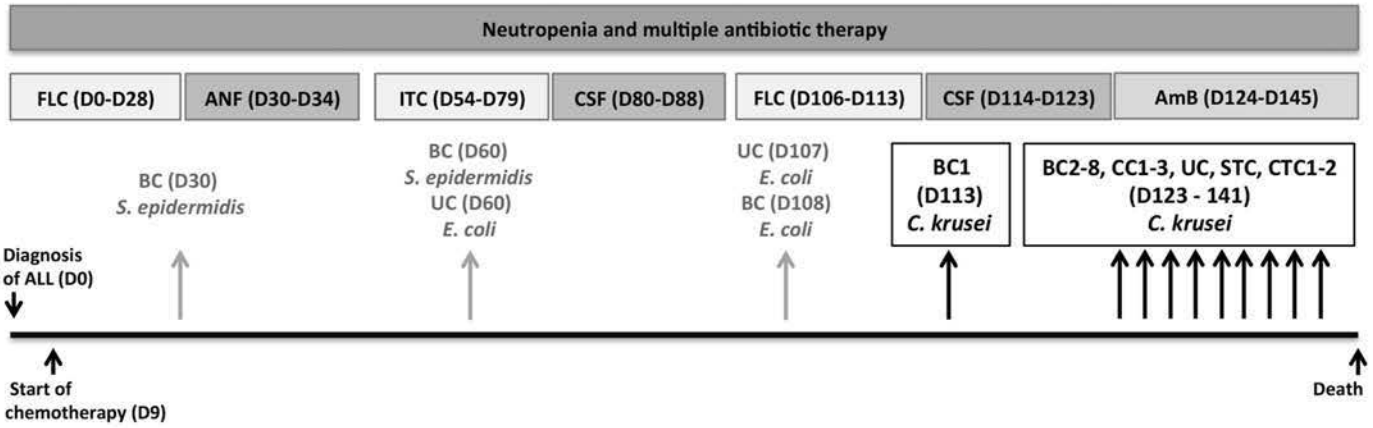


FIG 1 Time course of development of echinocandin resistance in different isolates from a patient suffering candidemia from *Candida krusei*. ALL, acute lymphocytic leukemia; D, days; FLC, fluconazole; ANF, anidulafungin; ITC, itraconazole; CSF, caspofungin; AmB, amphotericin B; BC, blood culture; CC, catheter culture; UC, urine culture; STC, soft tissue culture; CTC, catheter tip culture. The boxes represent the patient's isolates.

primers Arno1 (5′-GGCCAACACATACATACCTT-3′) and Arno2 (5′-GGTAGGATACTAACCACAGC-3′), previously described by Carlotti et al. (41). Each reaction mixture (50 μl) contained 5 μM (each) primers, PCR master mix (Life Science), and 1 μl of template DNA. Cycling conditions consisted of 4 min at 92°C followed by 32 cycles of 30 s at 92°C, 30 s at 55°C, and 2 min at 72°C, with a final step of 10 min at 72°C. Amplification products were resolved by electrophoresis through a 1.0% (wt/vol) agarose gel in Tris-acetate-EDTA (TAE) buffer for 4 h at 50 V, stained with ethidium bromide, and photographed under UV illumination. A 1-kb ladder (Promega) was used as a molecular size marker.

Six nonrelated *C. krusei* strains (strains A to F; see above) were used as control strains for typing. Two independent observers analyzed the patterns obtained with the polymorphic species-specific repetitive sequence CKRS-1 by visual grouping of the patterns. Also, cluster analysis was done by the unweighted-pair group method using average linkages (UPGMA), using the software PyElph, version 1.4 (http://pyelph.sourceforge.net/) (42).

For the second molecular typing method, multilocus sequence typing (MLST) was used. PCRs were used to amplify gene fragments of six genes (*NMT1*, *ADE2*, *LEU2*, *HIS3*, *TRP1*, and *LYS2*) by using previously described primers (43). Reaction volumes of 50 μl contained 100 ng of genomic DNA, 2.5 U *Taq* DNA polymerase (Applied Biosystems), 5 μl of 10× buffer (supplied with the enzyme), 2 mM $MgCl_2$, a 0.5 mM concentration of each deoxynucleoside triphosphate (Roche), and 1 mM (each) forward and reverse primers. A GeneAmp 9700 PCR system (Applied Biosystems) was used with a first cycle of denaturation for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 48°C/50°C for 1 min, and elongation at 72°C for 1 min, with a final extension step of 10 min at 72°C. The annealing temperature was 48°C for the *NMT1* and *ADE2* genes and 50°C for the *LEU2*, *HIS3*, *TRP1*, and *LYS2* genes. The amplified products were purified using Illustra Exoprostar 1-Step kits (GE Healthcare Life Science, Little Chalfont, United Kingdom). Both strands of purified gene fragments were sequenced on a CEQ8000 DNA analysis system (Beckman Coulter), using the same primers that were used in the PCR step. The sequence data were analyzed with DNASTAR software (Lasergene, Madison, WI). Heterozygosities were defined by the presence of two coincident peaks in the forward and reverse sequence chromatograms (43). The one-letter code for nucleotides from the International Union of Pure and Applied Chemistry (IUPAC) nomenclature was used to define results.

Finally, sequences were searched against those in the MLST *C. krusei* consensus sequence database (http://pubmlst.org/ckrusei/) to obtain an allele number for each locus. Once all six loci had allele numbers assigned, these were entered into the online database to determine the diploid sequence type (DST).

RESULTS

***Candida krusei* strain isolation.** On 6 February 2013, a 63-year-old female with acute lymphocytic leukemia (ALL) was admitted to the hospital to begin chemotherapy. During the following 3 months, despite having received prophylaxis with a broad-spectrum antibiotic (ciprofloxacin) and an antifungal drug (fluconazole), she developed three episodes of bacteremia (*Staphylococcus epidermidis* and *Escherichia coli*) and two urinary infections (*E. coli*) that were resolved by switching the antibiotic therapy to cefepime and vancomycin (Fig. 1). During prolonged neutropenia, the patient presented febrile relapse, so she received different antibiotic and antifungal schemes (itraconazole, anidulafungin, and caspofungin). On 31 May, the patient continued to have persistent fever and neutropenia, and a set of blood cultures (BC) was obtained. A *Candida krusei* (BC1) strain was isolated from these blood cultures. Caspofungin therapy (70 mg on the first day followed by 50 mg daily) was initiated. Blood cultures obtained during the following week were all negative. After 10 days of caspofungin therapy, a new set of blood cultures (BC2), catheter culture (CC1), urine culture (UC), and soft tissue culture (STC) were all positive for *C. krusei*. Caspofungin treatment was discontinued, and liposomal amphotericin B (AmB) therapy was begun at a dose of 5 mg/kg of body weight/day. The patient died 20 days later due to persistent candidemia (BC2 to BC8, CC2, CC3, CTC1, and CTC2) with multiple-organ failure (Fig. 1).

Antifungal susceptibility testing. Antifungal susceptibility testing showed that all isolates were susceptible to amphotericin B (MIC_{AmB}, 0.25 to 1 mg/liter) and voriconazole (MIC_{VRC}, 0.25 to 0.5 mg/liter) and had predictably high fluconazole MICs (MIC_{FLC}, 32 to 64 mg/liter). The first blood isolate (BC1), obtained pre-caspofungin therapy, was susceptible to anidulafungin (MIC_{ANF}, 0.03 mg/liter) and had a caspofungin MIC of 0.5 mg/liter and a micafungin MIC of 0.06 mg/liter. Isolates recovered after caspofungin therapy (BC2, BC3, BC5 to BC8, UC, STC, CC1 to CC3, and CTC1) showed elevated MICs for anidulafungin (0.125 to 1 mg/liter), caspofungin (1 to 4 mg/liter), and micafungin (0.5 to 1 mg/liter) (Table 1). Similar results were observed with the Etests for caspofungin (Fig. 2) and anidulafungin. Curiously, echinocandin MICs indicating susceptibility were observed for two

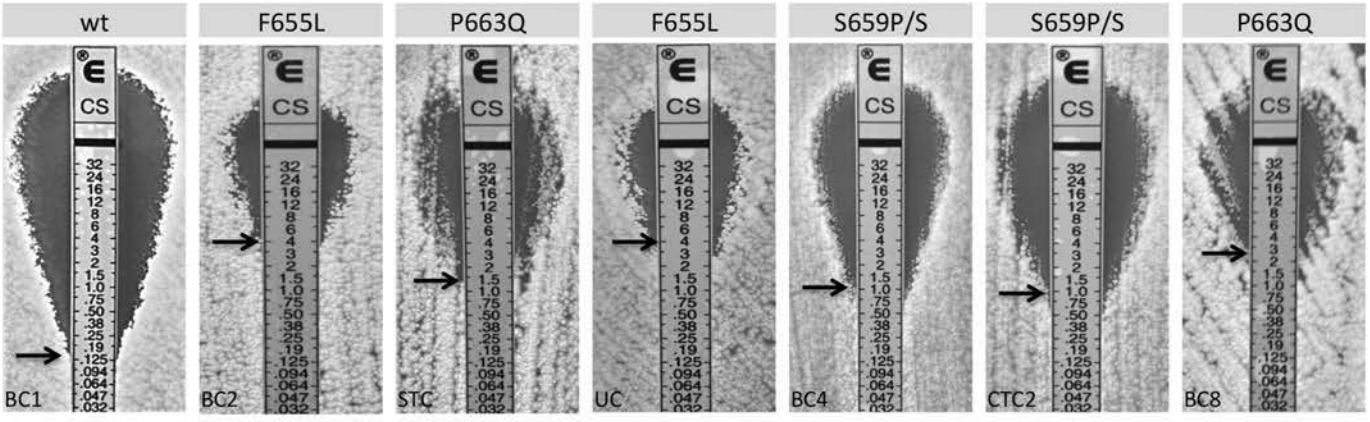


FIG 2 Caspofungin Etests of seven representative *Candida krusei* isolates. BC, blood culture; STC, soft tissue culture; UC, urine culture; CTC, catheter tip culture; wt, wild type; L, leucine; F, phenylalanine; P, proline; Q, glutamine; S, serine. The arrows represent the MICs.

isolates (BC4 and CTC2), both obtained post-caspofungin therapy.

Amplification and sequencing of the *FKS1* gene. Sequence analysis of the *FKS1*-HS1 region in the *C. krusei* ATCC 6258 and BC1 strains showed no differences. In contrast, all isolates recovered after caspofungin treatment showed mutations in hot spot 1 of the *FKS1* gene (*FKS1*-HS1) compared with the first isolate (BC1). *Candida krusei* strains isolated from blood (BC3 and BC6) and urine (UC) presented a missense mutation corresponding to an amino acid substitution of phenylalanine 655 to leucine (F655L) in the *FKS1*-HS1 region. Similarly, isolates recovered from blood (BC2, BC5, BC7, and BC8), catheter (CC1, CC2, and CC3), catheter tip (CTC1), and soft tissue (STC) cultures showed an amino acid change from proline 663 to glutamine (P663Q) compared with BC1. The analysis of the chromatograms generated from DNA sequencing revealed a single nucleotide base change at the site of the mutation, which is consistent with a homozygous change at *FKS1*-HS1 in mutant clinical isolates. A heterozygous mutation corresponding to an amino acid substitution of serine 659 to proline (S659P) was observed in the *FKS1*-HS1 region of blood culture 4 (BC4) and catheter tip culture 2 (CTC2). These isolates, along with BC1, presented an amino acid change from lysine 701 to methionine (L701M) as a result of a heterozygous mutation in a region outside the hot spots.

No changes were observed in hot spot 2 or 3 of *FKS1* in any of the isolates.

Molecular typing of *Candida krusei*. Given the diverse mutations found among isolates, molecular typing of all isolates was performed, using two different methods, in order to discern if the patient was infected by more than one *C. krusei* strain (41, 43).

PCR-based amplification of the species-specific repetitive polymorphic CKRS-1 sequence. The banding patterns obtained after polymorphic species-specific repetitive sequence CKRS-1 (*C. krusei* repeated sequence 1) amplification were analyzed visually. Band sizes ranged from 0.7 to 2 kb among all isolates tested. A unique band pattern was obtained with each of the six control isolates, showing a high discriminatory power of the technique (Fig. 3). However, no differences were observed in the band patterns obtained for the 15 strains isolated from the patient. Isolates showing only one band difference were considered identical. To confirm the visual reading of the gel, computer-assisted cluster analysis of profiles was performed with PyElph software, version 1.4. The dendrogram derived from the UPGMA clustering algorithm showed that all the patient isolates appeared to have the same genotype (genetic distance of 0.0) and were different from the nonrelated control strains (Fig. 3; see Fig. S1 in the supplemental material).

Multilocus sequence typing. For the 15 isolates of *C. krusei* obtained from different anatomical locations and with different *FKS1* mutations, the obtained DSTs were identical (DST 187) but were different from that of the ATCC 6258 control strain (DST 188) (Table 2).

DISCUSSION

Echinocandins are antifungal drugs that inhibit the enzyme 1,3-β-D-glucan synthase (GS) complex, whose catalytic subunit is encoded by three related genes: *FKS1*, *FKS2*, and *FKS3* (12). This mode of action makes them particularly interesting against yeasts with reduced susceptibility to azoles, such as *Candida glabrata* and *C. krusei*. The rising use of echinocandins, however, plays an important role in the species distribution of invasive infections (44) and in decreased echinocandin susceptibility after prolonged exposure to the drug, primarily in severely immunocompromised patients, in whom infections manifest as recurrent episodes of candidemia or invasive candidiasis (45, 46).

Currently, reduced echinocandin susceptibility remains rare and has been shown to occur through the following three mechanisms: (i) acquired *FKS* mutations, which confer reduced glucan synthase sensitivity and elevated echinocandin MICs and are associated with clinical failure (23); (ii) adaptive stress responses that result in elevated cell wall chitin content and paradoxical growth *in vitro* (47); and (iii) intrinsic *FKS* mutations, which are naturally occurring in *C. parapsilosis* and *C. guilliermondii* and result in elevated MICs but a lower level of reduced glucan synthase sensitivity than that with acquired *FKS* mutations (23, 48, 49). In most cases, alterations in *FKS* genes confer cross-resistance to all three echinocandins. However, some alterations cause more moderate MIC elevations, and not always for all related compounds. Additionally, several clinical studies have shown that the presence of an *FKS* mutation is the most important independent risk factor in predicting echinocandin therapeutic responses among patients with invasive candidiasis (31, 32, 50).

In the present case, rapid development of resistance was demonstrated in sequential and genetically related isolates of *C. krusei* recovered from a patient under caspofungin treatment. Clinical resistance was associated with increased echinocandin MICs and

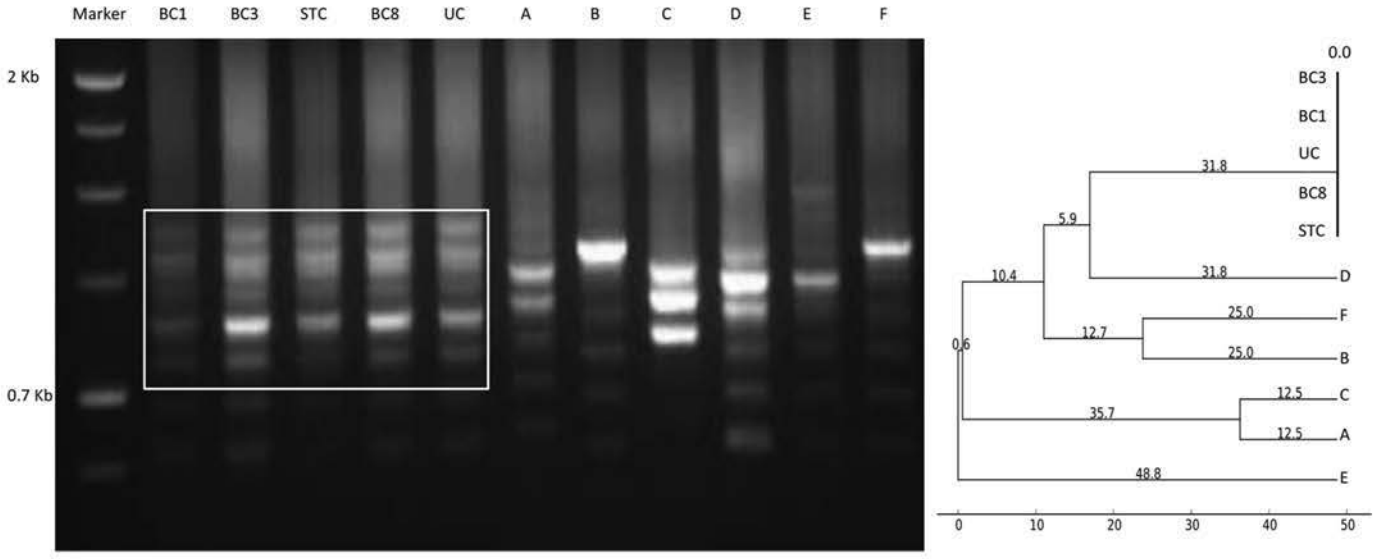


FIG 3 PCR-based amplification of the species-specific repetitive polymorphic region CKRS-1 of *Candida krusei* isolates. BC, blood culture; STC, soft tissue culture; UC, urine culture. The following six unrelated control strains were also analyzed: A, ATCC 6258; B, CNM-CL5642; C, CNM-CL5724; D, CNM-CL6695; E, CNM-CL6709; and F, CNM-CL7057. The box represents the isolates obtained from the patient. The phylogenetic tree was derived from the UPGMA clustering algorithm. The genetic distances are displayed on the branches of the tree.

was ultimately related to new mutations of the target enzyme (*FKS1*).

Sequencing of *FKS1* hot spot 1 showed that the *C. krusei* strain isolated prior to caspofungin therapy presented no amino acid substitutions compared to the *FKS1* sequence of the reference strain (ATCC 6258). However, all isolates of *C. krusei* recovered after only 10 days of treatment with caspofungin presented three different amino acid substitutions in hot spot 1 of Fks1p: F655L, S659P/S, and P663Q. This shows the rapid selection of mutant strains during antifungal treatment. None of the three amino acid changes have been reported previously for *C. krusei*. So far, only three clinical isolates of *C. krusei* with mutations in *FKS1* associated with reduced echinocandin susceptibility and treatment failure have been reported (18, 20, 21). As in a previous report on *C. albicans* (51), we found that isolates of *C. krusei* with the F655L change (corresponding to F641 in *C. albicans*) showed the highest anidulafungin MIC. In fact, the effect on echinocandin susceptibility produced by this mutation (F655L) has already been demonstrated for a clinical isolate of *C. tropicalis* with reduced echinocandin MICs through an elevated 50% inhibitory concentration (IC_{50}) in a kinetic enzyme assay (24), a method that has been critical for revealing the relationship between reduced whole-cell susceptibility and target site inhibition (19). Additionally, a heterozygous mutation at this position (F655C) was reported by Kahn et al. for a *C. krusei* isolate recovered post-caspofungin treatment, which presented an increase in the echinocandin MIC from 16- to 32-fold with respect to the first susceptible isolate (18).

Heterozygous amino acid changes within the *FKS*-HS region have been associated with caspofungin resistance in several yeast species, such as a *Saccharomyces cerevisiae* laboratory strain, *C.*

TABLE 2 Full multilocus sequence typing results for *Candida krusei* isolates

Isolate	DST	Allele no.					
		HIS3	*LEU2*	*NMT1*	*TRP1*	*ADE2*	*LYS2D*
BC1_31/05/13	187	3	7	1	16	6	10
BC2_11/06/13	187	3	7	1	16	6	10
CC1_11/06/13	187	3	7	1	16	6	10
BC3_14/06/13	187	3	7	1	16	6	10
CC2_14/06/13	187	3	7	1	16	6	10
STC_15/06/13	187	3	7	1	16	6	10
UC_15/06/13	187	3	7	1	16	6	10
CTC1_16/06/13	187	3	7	1	16	6	10
BC4_16/06/13	187	3	7	1	16	6	10
BC5_19/06/13	187	3	7	1	16	6	10
CTC2_23/06/13	187	3	7	1	16	6	10
BC6_23/06/13	187	3	7	1	16	6	10
BC7_26/06/13	187	3	7	1	16	6	10
CC3_26/06/13	187	3	7	1	16	6	10
BC8_29/06/13	187	3	7	1	16	6	10
ATCC 6258	188	4	7	1	4	4	10

albicans (18, 19), and *C. tropicalis* clinical isolates (24), indicating that the phenotypic expression of the mutation is dominant (33). In this regard, it is important that the degree of MIC elevation depends not only on ploidy but also on (i) the *Candida* species (20) and (ii) the position and specific amino acid substitution in the hot spot. These considerations may help to explain the anidulafungin-susceptible phenotype (MIC, 0.06 mg/liter) observed for two isolates of *C. krusei* (BC4 and CTC2) with an S659P/S heterozygous amino acid change. Further studies are under way in our laboratory to determine the impact of an S659P/S mutation in *C. krusei*.

The two isolates mentioned above (BC4 and CTC2), together with the first, susceptible isolate of *C. krusei* (BC1), also showed an L701M change, a substitution already reported for *C. krusei* (21, 52). However, its implication in resistance is unclear, as it is located outside the hot spots of *FKS1* and, in our case, was present in the initial isolate that showed lower echinocandin MICs (BC1).

In *C. parapsilosis*, a substitution in the distal proline of hot spot 1 (P660A) is responsible for the decreased susceptibility to the echinocandin drugs that is intrinsically observed in this species and related species, demonstrating the importance of this amino acid in the drug-enzyme interaction (48). In our case, a change at this position (P663Q) was the most frequently found mutation in the isolates from the patient, regardless of the body site origin. This could be a consequence of the random effect at the time of strain storage, as a single colony is kept to ensure the purity of the stored isolate. Therefore, it is likely that all *C. krusei* isolates with different mutations were present in the patient during the course of the disease and antifungal therapy and that the catheter was the common reservoir of infection (biofilm), reflecting the emergence of different isolates with a decreased echinocandin susceptibility phenotype under selective pressure. However, the study of the relationships of the different mutations to the caspofungin concentrations achieved at different body sites is an interesting matter that deserves further research. Additionally, 2 months before the development of the *C. krusei* candidemia, the patient received a 4-day course of anidulafungin. However, as the first isolated strain was susceptible to all echinocandins, it seems clear that the development of resistance occurred during treatment with caspofungin. Nevertheless, we cannot disregard the possibility that the use of anidulafungin as a preemptive treatment may have conditioned the development of resistance in some way.

The genetic discrimination among *C. krusei* isolates could offer some important clues to understanding transmission, pathogenesis, and resistance development. Several techniques have been used to document the genetic diversity of *C. krusei* clinical isolates, such as those employed here (41, 43). Both PCR-based and MLST techniques are accurate and useful for the typing of *C. krusei* isolates to clarify the epidemiology of nosocomial infections (41, 43). In our clinical case, the analysis of *C. krusei*'s genomic DNA and the analysis of fragment sequences of six genes revealed that all isolates, wild type and mutated, were genetically indistinguishable and were different from control strains. It seems likely that the mutant isolates recovered from this patient arose from a wild-type progenitor strain. Furthermore, these results establish that strains with different *FKS1* mutant alleles can be recovered from different sites within the same patient, as reported by Park et al. for a patient infected with the same strains of *C. albicans* but with different mutations in the *FKS* gene (19).

The best methodological parameters to be used for *in vitro* susceptibility testing of caspofungin are still under debate (38). The consequence is that susceptible strains are misclassified according to established clinical breakpoints, and this has led to limited therapeutic options, especially for *Candida* spp. with secondary or intrinsic resistance to fluconazole, such as *C. glabrata* and *C. krusei* (38).

At the time of isolate recovery, only the Etest for caspofungin was performed to test echinocandin susceptibility. The Etest method allowed us to easily determine the caspofungin MIC, and CLSI breakpoints helped to detect the MIC increase and the categorization of wild-type and mutant isolates. But previously published work by Arendrup and Pfaller (38) found that the caspofungin Etest for susceptible isolates of *C. glabrata* and *C. krusei* presents the risk of misclassification if the CLSI breakpoints are used. Therefore, we confirmed our finding by using BMD to establish the echinocandin susceptibility profile and to detect possible mutated isolates.

In summary, we provide molecular evidence of the rapid acquisition of clinical echinocandin resistance associated with increased echinocandin MICs *in vitro* and related to different mutations in hot spot 1 of the target enzyme (*FKS1*). The risk factors present, such as the underlying disease, use of multiple antibiotics, and antifungal prophylaxis, were decisive for the acquisition of infection by *C. krusei* and for the rapid development of resistance during caspofungin therapy. Therefore, these results highlight the need to monitor the development of resistance in *C. krusei* infections treated with echinocandin drugs. The categorization of isolates through susceptibility testing is an important tool to minimize unnecessary echinocandin use and treatment duration, helping to reduce selection pressure and the rise in echinocandin resistance.

ACKNOWLEDGMENTS

E.M. is supported by a project from the Spanish Fondo de Investigacion Sanitaria (FIS; grant PI12_02376) and by the Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for Research in Infectious Diseases (grant REIPI RD 12/0015). M.C.M. is funded by the Spanish Fondo de Investigacion Sanitaria through a Sara Borrell Fellowship (grant CD13/00198).

E.M. declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

1. **Guinea J.** 2014. Global trends in the distribution of *Candida* species causing candidemia. Clin Microbiol Infect **20**(Suppl 6):S5–S10. http://dx.doi.org/10.1111/1469-0691.12539.
2. **Pfaller M, Neofytos D, Diekema D, Azie N, Meier-Kriesche HU, Quan SP, Horn D.** 2012. Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance®) registry, 2004–2008. Diagn Microbiol Infect Dis **74**:323–331. http://dx.doi.org/10.1016/j.diagmicrobio.2012.10.003.
3. **Pfaller MA, Messer SA, Moet GJ, Jones RN, Castanheira M.** 2011. *Candida* bloodstream infections: comparison of species distribution and resistance to echinocandin and azole antifungal agents in intensive care unit (ICU) and non-ICU settings in the SENTRY antimicrobial surveillance program (2008–2009). Int J Antimicrob Agents **38**:65–69. http://dx.doi.org/10.1016/j.ijantimicag.2011.02.016.
4. **Scorzoni L, de Lucas MP, Mesa-Arango AC, Fusco-Almeida AM, Lozano E, Cuenca-Estrella M, Mendes-Giannini MJ, Zaragoza O.** 2013. Antifungal efficacy during *Candida krusei* infection in non-conventional models correlates with the yeast in vitro susceptibility profile. PLoS One **8**:e60047. http://dx.doi.org/10.1371/journal.pone.0060047.

5. **Lockhart SR, Iqbal N, Cleveland AA, Farley MM, Harrison LH, Bolden CB, Baughman W, Stein B, Hollick R, Park BJ, Chiller T.** 2012. Species identification and antifungal susceptibility testing of *Candida* bloodstream isolates from population-based surveillance studies in two U.S. cities from 2008 to 2011. J Clin Microbiol **50:**3435–3442. http://dx.doi.org/10.1128/JCM.01283-12.
6. **Cleveland AA, Farley MM, Harrison LH, Stein B, Hollick R, Lockhart SR, Magill SS, Derado G, Park BJ, Chiller TM.** 2012. Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008–2011. Clin Infect Dis **55:**1352–1361. http://dx.doi.org/10.1093/cid/cis697.
7. **Pfaller MA, Messer SA, Boyken L, Rice C, Tendolkar S, Hollis RJ, Diekema DJ.** 2003. Caspofungin against clinical isolates of fluconazole-resistant *Candida*. J Clin Microbiol **41:**5729–5731. http://dx.doi.org/10.1128/JCM.41.12.5729-5731.2003.
8. **Theuretzbacher U.** 2004. Pharmacokinetics/pharmacodynamics of echinocandins. Eur J Clin Microbiol Infect Dis **23:**805–812. http://dx.doi.org/10.1007/s10096-004-1228-z.
9. **Pappas PG, Kauffman CA, Andes D, Benjamin DK, Jr, Calandra TF, Edwards JE, Jr, Filler SG, Fisher JF, Kullberg BJ, Ostrosky-Zeichner L, Reboli AC, Rex JH, Walsh TJ, Sobel JD, Infectious Diseases Society of America.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis **48:**503–535. http://dx.doi.org/10.1086/596757.
10. **Ullmann AJ, Akova M, Herbrecht R, Viscoli C, Arendrup MC, Arikan-Akdagli S, Bassetti M, Bille J, Calandra T, Castagnola E, Cornely OA, Donnelly JP, Garbino J, Groll AH, Hope WW, Jensen HE, Kullberg BJ, Lass-Flörl C, Lortholary O, Meersseman W, Petrikkos G, Richardson MD, Roilides E, Verweij PE, Cuenca-Estrella M, ESCMID Fungal Infection Study Group.** 2012. ESCMID* guideline for the diagnosis and management of *Candida* diseases 2012: adults with haematological malignancies and after haematopoietic stem cell transplantation (HCT). Clin Microbiol Infect **18**(Suppl 7):S53–S67. http://dx.doi.org/10.1111/1469-0691.12041.
11. **Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, Meersseman W, Akova M, Arendrup MC, Arikan-Akdagli S, Bille J, Castagnola E, Cuenca-Estrella M, Donnelly JP, Groll AH, Herbrecht R, Hope WW, Jensen HE, Lass-Flörl C, Petrikkos G, Richardson MD, Roilides E, Verweij PE, Viscoli C, Ullmann AJ, ESCMID Fungal Infection Study Group.** 2012. ESCMID* guideline for the diagnosis and management of *Candida* diseases 2012: non-neutropenic adult patients. Clin Microbiol Infect **18**(Suppl 7):S19–S37. http://dx.doi.org/10.1111/1469-0691.12039.
12. **Perlin DS.** 2007. Resistance to echinocandin-class antifungal drugs. Drug Resist Updat **10:**121–130. http://dx.doi.org/10.1016/j.drup.2007.04.002.
13. **Debono M, Gordee RS.** 1994. Antibiotics that inhibit fungal cell wall development. Annu Rev Microbiol **48:**471–497. http://dx.doi.org/10.1146/annurev.mi.48.100194.002351.
14. **Pfaller MA, Messer SA, Woosley LN, Jones NR, Castanheira M.** 2013. Echinocandin and triazole antifungal susceptibility profiles for clinical opportunistic yeast and mold isolates collected from 2010 to 2011: application of new CLSI clinical breakpoints and epidemiological cutoff values for characterization of geographic and temporal trends of antifungal resistance. J Clin Microbiol **51:**2571–2581. http://dx.doi.org/10.1128/JCM.00308-13.
15. **Guinea J, Zaragoza O, Escribano P, Martín-Mazuelos E, Pemán J, Sánchez-Reus F, Cuenca-Estrella M, CANDIPOP Project, GEIH-GEMICOMED (SEIMC), REIPI.** 2014. Molecular identification and antifungal susceptibility of yeast isolates causing fungemia collected in a population-based study in Spain in 2010 and 2011. Antimicrob Agents Chemother **58:**1529–1537. http://dx.doi.org/10.1128/AAC.02155-13.
16. **Hakki M, Staab JF, Marr KA.** 2006. Emergence of a *Candida krusei* isolate with reduced susceptibility to caspofungin during therapy. Antimicrob Agents Chemother **50:**2522–2544. http://dx.doi.org/10.1128/AAC.00148-06.
17. **Pelletier R, Alarie I, Lagacé R, Walsh TJ.** 2005. Emergence of disseminated candidiasis caused by *Candida krusei* during treatment with caspofungin: case report and review of literature. Med Mycol **43:**559–564. http://dx.doi.org/10.1080/13693780500220415.
18. **Kahn JN, Garcia-Effron G, Hsu MJ, Park S, Marr KA, Perlin DS.** 2007. Acquired echinocandin resistance in a *Candida krusei* isolate due to modification of glucan synthase. Antimicrob Agents Chemother **51:**1876–1878. http://dx.doi.org/10.1128/AAC.00067-07.
19. **Park S, Kelly R, Kahn JN, Robles J, Hsu MJ, Register E, Li W, Vyas V, Fan H, Abruzzo G, Flattery A, Gill C, Chrebet G, Parent SA, Kurtz M, Teppler H, Douglas CM, Perlin DS.** 2005. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical *Candida* sp. isolates. Antimicrob Agents Chemother **49:**3264–3273. http://dx.doi.org/10.1128/AAC.49.8.3264-3273.2005.
20. **Jensen RH, Justesen US, Rewes A, Perlin DS, Arendrup MC.** 2014. Echinocandin failure case due to a yet unreported *FKS1* mutation in *Candida krusei*. Antimicrob Agents Chemother **58:**3550–3552. http://dx.doi.org/10.1128/AAC.02367-14.
21. **Prigitano A, Esposto MC, Cogliati M, Pitzurra L, Santamaria C, Tortorano AM.** 2014. Acquired echinocandin resistance in a *Candida krusei* blood isolate confirmed by mutations in the *fks1* gene. New Microbiol **37:**237–240.
22. **Johnson ME, Katiyar SK, Edlind TD.** 2011. New Fks hot spot acquired echinocandin resistance in *Saccharomyces cerevisiae* and its contribution to intrinsic resistance of *Scedosporium* species. Antimicrob Agents Chemother **55:**3774–3781. http://dx.doi.org/10.1128/AAC.01811-10.
23. **Beyda ND, Lewis RE, Garey KW.** 2012. Echinocandin resistance in *Candida* species: mechanisms of reduced susceptibility and therapeutic approaches. Ann Pharmacother **46:**1086–1096. http://dx.doi.org/10.1345/aph.1R020.
24. **Garcia-Effron G, Kontoyiannis AD, Lewis RE, Perlin DS.** 2008. Caspofungin-resistant *Candida tropicalis* strains causing breakthrough fungemia in patients at high risk for hematologic malignancies. Antimicrob Agents Chemother **52:**4181–4183. http://dx.doi.org/10.1128/AAC.00802-08.
25. **Garcia-Effron G, Lee S, Park S, Cleary JD, Perlin DS.** 2009. Effect of *Candida glabrata FKS1* and *FKS2* mutations on echinocandin sensitivity and kinetics of 1,3-beta-D-glucan synthase: implication for the existing susceptibility breakpoint. Antimicrob Agents Chemother **53:**3690–3699. http://dx.doi.org/10.1128/AAC.00443-09.
26. **Desnos-Ollivier M, Moquet O, Chouaki T, Guérin AM, Dromer F.** 2011. Development of echinocandin resistance in *Clavispora lusitaniae* during caspofungin treatment. J Clin Microbiol **49:**2304–2306. http://dx.doi.org/10.1128/JCM.00325-11.
27. **Fekkar A, Meyer I, Brossas JY, Dannaoui E, Palous M, Uzunov M, Nguyen S, Leblond V, Mazier D, Datry A.** 2013. Rapid emergence of echinocandin resistance during *Candida kefyr* fungemia treatment with caspofungin. Antimicrob Agents Chemother **57:**2380–2382. http://dx.doi.org/10.1128/AAC.02037-12.
28. **Lewis JS, Wiederhold NP, Wickes BL, Patterson TF, Jorgensen JH.** 2013. Rapid emergence of echinocandin resistance in *Candida glabrata* resulting in clinical and microbiologic failure. Antimicrob Agents Chemother **57:**4559–4561. http://dx.doi.org/10.1128/AAC.01144-13.
29. **Baixench MT, Aoun N, Desnos-Ollivier M, Garcia-Hermoso D, Bretagne S, Ramires S, Piketty C, Dannaoui EJ.** 2007. Acquired resistance to echinocandins in *Candida albicans*: case report and review. J Antimicrob Chemother **59:**1076–1083. http://dx.doi.org/10.1093/jac/dkm095.
30. **Durán-Valle MT, Gago S, Gómez-López A, Cuenca-Estrella M, Jiménez Díez-Canseco L, Gómez-Garcés JL, Zaragoza O.** 2012. Recurrent episodes of candidemia due to *Candida glabrata* with a mutation in hot spot 1 of the FKS2 gene developed after prolonged therapy with caspofungin. Antimicrob Agents Chemother **56:**3417–3419. http://dx.doi.org/10.1128/AAC.06100-11.
31. **Shields RK, Hong Nguyen M, Press EG, Updike CL, Clancy CJ.** 2013. Caspofungin MICs correlate with treatment outcomes among patients with *Candida glabrata* invasive candidiasis and prior echinocandin exposure. Antimicrob Agents Chemother **57:**3528–3535. http://dx.doi.org/10.1128/AAC.00136-13.
32. **Shields RK, Nguyen MH, Press EG, Kwa AL, Cheng S, Du C, Clancy CJ.** 2012. The presence of an *FKS* mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to *Candida glabrata*. Antimicrob Agents Chemother **56:**4862–4869. http://dx.doi.org/10.1128/AAC.00027-12.
33. **Balashov SV, Park S, Perlin DS.** 2006. Assessing resistance to the echinocandin antifungal drug caspofungin in *Candida albicans* by profiling mutations in *FKS1*. Antimicrob Agents Chemother **50:**2058–2063. http://dx.doi.org/10.1128/AAC.01653-05.
34. **Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope W, EUCAST-AFST.** 2012. EUCAST technical note on the EUCAST definitive document EDef 7.2: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2

(EUCAST-AFST). Clin Microbiol Infect **18:**E246–E247. http://dx.doi.org/10.1111/j.1469-0691.2012.03880.x.

35. **Lass-Flörl C, Arendrup MC, Rodriguez-Tudela JL, Cuenca-Estrella M, Donnelly P, Hope W, European Committee on Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility Testing.** 2011. EUCAST technical note on amphotericin B. Clin Microbiol Infect **17:**E27–E29. http://dx.doi.org/10.1111/j.1469-0691.2011.03644.x.
36. **Arendrup MC, Rodriguez-Tudela JL, Lass-Flörl C, Cuenca-Estrella M, Donnelly JP, Hope W, EUCAST-AFST.** 2011. EUCAST technical note on anidulafungin. Clin Microbiol Infect **17:**E18–E20. http://dx.doi.org/10.1111/j.1469-0691.2011.03647.x.
37. **Clinical and Laboratory Standards Institute.** 2012. Reference method for broth dilution antifungal susceptibility testing of yeasts; fourth informational supplement. Document M27-S4, vol 32, no 17. CLSI, Wayne, PA.
38. **Arendrup MC, Pfaller MA.** 2012. Caspofungin Etest susceptibility testing of *Candida* species: risk of misclassification of susceptible isolates of *C. glabrata* and *C. krusei* when adopting the revised CLSI caspofungin breakpoints. Antimicrob Agents Chemother **56:**3965–3968. http://dx.doi.org/10.1128/AAC.00355-12.
39. **Espinel-Ingroff A, Arendrup MC, Pfaller MA, Bonfietti LX, Bustamante B, Canton E, Chryssanthou E, Cuenca-Estrella M, Dannaoui E, Fothergill A, Fuller J, Gaustad P, Gonzalez GM, Guarro J, Lass-Flörl C, Lockhart SR, Meis JF, Moore CB, Ostrosky-Zeichner L, Pelaez T, Pukinskas SR, St-Germain G, Szeszs MW, Turnidge J.** 2013. Interlaboratory variability of caspofungin MICs for *Candida* spp. using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? Antimicrob Agents Chemother **57:**5836–5842. http://dx.doi.org/10.1128/AAC.01519-13.
40. **Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ.** 1998. Multiple sequence alignment with Clustal X. Trends Biochem Sci **23:**403–405. http://dx.doi.org/10.1016/S0968-0004(98)01285-7.
41. **Carlotti A, Chaib F, Couble A, Bourgeois N, Blanchard V, Villard J.** 1997. Rapid identification and fingerprinting of *Candida krusei* by PCR-based amplification of the species-specific repetitive polymorphic sequence CKRS-1. J Clin Microbiol **35:**1337–1343.
42. **Pavel AB, Vasile CI.** 2012. PyElph—a software tool for gel images analysis and phylogenetics. BMC Bioinformatics **13:**9. http://dx.doi.org/10.1186/1471-2105-13-9.
43. **Jacobsen MD, Gow NA, Maiden MC, Shaw DJ, Odds FC.** 2007. Strain typing and determination of population structure of *Candida krusei* by multilocus sequence typing. J Clin Microbiol **45:**317–323. http://dx.doi.org/10.1128/JCM.01549-06.
44. **Lortholary O, Desnos-Ollivier M, Sitbon K, Fontanet A, Bretagne S, Dromer F, French Mycosis Study Group.** 2011. Recent exposure to caspofungin or fluconazole influences the epidemiology of candidemia: a prospective multicenter study involving 2,441 patients. Antimicrob Agents Chemother **55:**532–538. http://dx.doi.org/10.1128/AAC.01128-10.
45. **Pfeiffer CD, Garcia-Effron G, Zaas AK, Perfect JR, Perlin DS, Alexander BD.** 2010. Breakthrough invasive candidiasis in patients on micafungin. J Clin Microbiol **48:**2373–2380. http://dx.doi.org/10.1128/JCM.02390-09.
46. **Sun HY, Singh N.** 2010. Characterization of breakthrough invasive mycoses in echinocandin recipients: an evidence-based review. Int J Antimicrob Agents **35:**211–218. http://dx.doi.org/10.1016/j.ijantimicag.2009.09.020.
47. **Walker LA, Gow NAR, Munro CA.** 2010. Fungal echinocandin resistance. Fungal Genet Biol **47:**117–126. http://dx.doi.org/10.1016/j.fgb.2009.09.003.
48. **Garcia-Effron G, Katiyar SK, Park S, Edlind TD, Perlin DS.** 2008. A naturally occurring proline-to-alanine amino acid change in Fks1p in *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* accounts for reduced echinocandin susceptibility. Antimicrob Agents Chemother **52:**2305–2312. http://dx.doi.org/10.1128/AAC.00262-08.
49. **Barchiesi F, Spreghini E, Tomassetti S, Della Vittoria A, Arzeni D, Manso E, Scalise G.** 2006. Effects of caspofungin against *Candida guilliermondii* and *Candida parapsilosis*. Antimicrob Agents Chemother **50:**2719–2727. http://dx.doi.org/10.1128/AAC.00111-06.
50. **Alexander BD, Johnson MD, Pfeiffer CD, Jiménez-Ortigosa C, Catania J, Booker R, Castanheira M, Messer SA, Perlin DS, Pfaller MA.** 2013. Increasing echinocandin resistance in *Candida glabrata*: clinical failure correlates with presence of *FKS* mutations and elevated minimum inhibitory concentrations. Clin Infect Dis **56:**1724–1732. http://dx.doi.org/10.1093/cid/cit136.
51. **Perlin DS.** 2014. Echinocandin resistance, susceptibility testing and prophylaxis: implications for patient management. Drugs **74:**1573–1585. http://dx.doi.org/10.1007/s40265-014-0286-5.
52. **Desnos-Ollivier M, Bretagne S, Raoux D, Hoinard D, Dromer F, Dannaoui E.** 2008. Mutations in the *fks1* gene in *Candida albicans*, *C. tropicalis*, and *C. krusei* correlate with elevated caspofungin MICs uncovered in AM3 medium using the method of the European Committee on Antibiotic Susceptibility Testing. Antimicrob Agents Chemother **52:**3092–3098. http://dx.doi.org/10.1128/AAC.00088-08.