UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA

TESIS DOCTORAL

Detección de resistencia a azoles en Aspergillus fumigatus sensu lato: simplificación metodológica parala adecuación al laboratorio de Microbiología Clínica

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Julia Serrano Lobo

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Memoria para la obtención del Grado de Doctora, presentada por

Julia Serrano Lobo

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1. Azole and Amphotericin B MIC Values against *Aspergillus fumigatus*: High Agreement between Spectrophotometric and Visual Readings Using the EUCAST EDef 9.3.2 Procedure. **Serrano-Lobo J**, Gómez A, Sánchez-Yebra W, Fajardo M, Lorenzo B, Sánchez-Reus F, Vidal I, Fernández-Torres M, Sánchez-Romero I, Ruiz de Alegría-Puig C, Del Pozo JL, Muñoz P, Escribano P, Guinea J. *Antimicrobial Agents and Chemotherapy.* (2020). 65(1): e01693-20. doi: 10.1128/AAC.01693-20.

2. Spectrophotometric azole and amphotericin B MIC readings against *Aspergillus fumigatus sensu lato* using the EUCAST 9.3.2 methodology. Are ≥90 and ≥95% fungal growth inhibition endpoints equally suitable? **Serrano-Lobo J**, Gómez A, Muñoz P, Escribano P, Guinea J. *Medical Mycology*. (2021). 60(1): myab072. doi: 10.1093/mmy/myab072.

3. Azole-Resistant *Aspergillus fumigatus* Clinical Isolate Screening in Azole-Containing Agar Plates (EUCAST E.Def 10.1): Low Impact of Plastic Trays Used and Poor Performance in Cryptic Species. **Serrano-Lobo J**, Gómez A, Rodríguez-Sánchez B, Muñoz P, Escribano P, Guinea J; *ASPE*IN Study Group. *Antimicrobial Agents and Chemotherapy*. (2021). 65(8): e0048221. doi: 10.1128/AAC.00482-21.

4. Screening of azole resistance in *Aspergillus fumigatus* using the EUCAST E.Def 10.2 azole-containing agar method: a single study suggests that filtration of conidial suspensions prior to inoculum preparation may not be needed. **Serrano-Lobo J**, Gómez A, Reigadas E, Muñoz P, Escribano P, Guinea J; *ASPE*IN Study Group. *Mycoses*. (2022). 65(11): 1045-1049. doi: 10.1111/myc.13492.

5. Gradient diffusion strips for detecting azole resistance in *Aspergillus fumigatus sensu lato*. **Serrano-Lobo J**, Gómez A, Reigadas E, Muñoz P, Escribano P, Guinea J; *ASP*EIN Study Group. *Mycoses*. (2023). 66(3): 196-201. doi: 10.1111/myc.13541.

6. Azole resistance screening in *Aspergillus fumigatus sensu stricto* using the azole-containing agar method (EUCAST E.Def 10.2): conidial suspension filtration and inoculum adjustment before inoculum preparation may not be needed. **Serrano-Lobo J**, Reigadas

E, Muñoz P, Escribano P, Guinea J; *ASPE*IN Study Group. JCM. (2024). 31: e0036924. doi: 10.1128/jcm.00369-24.

7. Effective Detection of Azole Resistance in *Aspergillus fumigatus sensu stricto* Using a Gradient Diffusion Plastic Strip: A Comparison of Filtered adjusted vs. Unfiltered unadjusted Inocula. **Serrano-Lobo J**, Reigadas E, Muñoz P, Escribano P, Guinea J; *ASPE*IN Study Group. Manuscrito en revisión.

ÍNDICE

RESUMEN

Detección de resistencia a azoles en *Aspergillus fumigatus sensu lato*: simplificación metodológica para la adecuación al laboratorio de Microbiología Clínica.

La resistencia a azoles en *Aspergillus fumigatus* ha ido cobrando gran relevancia por el aumento de su incidencia de forma global. La detección rápida de cepas de *A. fumigatus* resistentes a azoles es una necesidad para mejorar el manejo del paciente afectado y su pronóstico, ya que la mortalidad es significativamente superior a la de los pacientes infectados por cepas sensibles.

Esta tesis ha pretendido convertir las pruebas para el estudio de la sensibilidad a los antifúngicos en *A. fumigatus* en métodos simplificados y disponibles en los hospitales mediante estudios estructurados en los siguientes tres capítulos: **Capítulo I**, Comparación de las lecturas visuales y espectrofotométricas de las CMIs de azoles y anfotericina B frente a cepas de *A. fumigatus*; **Capítulo II**, Evaluación y simplificación del método de placas de agar con azoles (EUCAST E.Def 10.2) para la detección de cepas de *A. fumigatus* resistentes a azoles; **Capítulo III**, Evaluación de las tiras de difusión en gradiente para la detección de cepas de *A. fumigatus* resistentes a azoles.

Con el procedimiento estándar de microdilución de EUCAST se ha recomendado exclusivamente la lectura visual de las CMIs de azoles y anfotericina B frente a especies de *Aspergillus*. Sin embargo, ésta es subjetiva y puede conllevar variabilidad inter-observador, haciendo necesaria la implementación de un método más eficaz para la determinación de la CMI. En el Capítulo I, se compararon las lecturas espectrofotométricas y visuales de las CMIs de azoles y anfotericina B frente a *A. fumigatus sensu lato*, observándose que ambas formas de obtener la CMI mostraban altos acuerdos esenciales y categóricos, utilizando el parámetro de inhibición del crecimiento fúngico tanto del 90% como del 95%. Por lo tanto, la lectura espectrofotométrica podría ser una buena alternativa a la determinación visual de la CMI. Tras la publicación de estos resultados, y otros similares procedentes de otros laboratorios, se incorporó al actual procedimiento EUCAST E. Def 9.4 la posibilidad de obtener las CMIs de anfotericina B y azoles frente a *A. fumigatus* mediante lectura espectrofotométrica.

Las placas de agar con azoles permiten estudiar la sensibilidad antifúngica en *A. fumigatus*. Existen placas de agar con azoles comercializados sólo en unos pocos países, por lo que, con frecuencia, es necesario prepararlas de forma casera. Además, el procedimiento estandarizado para la realización de esta prueba requiere filtrar y ajustar las suspensiones de conidias antes de inocularlas, lo que conlleva tiempo y aumento en la carga de trabajo del laboratorio. En el Capítulo II se evaluó el rendimiento de este método y si, por un lado, el tipo de plástico utilizado para preparar placas de agar con azoles, y por otro, omitir los pasos de filtración y ajuste del inóculo, influían negativamente en el procedimiento. Se confirmó el buen rendimiento de las placas que contienen azoles para detectar la presencia de resistencia en *A. fumigatus sensu stricto* y que permitían inferir incluso la presencia de las mutaciones más frecuentes que confieren resistencia. Sin embargo, su rendimiento fue pobre frente a especies crípticas del complejo. Se concluyó que omitir tanto la filtración de la suspensión conidial como el posterior ajuste del inóculo, así como la composición de las placas utilizadas, no influían negativamente en el rendimiento del método.

Las tiras de difusión en gradiente son un método comúnmente utilizado en los hospitales, pero está limitado por la ausencia de puntos de corte bien establecidos para obtener una correcta interpretación de los valores de CMIs. En el Capítulo III se evaluó el rendimiento de las tiras de difusión en gradiente para detectar resistencia a azoles y se propusieron puntos de corte para clasificar a las cepas como resistentes; además, se evaluó si omitir los pasos de filtración y ajuste del inóculo influía negativamente en el rendimiento. Se concluyó que este es un método útil de cribado de resistencia a azoles en cepas de *A. fumigatus sensu stricto* cuando las CMIs obtenidas son: itraconazol >1 mg/L, voriconazol >0,5 mg/L, y posaconazol >0,25 mg/L. Sin embargo, el rendimiento frente a especies crípticas fue deficiente. La omisión de la filtración y el ajuste de las suspensiones conidiales no influyeron negativamente en el rendimiento del método.

En resumen, esta tesis ha permitido simplificar y hacer más accesible la metodología disponible para la detección de resistencia a azoles en *A. fumigatus sensu stricto* (microdilución en caldo, placas de agar con azoles, y las tiras de difusión en gradiente) para adecuarla a la actividad del laboratorio de Microbiología Clínica. Sin embargo, estos

procedimientos no resultaron adecuados en el caso de las especies crípticas. La lectura espectrofotométrica de las CMIs obtenidas por microdilución aporta objetividad, automatización y rapidez. El rendimiento de las placas de agar con azoles no se ve afectado por el tipo de plástico utilizado. Ni el método de las placas de agar con azoles ni el de las tiras de difusión en gradiente, se ven afectados por la omisión de la filtración y el ajuste de las suspensiones conidiales. Por último, se han establecido puntos de corte para interpretar las CMIs de los azoles con el uso de tiras de difusión en gradiente y permitir una correcta clasificación de las cepas.

SUMMARY

Detection of azole resistance in *Aspergillus fumigatus sensu lato*: methodological simplification for Clinical Microbiology Laboratories

Azole resistance in *Aspergillus fumigatus* has gained traction due to the global increasing incidence. Quick detection of azole-resistant *A. fumigatus* strains is key to improving patient management and prognosis, given that mortality is significantly higher in patients infected by resistant strains compared to those infected by susceptible strains.

This thesis aims to address key questions regarding current antifungal susceptibility tests for *A. fumigatus*, and structured across three chapters: **Chapter I**, Comparison of visual and spectrophotometric readings of azole and amphotericin B MICs against *A. fumigatus* strains; **Chapter II**, Evaluation and simplification of the azole agar plate method (EUCAST E.Def 10.2) for detecting azole-resistant *A. fumigatus* strains; and **Chapter III**, Evaluation of gradient diffusion strips for detecting azole-resistant *A. fumigatus* strains.

The EUCAST microdilution standard procedure recommended only visual inspection for assessing azole and amphotericin B MICs against *Aspergillus* species. However, this reading might be subjective and lead to inter-observer variability, thus making it necessary to implement a more efficient method for MIC assessment. In Chapter I, we compared spectrophotometric and visual readings of azole and amphotericin B MICs against *A. fumigatus sensu lato*. Both methods demonstrated high essential and categorical agreement values by using 90% and 95% fungal growth inhibition endpoints, what proved that spectrophotometric reading is a reliable alternative to visual MIC assessment. In line with similar data reported from laboratories elsewhere, spectrophotometric reading was then incorporated into the current EUCAST E.Def 9.4 procedure for interpreting MICs of amphotericin B and azoles against *A. fumigatus*.

Azole agar plates pose another method for antifungal susceptibility testing in *A. fumigatus*. Azole agar plates are commercially available, but not everywhere, what makes in-house plates preparation a necessity. Moreover, the standard procedure requires filtration of the conidial suspension and adjustment prior to inoculum preparation, which is time-consuming and increases the laboratory workload. In Chapter

II, we assessed the impact of the type of plastic used in azole agar plates and the omission of filtration and inoculum adjustment steps on the performance of the procedure. Azole-containing plates demonstrated good performance in detecting azole resistance in *A. fumigatus* sensu stricto, results were less accurate against cryptic species though. The type of plastic used did not impact sensitivity or specificity values. Finally, omitting conidial suspension filtration and inoculum adjustment steps did not impact negatively the performance of the method.

Gradient diffusion plastic strips, a widely used method in hospitals, are limited by the lack of established cut-off values and ambiguous MIC interpretation. In Chapter III, we assessed the efficacy of gradient diffusion plastic strips in detecting azole resistance, proposed cut-off values for resistance classification, and assessed the impact of omitting filtration and inoculum adjustment steps on performance. Results suggested that the method is useful to screen for azole resistance in *A. fumigatus sensu stricto* if MIC values are higher than >1 mg/L (itraconazole), >0.5 mg/L (voriconazole), and >0.25 mg/L (posaconazole). However, performance was poor against cryptic species. Omitting filtration and conidial suspension adjustments did not negatively impact method accuracy.

In summary, this thesis proved that broth microdilution, azole agar plates, and gradient diffusion plastic strips proved useful in detecting azole resistance in *A. fumigatus sensu stricto*, with potential for simplification to meet the needs of Clinical Microbiology laboratories. However, the methods were less useful in cryptic species. Spectrophotometric reading in microdilution procedures improves objectivity, automation, and turnaround. The performance of azole agar plates was unaffected by the type of plastic used, and omitting filtration and inoculum adjustments did not impact the accuracy of azole agar plate or gradient diffusion plastic strip either.

1. INTRODUCCIÓN

1.1. Género Aspergillus y Aspergillus fumigatus

El género *Aspergillus,* de la familia *Aspergillaceae*, orden Eurotiales, incluye más de 400 especies agrupadas en seis subgéneros (*Circumdati, Nidulantes, Fumigati, Aspergillus, Cremei* y *Polypaecilum*), divididos a su vez en secciones (Figura 1) (Kocsube et al., 2016; Houbraken et al., 2020).

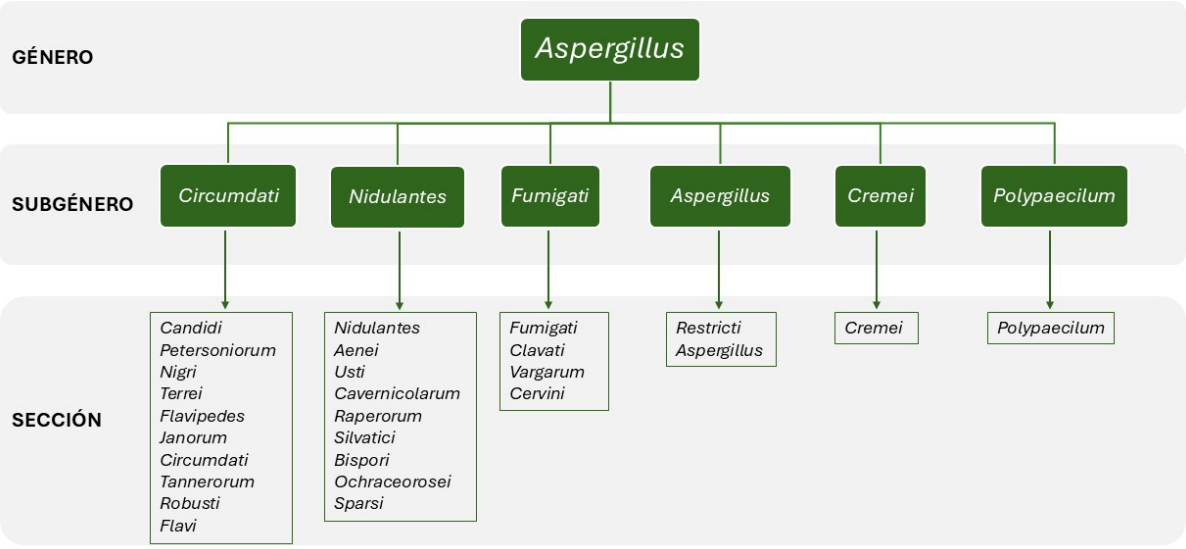

Figura 1. Filograma del género *Aspergillus*.

Sin embargo, tan solo unas 30 especies han demostrado ser capaces de causar infección en humanos, entre las que destacan *Aspergillus fumigatus*, *A. flavus*, *A. terreus, A. niger* y *A. nidulans* (Tabla 1) (Sugui et al., 2014; Pathakumari et al., 2020).

Dentro del subgénero y sección *Fumigati*, se encuentran *A. fumigatus sensu stricto,* la especie dominante, y las denominadas especies crípticas, que representan el 10 - 15% de las cepas dentro de la sección. Las especies crípticas son difíciles de diferenciar de *A. fumigatus sensu stricto* por métodos morfológicos, entre ellas las más frecuentes son *A. lentulus*, *A. novofumigatus, A. fumigatiaffinis, A. thermomutatus, A. udagawae*, *A. viridinutans, A. felis, A. fischeri* y *A. hiratsukae* (Alastruey-Izquierdo et al., 2013; Alastruey-Izquierdo et al., 2018; Balajee et al., 2009).

Tabla 1. Principales especies relacionadas con infección en humanos.

Especies	Sección				
	Fumigati	***Flavi***	***Terrei***	***Nigri***	***Nidulantes***
	A. fumigatus	*A. flavus*	*A. terreus*	*A. niger*	*A. nidulans*
	A. lentulus	*A. alliaceus*	*A. alabamensis*	*A. tubingensis*	*A. tetrazonus*
	A. novofumigatus	*A. tamarii*	*A. carneus*	*A. awamori*	
	A. fumigatiaffinis			*A. acidus*	
	A. thermomutatus				
	A. udagawae				
	A. viridinutans				

1.2. Epidemiología y formas clínicas de la infección causada por *A. fumigatus*

El género *Aspergillus* se encuentra distribuido de forma ubicua en la naturaleza, predominando su crecimiento sobre materia orgánica en descomposición (Latge y Chamilos, 2019).

Las especies del género *Aspergillus* poseen en sus hifas unas estructuras especializadas denominadas conidióforos, donde se producen las conidias que permiten la reproducción asexual del hongo. Estas conidias, de alrededor de 2,5 µm de tamaño, son liberadas al exterior con el fin de ser dispersadas y asegurar la supervivencia en el ambiente. Los humanos inhalamos de forma indirecta entre 100-1000 conidias al día que, gracias a su reducido tamaño, son capaces de penetrar hasta los alveolos pulmonares. En las vías aéreas estas conidias son eliminadas del organismo por las primeras barreras de defensa que comprenden la inmunidad innata, principalmente por los macrófagos alveolares, y los neutrófilos, encargados de destruir las hifas que lleguen a producirse a partir de la germinación de las conidias. Estos sistemas de aclaramiento innatos son extraordinariamente eficaces, de manera que los individuos con un sistema

inmunitario íntegro no se ven afectados por el hongo. En aquellos casos en los que el sistema inmune esté comprometido, las conidias pueden filamentar dentro de los pulmones causando infecciones invasivas (Baltussen et al., 2020).

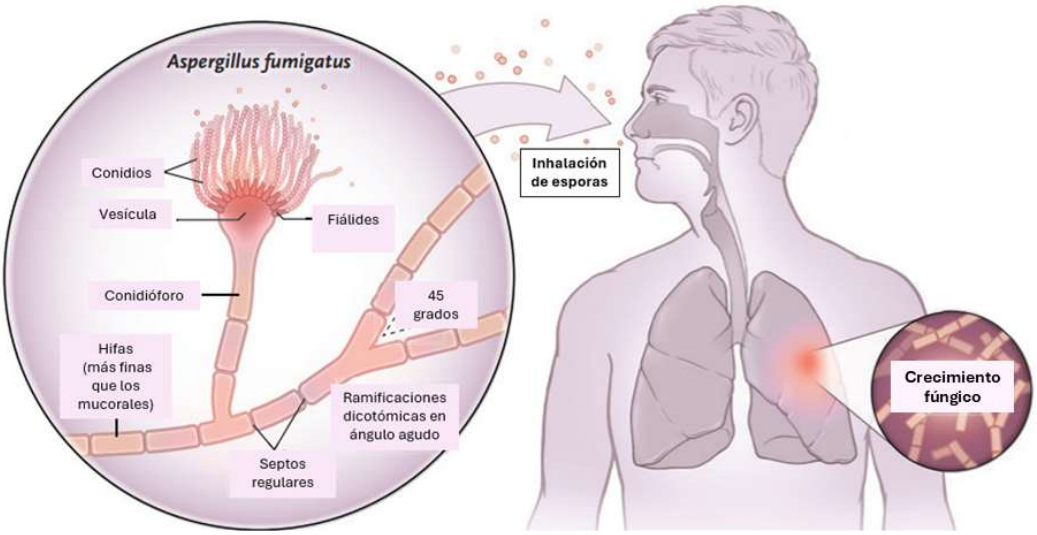

Figura 2. Ciclo de infección y estados morfológicos por los que pasa *A. fumigatus* tras ser inhalado por un individuo inmunodeprimido. Adaptación de la figura de Thompson y Young, 2021.

Las enfermedades causadas por especies del género *Aspergillus* se denominan de manera general como aspergilosis. *A. fumigatus* es la especie de mayor relevancia clínica, ya que es el principal agente causal de aspergilosis (Lestrade et al., 2018; Peri et al., 2018; Guinea et al., 2010; Verweij et al., 2020b).

Algunas características peculiares de *A. fumigatus* favorecen que sea la especie más patógena del grupo: produce conidias de menor tamaño (2-3 µm) que otras especies de *Aspergillus*, además estas son pigmentadas y muy hidrofóbicas, lo que favorece su dispersión y las protege de agentes externos (como la luz, los agentes ionizantes y la lisis enzimática); su tasa de crecimiento es muy elevada (2-6 días), lo que le permite colonizar múltiples nichos de forma rápida, y es capaz de soportar un amplio rango de temperatura creciendo muy bien a 37°C; secreta muchos metabolitos secundarios que tienen un papel muy importante en la patogenicidad tales como enzimas (proteasas, elastasas, catalasas y fosfolipasas) o micotoxinas (gliotoxina, fumagilina, ácido helvolico, fumitremorgina A y Asp-hemolisina); en respuesta a la baja biodisponibilidad de nutrientes en el medio, por ejemplo, en el huésped, es capaz de adaptarse y obtener

todos los nutrientes que necesita para reproducirse en el lugar de la infección; tiene capacidad de formar biopelículas, lo que le confiriere protección frente a agentes físicos y químicos, y le confiere mayor resistencia a los antifúngicos (van de Veerdonk et al., 2017). Dada su ubicuidad, su capacidad de producir enfermedades invasivas y los niveles crecientes de resistencia a los azoles, la Organización Mundial de la Salud incluyó a *A. fumigatus* dentro de la lista de patógenos fúngicos prioritarios (WHO FPPL) en 2022.

Los cuadros clínicos que se manifiestan como consecuencia de las infecciones producidas por *A. fumigatus* varían en gravedad y van desde la aspergilosis broncopulmonar alérgica (ABPA) hasta la aspergilosis invasiva, que es el cuadro de mayor gravedad, sobre todo cuando se afecta el sistema nervioso central, el aspergiloma, y la aspergilosis pulmonar crónica (APC) (Latge y Chamilos, 2019; Thompson y Young, 2021).

La ABPA es la forma más leve de aspergilosis y cursa como una reacción inflamatoria exagerada producida de forma secundaria a una hipersensibilización del pulmón tras la exposición a *A. fumigatus*. Se diagnostica fundamentalmente en pacientes con asma o fibrosis quística. El aspergiloma es una colonización por parte del hongo de cavidades del parénquima pulmonar preexistentes y lo compone un conjunto de hifas de *A. fumigatus*, células inflamatorias, fibrina, moco y epitelio muerto. La APC se desarrolla en pacientes con patologías pulmonares crónicas, como el enfisema o la enfermedad pulmonar obstructiva crónica (Guinea et al., 2010), y cursa como una invasión lenta y prolongada en el tiempo del parénquima pulmonar. Se diferencia del aspergiloma por la aparición de nódulos pulmonares persistentes y el engrosamiento de la pleura (Thompson y Young, 2021). La aspergilosis invasiva es la forma más grave de la aspergilosis y el cuadro con peor pronóstico. La gravedad se correlaciona inversamente con el estado inmunológico del huésped y los factores de riesgo incluyen: neutropenia profunda y/o prolongada; defectos en la inmunidad mediada por células; y la recepción de terapia inmunosupresora.

Las tasas más elevadas de incidencia de la aspergilosis invasiva se dan en la población de enfermos con enfermedad granulomatosa crónica debido al deterioro funcional de la NADPH oxidasa, necesaria para la eliminación de las conidias del hongo tras su fagocitosis por los macrófagos (King et al., 2016). Estos pacientes presentan

predominantemente formas invasoras pulmonares, osteomielitis, e infecciones cerebrales focales. Las neoplasias hematológicas y su tratamiento intensivo, incluido el trasplante, también constituyen una población muy vulnerable. En particular, la leucemia mieloide aguda y su neutropenia prolongada durante la quimioterapia de inducción y el trasplante alogénico de células madre hematopoyéticas, especialmente con la enfermedad injerto contra receptor (Garner et al., 2021). En los pacientes sometidos a trasplante de órgano sólido las incidencias varían en función del órgano trasplantado, y los pacientes en unidades de cuidados intensivos también son una población actualmente en riesgo.

La enfermedad invasiva también se ha relacionado con infecciones virales respiratorias, incluyendo la gripe (IAPA), el virus respiratorio sincitial y, más recientemente, el SARS-CoV-2 (CAPA), entre otros. Se cree que la aspergilosis invasiva tras estas infecciones virales respiratorias es secundaria a la lesión epitelial de las vías respiratorias, lo que permitiría la invasión por *Aspergillus* (Cadena et al., 2021).

Las formas invasivas de aspergilosis presentan alta morbilidad y mortalidad relacionadas con la dificultad para diagnosticarlas y, en consecuencia, el retraso en el inicio de una terapia antifúngica adecuada.

1.3. Diagnóstico de la aspergilosis invasiva

El diagnóstico de la aspergilosis es complejo y es necesario un alto índice de sospecha para realizarlo, ya que un paciente inmunocomprometido puede estar relativamente asintomático. El diagnóstico temprano de la aspergilosis invasiva es un desafío, y debe basarse en la integración de criterios del huésped, datos clínicos/radiológicos y microbiológicos.

Siguiendo las recomendaciones diagnósticas para la aspergilosis invasiva, elaboradas por la Organización Europea para la Investigación y Tratamiento del Cáncer y el Grupo de Estudio de Micosis (EORTC/MSG), se diferencian caso posible, probable y probado, siendo este último el que reúne los criterios diagnósticos más estrictos (Donnelly et al., 2020).

La infección fúngica invasiva (IFI) probada es independiente del estado inmunitario de los pacientes y su enfermedad subyacente. Requiere de la demostración de la invasión de hifas objetivada en el examen microscópico de una biopsia de tejido o muestra obtenida por aspiración con aguja; alternativamente se puede aislar el hongo en cultivo a partir de una muestra obtenida mediante un procedimiento estéril de una zona normalmente estéril y clínica o radiológicamente compatible con un proceso de enfermedad infecciosa.

Por el contrario, las IFI probables y posibles requieren un factor de riesgo del huésped para el desarrollo de la enfermedad (por ejemplo, neutropenia prolongada). La aspergilosis invasiva probable requiere la presencia de al menos un factor de riesgo del huésped, un hallazgo clínico/radiológico coherente con la enfermedad, y evidencia micológica. La aspergilosis invasiva posible, requiere la presencia de al menos un factor de riesgo del huésped y un hallazgo clínico/radiológico coherente con la enfermedad, pero sin evidencia micológica.

La tomografía computada de alta resolución es la prueba de imagen por excelencia (Donnelly et al., 2020; Ullmann et al., 2018). Los hallazgos radiológicos clásicos en la aspergilosis angioinvasiva suelen incluir macronódulos superiores a 1 cm, que pueden estar rodeados de un halo de atenuación en vidrio deslustrado. Este signo, denominado "signo del halo", es la evidencia del daño local y la respuesta inflamatoria desencadenada por la invasión de *Aspergillus*.

Entre las evidencias micológicas se incluyen: visualización microscópica de hifas septadas en muestras respiratorias, recuperación de *Aspergillus* en cultivo de muestras respiratorias; antígeno de galactomanano positivo en plasma, suero, lavado broncoalveolar o líquido cefalorraquídeo; y pruebas de reacción en cadena de la polimerasa (PCR) de *Aspergillus* en plasma, suero, sangre completa o lavado broncoalveolar (Donnelly et al., 2020).

Aunque el cultivo microbiológico tiene importantes limitaciones, como su baja sensibilidad y la dificultad de establecer su significancia clínica (Bouza et al., 2005), la recuperación de colonias de *Aspergillus* proporciona una información valiosa porque permite llevar a cabo la identificación a nivel de especie y estudios de sensibilidad

antifúngica. *Aspergillus* spp. crece en medios de cultivo rutinarios, aunque el uso de medios específicos para hongos, como agar Sabouraud, aumenta su recuperación (Horvath y Dummer, 1996). La recuperación de *Aspergillus* spp. de muestras no estériles debe interpretarse con precaución porque la colonización asintomática es común incluso en poblaciones de riesgo. La terapia previa, ya sea como profilaxis o tratamiento, también puede disminuir el rendimiento de los cultivos (Perfect et al., 2001). La espectrometría de masas por ionización/desorción láser asistida por matriz de tiempo de vuelo (MALDI-TOF MS) ha demostrado ser útil para la identificación rápida de diferentes especies de *Aspergillus*, lo cual resulta especialmente importante para identificar algunas que muestran baja sensibilidad a los azoles, como por ejemplo para la discriminación entre *A. fumigatus* y las especies crípticas (Zvezdanova et al., 2022; Takeda et al., 2022). Para la identificación correcta de las especies de *Aspergillus*, se recomienda la secuenciación del gen de la *β-tubulina* y de la *calmodulina* (Houbraken et al., 2020).

La obtención de una muestra de tejido mediante una técnica invasiva puede ser difícil o estar contraindicada en pacientes con trombocitopenia, trastornos de la coagulación o inestabilidad hemodinámica. Dentro de las pruebas diagnósticas no invasivas se encuentran la detección de componentes de la pared celular fúngica: (1,3)-β-D glucano, cuya sensibilidad es variable y depende del estado inmunológico del huésped, el lugar de infección, la profilaxis o tratamiento antifúngico previo, el tipo de muestra y el laboratorio que realiza la prueba; y el galactomanano, el cual, en suero aumenta con recuentos bajos de neutrófilos, mientras que en el lavado broncoalveolar es menos dependiente de factores del huésped. Un índice de galactomanano sérico más alto que el nivel basal, y que permanezca elevado en el tiempo, se ha asociado con mayor mortalidad. La reactividad cruzada con otros hongos (por ejemplo, *Fusarium* o *Penicillium*) está descrita. Una comparación directa entre la prueba de 1,3-β-d-glucano y la prueba de galactomanano ha sugerido una mayor sensibilidad para la prueba de 1,3-β-d-glucano y una mayor especificidad para el galactomanano (Thompson y Young, 2021).

Las PCR de *Aspergillus,* evaluadas principalmente en pacientes hematológicos, pueden ser de utilidad en pacientes con una alta sospecha y cultivos negativos ya que se pueden realizar sobre muestra directa de plasma, suero y lavado broncoalveolar, siendo esta última la que da mejores resultados. La PCR comercial AsperGenius® es capaz de detectar tanto género como algunas especies (*A. fumigatus*, *A. flavus* y *A. terreus*) y Mycogenie® detecta *A. fumigatus*; además de ciertas mutaciones asociadas con la resistencia a azoles, las cuales serán comentadas más adelante (White et al., 2017; Chong et al., 2015; Mikulska et al., 2019).

Los factores del huésped del consenso EORTC no son aplicables a pacientes no inmunodeprimidos ya que para estas existen criterios específicos (Bassetti et al., 2021; Verweij et al., 2020b; Koehler et al., 2021). En los últimos años han surgido distintas definiciones para poder estandarizar los criterios diagnósticos:

- La IFI de brecha, según el Grupo de Estudio de Micosis Educación e Investigación (MSG-ERC) y la Confederación Europea de Micología Médica (ECMM), se define como cualquier IFI que ocurra durante la exposición a un antifúngico, incluyendo hongos fuera del espectro de actividad de este. El momento de la IFI de brecha se definió como el momento en el que apareció el primer signo o síntoma atribuible clínicamente, el primer hallazgo micológico o el primer hallazgo radiológico (Cornely et al., 2019).
- La aspergilosis pulmonar asociada a influenza (IAPA) probada, fue definida por Verweij y colaboradores, como un paciente que requiere ingreso en UCI por tener dificultad respiratoria con detección positiva de virus Influenza durante ese ingreso, más evidencia histológica de hifas septadas invasoras, y evidencia micológica de *Aspergillus* obtenida por PCR o cultivo a partir de tejido (Verweij et al., 2020b).
- La aspergilosis pulmonar asociada a COVID-19 (CAPA) probada, según la guía de 2020 de la Confederación Europea de Micología Médica (ECMM) y la Sociedad Internacional de Micología Humana y Animal (ISHAM), se define como un paciente que requiere ingreso en UCI por dificultad respiratoria con una prueba de RT-PCR positiva para SARS-CoV-2 temporalmente relacionada

con ese ingreso, más la detección histopatológica o microscópica directa de elementos fúngicos morfológicamente compatibles con *Aspergillus* spp., mostrando invasión en los tejidos con daño tisular asociado, o con *Aspergillus* aislado o detectado por microscopía, en estudios de histología o por PCR a partir de material obtenido por aspiración estéril o biopsia de un sitio pulmonar (Koehler et al., 2021).

En resumen, el diagnóstico de la aspergilosis invasiva es una tarea complicada debido a múltiples motivos: la sintomatología es inespecífica, la toma de muestras respiratorias o de tejido es complicada y puede estar contraindicada, los cultivos a menudo tienen baja sensibilidad, los signos radiográficos clásicos generalmente están ausentes en poblaciones no inmunodeprimidas, puede haber dificultades para obtener tomografías computarizadas en lugar de radiografías de tórax, y la discriminación entre la colonización por *Aspergillus* y la infección es problemática (Bassetti et al., 2021).

1.4. Tratamiento de la aspergilosis

Los azoles son la base del tratamiento y la prevención de las enfermedades causadas por *Aspergillus* spp. y representan las únicas opciones por vía oral, junto con la olorofima, aunque esta última no está aún aprobada para su uso. Por este motivo, las tasas de mortalidad en pacientes infectados por *A. fumigatus* resistente a los azoles son significativamente superiores a las de los sujetos infectados por cepas sensibles a estos (van der Linden et al., 2015).

Los azoles son un grupo de antifúngicos cuyo modo de acción se basa en el bloqueo de la síntesis del ergosterol mediante la inhibición competitiva de la enzima 14 α-esterol desmetilasa, que pertenece a la familia de proteínas citocromo P450 cyp51.

La anfotericina B es un polieno con un amplio espectro de acción, cuyo mecanismo de acción se basa en la unión de la molécula a la membrana plasmática de la célula fúngica, donde interacciona con el ergosterol, generando poros y dando lugar a la muerte celular por choque osmótico. Además, se piensa que puede haber otros mecanismos de acción que todavía no han sido clarificados (Carolus et al., 2020). La

anfotericina B liposomal (L-AmB) es la formulación más utilizada, la cual reduce su alta nefrotoxicidad.

La olorofima, es un antifúngico que pertenece a la nueva clase, las orotomidas, e inhibe la enzima dihidroorotato deshidrogenasa, lo que evita la biosíntesis de pirimidina. La olorofima es un antifúngico con potente actividad frente a especies de *Aspergillus* incluyendo a las cepas de *A. fumigatus* resistente a azoles.

Según las guías de la Sociedad Europea de Microbiología Clínica y Enfermedades Infecciosas (ESCMID), se recomienda el tratamiento con itraconazol para el manejo de pacientes con APC y ABPA. El voriconazol y el isavuconazol están indicados como tratamiento de primera línea para la aspergilosis invasiva pulmonar. El voriconazol, además, se recomienda como terapia primaria en pacientes con compromiso del sistema nervioso central y en pacientes con formas pulmonares crónicas. El posaconazol se recomienda como profilaxis antifúngica durante la neutropenia prolongada en pacientes de alto riesgo, o como terapia de rescate en pacientes intolerantes o no respondedores. L-AmB se recomienda en situaciones en las que los azoles estén contraindicados, como en el caso de infecciones causada por cepas resistentes, y como terapia de rescate (Ullmann et al., 2018).

Cuando las tasas de resistencia a azoles superan el 10%, los regímenes de tratamientos recomendados incluyen voriconazol/isavuconazol en combinación con una equinocandina o L-AmB, o monoterapia con L-AmB. Este régimen podría desescalarse una vez que se haya comprobado la sensibilidad a los azoles (Ullmann et al., 2018; Verweij et al., 2015).

1.5. Susceptibilidad de *A. fumigatus* a los antifúngicos

Las secciones *Terrei, Flavi* y *Nidulantes* presentan resistencia intrínseca a la anfotericina B y la sección *Usti*, presentan un perfil de multirresistencia a azoles y CMIs altas a anfotericina B. En estas secciones no hay diferencias significativas entre especies (Van Der Linden et al., 2011b; Gautier et al., 2016).

Sin embargo, dentro de la sección *Fumigati*, el perfil de susceptibilidad no es uniforme. *A. fumigatus sensu stricto* es habitualmente sensible a anfotericina B, voriconazol, itraconazol, posaconazol e isavuconazol. Mientras que, dentro de las especies crípticas, algunas muestran resistencia intrínseca a la anfotericina B y a los azoles, como *A. lentulus* y *A. fumigatiaffinis*, otras suelen ser sensibles a anfotericina B pero resistentes a los azoles, como *A. viridutans* y *A. thermomutatus,* y otras son sensibles tanto a anfotericina B como a los azoles, como *A. hiratsukae* (Tabla 2) (Perlin et al., 2017).

Tabla 2. Resistencia antifúngica en especies crípticas de *A. fumigatus*. Rojo=resistente al fármaco. Blanco=sensible al fármaco. Gris=elevada concentración mínima inhibitoria del fármaco.

ESPECIES	ANFOTERICINA B	ITRACONAZOL	VORICONAZOL	POSACONAZOL
A. lentulus				
A. fumigatiaffinis				
A. udagawae				
A. viridutans				
A. thermomutatus				
A. hiratsukae				

1.6. Rutas de desarrollo de la resistencia a azoles en *A. fumigatus*

Los triazoles no son mutagénicos, pero la variación genética, incluidas las mutaciones de resistencia a los triazoles, puede surgir a través de mutaciones espontáneas, recombinación mitótica o recombinación meiótica. Cuando una población de conidias de *Aspergillus* se expone a la presión de selección de triazoles, las cepas que albergan una mutación de resistencia prosperarán en comparación con las cepas de tipo salvaje y se volverán dominantes en la población. Este proceso de selección de resistencia puede ocurrir en un paciente que recibe un tratamiento prolongado con triazoles o en el medio ambiente, donde los fungicidas azólicos con actividad frente a *A.*

fumigatus pueden ejercer presión de selección (Anderson et al., 2005; Rogers et al., 2022).

El desarrollo de resistencia a los azoles en un paciente ocurre en aquellos que reciben un tratamiento o profilaxis prolongada, y en aquellos con condiciones predisponentes como cavidades pulmonares preexistentes (aspergiloma) y fibrosis quística. La presencia de una cavidad permite que se dé esporulación asexual, y con una exposición crónica a los azoles pueden ocurrir numerosas mutaciones espontáneas en las conidias. Los pacientes con APC pueden estar infectados con numerosas cepas de *A. fumigatus* genéticamente distintas, que incluyen tanto cepas de tipo salvaje como aquellas que albergan diferentes mutaciones de resistencia. Esto no se observa en pacientes con aspergilosis invasiva, donde forman exclusivamente hifas, no esporulan, y no desarrollan variación genética derivada de la esporulación asexual (Camps et al., 2012).

El desarrollo de resistencia a los azoles en el ambiente, ocurre presumiblemente como consecuencia de la exposición a fungicidas azólicos utilizados en agricultura que tienen una estereoquímica similar a los azoles utilizados en clínica. En este caso, los pacientes se infectan al inhalar conidias que ya albergan mecanismos de resistencia (Wiederhold y Verweij, 2020).

1.7. Mecanismos de resistencia a azoles en *A. fumigatus*

Los mecanismos por los que se puede producir la resistencia a azoles en *A. fumigatus* se pueden englobar en dos grandes grupos:

1) Mecanismos de resistencia dependientes del gen *cyp51A.*

2) Mecanismos de resistencia independientes del gen *cyp51A.*

1.7.1. Mecanismos de resistencia a azoles dependientes de *cyp51A*

En *A. fumigatus*, los mecanismos de resistencia a los triazoles están principalmente asociados con alteraciones en el gen *cyp51A* que codifica la lanosterol 14α-desmetilasa,

una enzima responsable del paso final de la vía de biosíntesis del ergosterol. El ergosterol es el esterol mayoritario de las membranas fúngicas y es fundamental para su permeabilidad y fluidez, siendo esencial para el crecimiento y supervivencia del hongo. En *A. fumigatus* están presente tanto la proteína Cyp51A como la Cyp51B, donde Cyp51B se expresa de manera constitutiva y la expresión de Cyp51A es inducible. Las mutaciones puntuales en la lanosterol 14α-desmetilasa pueden provocar cambios en los aminoácidos que resultan en modificaciones de los canales de acceso de los ligandos a través de los cuales los azoles acceden al sitio activo de la enzima y se unen a la molécula de hemo. Los puntos calientes para las sustituciones de aminoácidos incluyen G54, G138, M220 y G448, que se corresponden con fenotipos específicos de resistencia a azoles. Estas mutaciones de resistencia se encuentran comúnmente en pacientes con exposición prolongada previa a la terapia con triazoles (Garcia-Rubio et al., 2017).

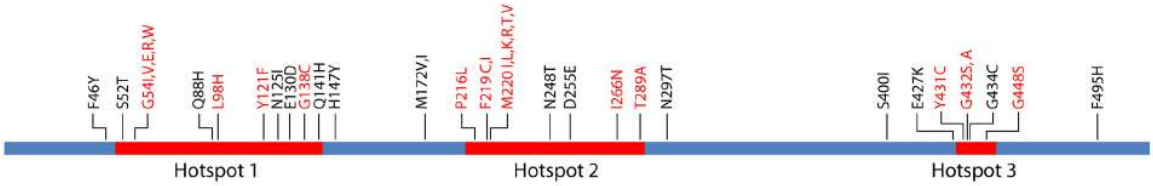

Figura 3. Sustituciones conocidas de aminoácidos en la proteína cyp51A; en rojo se muestran aquellas que confieren resistencia. Figura de Dudakova et al, 2017.

Las sustituciones no sinónimas de la proteína Cyp51A de *A. fumigatus*, como L98H, Y121F y T289A, suelen ir acompañadas de repeticiones en tándem (TR) en el promotor del gen *cyp51A*, que regulan al alza su expresión. La más habitual es la duplicación de 34 pares de bases en el promotor del gen junto a L98H (TR_{34}-L98H), que está relacionada con resistencia a todos los azoles. Sin embargo, cuando TR_{34}-L98H se acompaña de las sustituciones S297T y F495I se han observado CMIs más bajas de voriconazol (Rogers et al., 2022). También es común la duplicación de 46 pares de bases en el promotor con Y121F y T289A (TR_{46}-Y121F-T289A), asociada a resistencia a voriconazol e isavuconazol y CMIs elevadas a itraconazol y posaconazol (van der Linden et al., 2013; Pelaez et al., 2015). La sustitución Y121F ha sido descrita en solitario también con un perfil de resistencia similar a cuando aparece en la combinación anterior (Lescar et al., 2014).

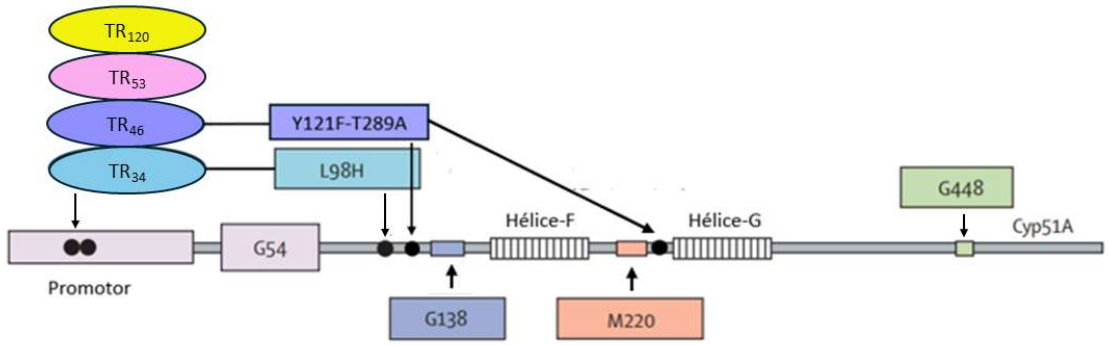

Figura 4. Gen *cyp51A* de *A. fumigatus* con las principales mutaciones asociadas a la resistencia a azoles. Figura adaptada de Verweij, P. E. et al, 2009.

Otro mecanismo encontrado, aunque con menos frecuencia, consiste en una duplicación de 53 pares de bases en el promotor de la diana (TR_{53}), sin modificaciones adicionales en el gen, y que se asocia con resistencia a todos los azoles (Alvarez-Moreno et al., 2017). Estas tres modificaciones 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 agricultura (Snelders et al., 2012), observándose en cepas aisladas de pacientes que no habían estado en terapia antifúngica anteriormente (Verweij et al., 2007). Se ha descrito también una repetición de 120 pares de bases en tándem en el promotor de *cyp51A* sin mutaciones adicionales en el gen, probablemente relacionada con exposición previa a la terapia con triazoles ya que nunca se ha encontrado en el ambiente, y relacionada con resistencia a todos los azoles (Hare et al., 2019).

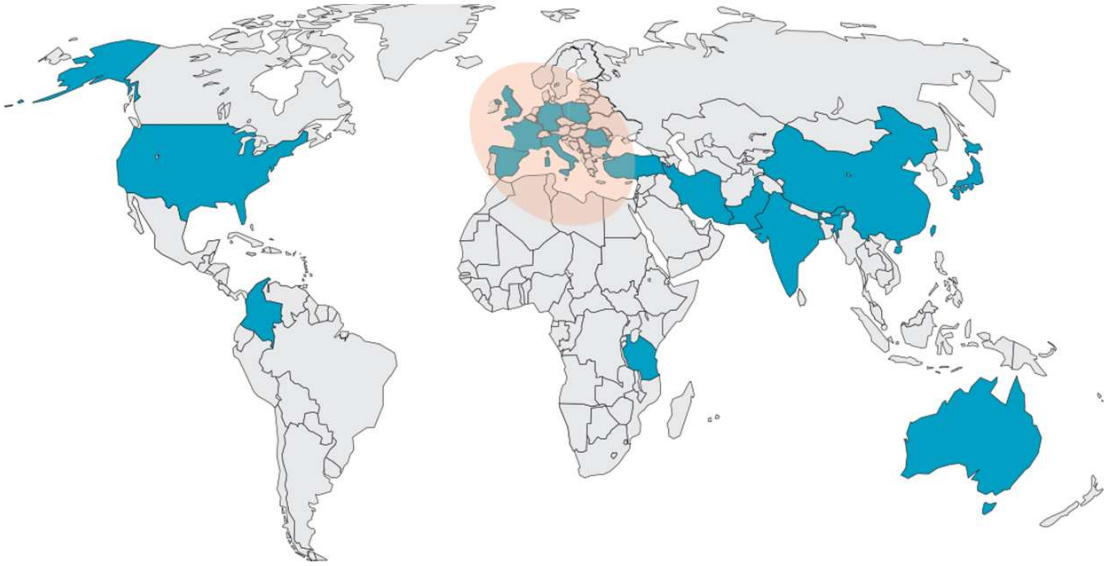

Figura 5. Países en los que se han notificado cepas de *A. fumigatus* resistentes a azoles con sustituciones TR_{34}-L98H o TR_{46}-Y121F-T289A. El óvalo naranja marca la región con mayor carga de resistencia. Figura adaptada de Perlin et al, 2017.

Además de estas modificaciones en el gen *cyp51A* responsables de los mecanismos de resistencia, también se han descrito otras mutaciones puntuales, aunque no se ha establecido una relación directa entre éstas y la resistencia a azoles. La combinación de tres (F46Y-M172V-E427K) y cinco (F46Y-M172V-N248K-D255E-E427K) mutaciones puntuales, respectivamente, se han asociado a perfiles de sensibilidad a azoles variables (Escribano et al., 2011; Garcia-Rubio et al., 2018). Sin embargo, las cepas de *A. fumigatus* con estas combinaciones de polimorfismos se han descrito en pacientes bajo tratamiento con voriconazol, lo que sugiere que podrían estar seleccionándose por la exposición continuada de este azol (Alanio et al., 2012).

Tabla 3. Mutaciones en el gen *cyp51A* y las resistencias reportadas que conllevan.

Mutación *cyp51A*	TR_{34}-L98H TR_{120}-F46Y-M172V-E427K	TR_{46}-Y121F-T289A	TR_{53} G432	G54 P216 F219 M220	N22 M236 P394 T440 G448 Y491	Y121	G138 Q141 H147 A284 S297 Y431 G434 F495
Fenotipo de resistencia	Pan-azol	Voriconazol Posaconazol	Itraconazol Voriconazol	Itraconazol Posaconazol	Itraconazol	Voriconazol	Pan-azol

Se han desarrollado PCR comerciales que son capaces de detectar directamente en muestras clínicas la presencia de *A. fumigatus*, así como las sustituciones más frecuentemente asociadas con la resistencia a azoles (AsperGenius®, Mycogenie®): TR_{34}-L98H o TR_{46}-Y121F-T289A (en el caso de AsperGenius®) (Pelzer et al., 2020; Mikulska et al., 2019). El inconveniente de estos ensayos es que sólo pueden detectar un limitado número de modificaciones genéticas y tienen una baja sensibilidad (Pelzer et al., 2020; Buil et al., 2018).

1.7.2. Mecanismos de resistencia a azoles independientes de *cyp51A*

A pesar de que la resistencia a azoles en *A. fumigatus* se atribuye principalmente a las alteraciones en el gen *cyp51A*, se han propuesto otros mecanismos.

Entre estos se encuentran, por un lado, la sobreexpresión de las bombas de eflujo, que disminuyen la concentración efectiva del azol. Estas bombas se clasifican en dos clases principales: la superfamilia de los grandes facilitadores (MFS) y las proteínas de casete de unión a ATP (ABC) (Cannon et al., 2009). Por otro lado, la sobreexpresión de los genes *cyp51B* (Buied et al., 2013) y *cyp51A.* Esta última mediada por modificaciones en genes transcripcionales, como la alteración de SrbA, una proteína de unión al elemento regulador del esterol (SREBP), que se une a la región promotora del gen

cyp51A favoreciendo su transcripción. Esto se ha asociado con un aumento en la producción de ergosterol y una disminución en la tolerancia a los azoles. Asimismo, la mutación P88L en el factor de transcripción HapE que inhibe su acción represora sobre el gen *cyp51A*, lo que también desencadena su sobreexpresión, y como consecuencia, una reducción de la sensibilidad a azoles (Gsaller et al., 2016). Además, mutaciones en genes implicados en la síntesis del ergosterol, como *hmg1*, también se han relacionado con la resistencia a azoles (Losada et al., 2015). Y, recientemente, se identificó el cofactor negativo dos (Nct2), formado por los subfactores NctBA y B, como un regulador de la resistencia a los triazoles al modular los niveles de expresión de los factores de transcripción asociados con la biosíntesis del ergosterol (Rogers et al., 2022).

No obstante, a día de hoy, la relación de estos nuevos mecanismos propuestos con la resistencia a azoles no es clara, especialmente sus implicaciones clínicas, ya que también se han encontrado en cepas sensibles a los azoles.

Dado que los triazoles son el tratamiento de elección para la aspergilosis (Ullmann et al., 2018; Patterson et al., 2016), la resistencia en *A. fumigatus* plantea un problema (van der Linden et al., 2011a). Los pacientes infectados con cepas de *A. fumigatus* resistentes a los azoles presentan mayor mortalidad que aquellos infectados con cepas sensibles (Chong et al., 2016; Lestrade et al., 2019a; Lestrade et al., 2019b) lo que hace que la detección de resistencia sea importante para mejorar el manejo de los pacientes.

1.8. Distribución global de la resistencia a azoles

La resistencia a los azoles en las cepas clínicas de *A. fumigatus sensu stricto* se ha detectado de manera creciente en todo el mundo durante los últimos 20 años.

El primer estudio sistemático a gran escala fue el estudio SCARE, realizado por van der Linden y colaboradores entre 2009 y 2011. En este estudio se determinaron las tasas de resistencia a azoles en centros de 22 países, incluyendo 19 países europeos, EEUU, Brasil y Australia, con una prevalencia global del 3,2% y con un predominio de la sustitución TR_{34}-L98H. Sin embargo, hubo grandes diferencias entre regiones,

notificándose en algunos centros tasas de resistencia de hasta el 26,1% (van der Linden et al., 2015).

La resistencia a azoles ha sido reportada de forma relativamente común en el norte de Europa en las últimas décadas. En los Países Bajos se ha notificado un aumento de la prevalencia del 1,7-6% entre 1997 y 2007 al 8-15% entre 2013 y 2018 (Snelders et al., 2008; Lestrade et al., 2020). En Reino Unido, un estudio realizado por el Centro de Referencia de Micología de Manchester, entre 2008 y 2009, reportó una tasa de resistencia del 28% (Bueid et al., 2010). En Dinamarca, según un programa nacional de vigilancia realizado entre 2018 y 2020 se reportaron tasas de resistencia del 6,1% (Risum et al., 2022), y en Bélgica, un estudio multicéntrico retrospectivo realizado entre 2011 y 2012 en 18 hospitales, mostró una prevalencia del 5,5% (Vermeulen et al., 2015).

En Estados Unidos, un programa de vigilancia entre realizado entre los años 2015 y 2017 mostró una prevalencia de resistencia a azoles en *A. fumigatus* del 1,4%, siendo un tercio de ella asociada a la presencia de cepas con las sustituciones TR_{34}-L98H (Berkow et al., 2018). En América Latina los datos son escasos, en Perú, un estudio prospectivo realizado en cepas de pacientes con aspergilosis pulmonar crónica mostró una prevalencia de resistencia del 2%, y en Brasil, un estudio retrospectivo de cuatro años notificó una prevalencia de resistencia del 1% (Bustamante et al., 2020; Pontes et al., 2020).

En África los datos también son limitados, algunos estudios sugieren una prevalencia de resistencia a azoles del 1,3% en muestras clínicas (Amona et al., 2022).

La prevalencia de resistencia en estudios realizados en Asia oscila entre un 3% y un 8% (4% en China, 8% en Taiwán, 3% en Japón) (Deng et al., 2017; Wang et al., 2018; Toyotome et al., 2020).

En Australia, en un estudio con un bajo número de cepas, el 2% de los *A. fumigatus* fueron resistentes a los azoles (Talbot et al., 2018).

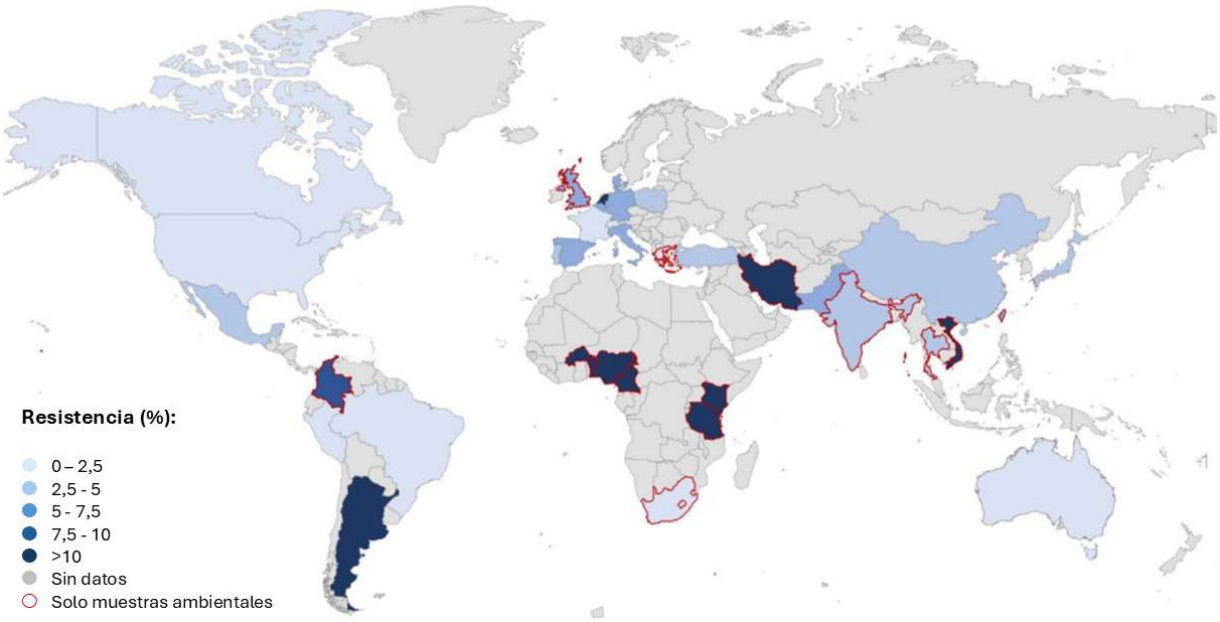

Figura 6. Distribución mundial de la resistencia a los azoles en cepas clínicas y ambientales de *A. fumigatus*. Figura adaptada de Bosetti y Neofytos, 2023.

En España se han realizado varios estudios con el fin de vigilar la resistencia a azoles en *A. fumigatus*. En los estudios realizados en 2010-2011 y 2016, denominados FILPOP, realizados con cepas procedentes de 29 y 10 hospitales, respectivamente, se encontró un 0,6% de resistencia a azoles en *A. fumigatus sensu stricto* en el primer periodo y un 1,2% en el segundo periodo. En este segundo estudio, dos cepas tenían las sustituciones TR_{34}-L98H y procedían de pacientes de Sevilla y Donostia (Alastruey-Izquierdo et al., 2013; Alastruey-Izquierdo et al., 2018).

En 2019 se llevó a cabo otro estudio, denominado *ASPE*IN, en el que se recogieron un número mayor de cepas de *A. fumigatus* (n=847) procedentes de 29 hospitales españoles, revelando una tasa de resistencia inesperadamente alta en *A. fumigatus sensu stricto* (5,5%). Las sustituciones TR_{34}-L98H fueron las predominantes y las cepas resistentes procedían principalmente de las zonas septentrional y mediterránea de la España peninsular (Escribano et al., 2021).

Tabla 4. Estudios multicéntricos sobre la prevalencia de la resistencia a azoles en *A. fumigatus* en cepas clínicas. Subrayadas las sustituciones predominantes.

Autor, año publicación	Periodo de estudio	País	Resistencia a azoles	Mutaciones en *cyp51A* relacionadas con la resistencia
Snelders et al., 2008	1994-2007	Países Bajos	1.7%-6%	$\underline{TR_{34}\text{-}L98H}$, M220
van der Linden et al., 2015	2009-2011	19 países europeos, EEUU, Australia, Brasil	3,2%	$\underline{TR_{34}\text{-}L98H}$, TR_{46}-Y121F-T289A, G54, M220
Vermeulen et al., 2015	2011-2012	Bélgica	5,5%	$\underline{TR_{34}\text{-}L98H}$, TR_{46}-Y121F-T289A
Berkow et al., 2018	2015-2017	EEUU	1,4%	$\underline{TR_{34}\text{-}L98H}$±S297T-F495I, P216L, F46Y-M172V-N248T-D255E-E427K
Lestrade et al., 2020	2013-2018	Países Bajos	8%-15%	$\underline{TR_{34}\text{-}L98H}$±S297T-F495I, TR_{46}-Y121F-T289A
Risum et al., 2022	2018-2020	Dinamarca	6,1%	$\underline{TR_{34}\text{-}L98H}$, G54, P216S, F219L, M220, G432S, G448S y Y121F
Alastruey-Izquierdo et al., 2013	2010-2011	España	0,6%	No encontradas
Alastruey-Izquierdo et al., 2018	2016	España	1,2%	TR_{34}-L98H
Escribano et al., 2021	2019	España	5,5%	$\underline{TR_{34}\text{-}L98H}$, G54, TR_{46}-Y121F-T289A, F46Y-M172V-N248T-D255E±E416Q-E427K

En pacientes hematológicos se han llevado a cabo algunos estudios sobre la prevalencia de las infecciones por *A. fumigatus* resistentes a azoles. En Alemania, un estudio realizado en dos centros entre 2012 y 2013 en pacientes hematológicos con aspergilosis invasiva por *A. fumigatus*, reportó tasas alarmantemente altas del 29.6%, asociada principalmente a la presencia de cepas con sustituciones TR_{34}-L98H y TR_{46}-Y121F-T289A (Steinmann et al., 2015). Sin embargo, en Francia, en un estudio realizado entre 2006 y 2009 en pacientes hematológicos con aspergilosis invasiva, la prevalencia de la resistencia a azoles fue del 0,85%, debido a la presencia de una cepa con la sustitución TR_{34}-L98H (Alanio et al., 2011). Y del mismo modo, en Italia, un estudio multicéntrico realizado en 18 centros, entre 2014 y 2016, reportó una tasa del 0,8%, debido a la presencia de una cepa con la sustitución G432S (Prigitano et al., 2020).

1.9. Métodos de estudio de la sensibilidad antifúngica en *A. fumigatus*

Las pruebas de sensibilidad antifúngica se realizan especialmente cuando las cepas aisladas representan infecciones invasivas, recurrentes, o no respondedoras a la terapia y se sospecha la existencia de resistencia adquirida, o cuando la sensibilidad no puede predecirse solo con la identificación a nivel de especie. También son importantes en la vigilancia de la resistencia y estudios epidemiológicos. Las pautas de la ESCMID de 2018 respaldan la identificación a nivel de sección para todas las cepas clínicamente significativas de *Aspergillus* y las pruebas de sensibilidad antifúngica de las cepas tanto para el manejo clínico de los pacientes como con fines epidemiológicos (Ullmann et al., 2018).

Un aspecto importante, y que aplica a todos los métodos de estudio de la sensibilidad, es que los cultivos pueden albergar varios fenotipos de resistencia y se deben analizar múltiples colonias para detectarla.

A continuación, se citan algunos conceptos importantes relacionados con la interpretación de la sensibilidad a los antimicrobianos:

- **CMI - Concentración Mínima Inhibitoria:** concentración más baja, expresada en mg/L, de un agente que inhibe el crecimiento de un microorganismo.

- **WT - *Wild type*:** población de microorganismos que no tiene mecanismos de resistencia adquiridos para un agente antimicrobiano concreto.
- **NWT - *Non-wild type*:** población de microorganismos que tiene mecanismos de resistencia adquiridos para un agente antimicrobiano concreto.
- **ECOFF - *epidemiological cut-off value***: es la CMI que separa la población WT de la NWT.
- **S - Sensible, régimen de dosificación estándar:** un microorganismo se categoriza como "Sensible, régimen de dosificación estándar" cuando hay una alta probabilidad de éxito terapéutico utilizando un régimen de dosificación estándar del agente.
- **I - Sensible, exposición aumentada**: un microorganismo se categoriza como "Sensible, exposición aumentada" cuando hay una alta probabilidad de éxito terapéutico cuando la exposición al agente se incrementa o bien ajustando el régimen de dosificación o por su elevada concentración fisiológica en el lugar de infección. La exposición se refiere a el modo de administración, la dosis, el intervalo de dosificación, el tiempo de infusión, la distribución y la excreción en la que agente antimicrobiano influirán en el microorganismo infectante en el sitio de la infección.
- **R - Resistente:** un microorganismo se categoriza como "Resistente" cuando hay una alta probabilidad de fracaso terapéutico incluso cuando hay una exposición aumentada.
- **ATU - Área de Incertidumbre Técnica:** es un concepto para advertir a los laboratorios de que el valor de CMI se encuentra en un área donde hay dificultades de interpretación. No es una categoría como S, I y R. Se asume que la prueba de sensibilidad se ha realizado correctamente y que el valor obtenido es correcto en sí mismo, pero requiere de pruebas adicionales para la interpretación de la CMI. En el caso de *A. fumigatus*, la interpretación de las CMI en ATU se realiza de la siguiente manera: posaconazol (se considera resistente si la cepa también es resistente a itraconazol), isavuconazol (se considera resistente si la cepa también es resistente a voriconazol).

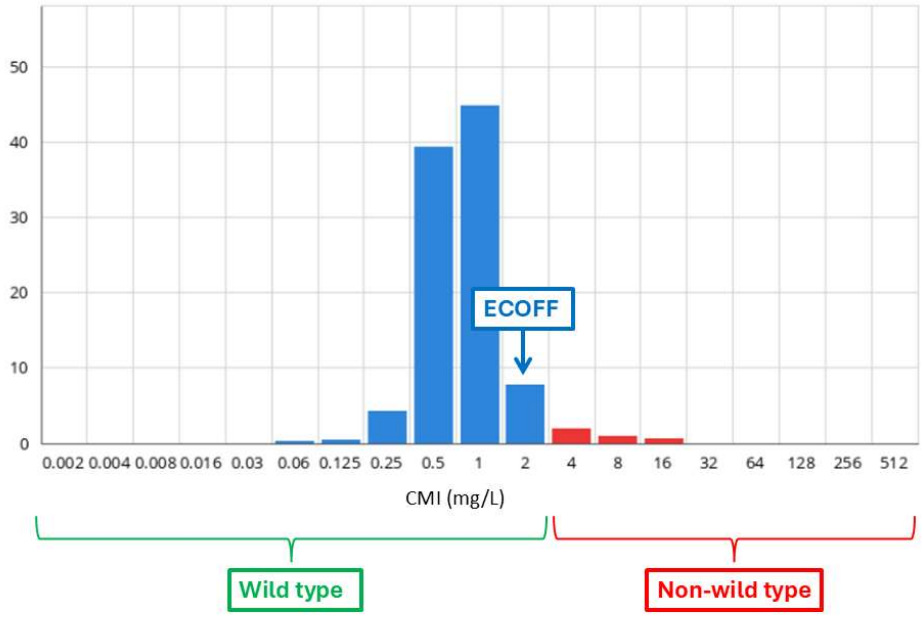

Figura 7. Representación de los conceptos de ECOFF, WT y NWT en la distribución de CMIs mediante microdilución en caldo de isavuconazol frente a *A. fumigatus* (https://www.mic.eucast.org).

1.9.1. Microdilución en caldo: procedimiento EUCAST E. Def 9.4.

Los métodos de microdilución en caldo se utilizan para establecer las CMIs de los agentes antimicrobianos. Con estos métodos se mide la actividad *in vitro* de un fármaco frente a un organismo en cultivo líquido que contiene concentraciones seriadas de fármaco.

Hay dos métodos estandarizados de microdilución en caldo para hongos filamentosos: el método descrito por el Comité Europeo de Pruebas de Sensibilidad a los Antimicrobianos (EUCAST) y el del Instituto de Normas Clínicas y de Laboratorio (CLSI). En Europa el más utilizado actualmente es el método de EUCAST.

El método estandarizado de EUCAST para el estudio de la sensibilidad a antifúngicos en hongos filamentosos es el E.Def. 9.4 cuya versión actualizada fue publicada en marzo de 2022 (Guinea et al., 2022). Este método describe la metodología específica para estos estudios en *Aspergillus* spp. y, en el caso de *A. fumigatus*, los puntos de corte han sido descritos y estandarizados frente a los principales antifúngicos de uso clínico. Estos

puntos de corte clínicos (v10) pueden ser consultados en el siguiente enlace: https://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals) (Tabla 5).

Tabla 5. Puntos de corte clínicos de anfotericina B y azoles frente a *A. fumigatus* [EUCAST (v10)].

ANTIFÚNGICO	PUNTOS DE CORTE (mg/L)		
	S≤	R ≥	ATU
Anfotericina B	1	2	No aplica
Itraconazol	1	2	2
Voriconazol	1	2	2
Posaconazol	0,125	0,5	0,25
Isavuconazol	1	4	2

Desafortunadamente, los procedimientos de microdilución son lentos, técnicamente exigentes, y requieren personal bien entrenado, lo que puede dificultar que estas pruebas de sensibilidad antifúngica estén ampliamente disponibles en los laboratorios de Microbiología Clínica.

1.9.2. Placas de agar con azoles: procedimiento EUCAST E.Def 10

Como alternativa a la microdilución en caldo, se pueden utilizar placas de agar que contienen azoles para cribar y detectar de forma rutinaria la resistencia en *A. fumigatus* en los laboratorios de Microbiología Clínica. El procedimiento se basa en la capacidad de las cepas resistentes a los azoles para crecer en placas con pocillos de agar que contienen estos fármacos (itraconazol, voriconazol y posaconazol) (Figura 8).

Las placas de agar que contienen azoles, que están también disponibles comercialmente en algunos países (VIPcheck©, BovenLeeuwen, Países Bajos), han demostrado ser útiles para detectar la presencia de cepas de *A. fumigatus* resistentes a los azoles en el ambiente y en entornos clínicos (van der Linden et al., 2015; Chowdhary et al., 2014; Mortensen et al., 2010; Arendrup et al., 2017a; Buil et al., 2017; Tsitsopoulou et al., 2018; Chen et al., 2019).

Una validación multicéntrica del método utilizando placas VIPcheck© y placas caseras realizadas en los propios laboratorios participantes permitió que EUCAST desarrollara un método nuevo (E.Def 10) e implementara el procedimiento para su uso rutinario (Arendrup et al., 2018; Buil et al., 2017; Guinea et al., 2019).

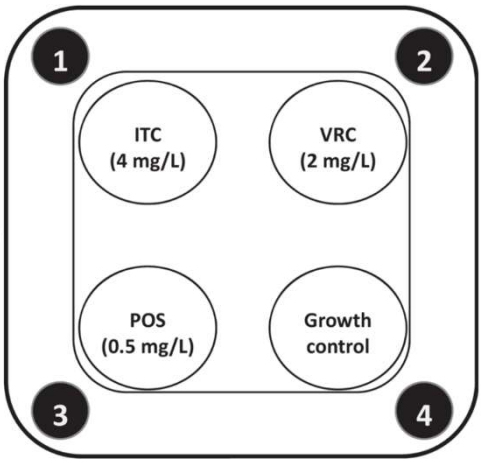

Figura 8. Representación de la placa de agar de cuatro pocillos con los pocillos que contienen 1 mL de agar que contiene azoles (pocillos 1–3) y agar sin antifúngicos (pocillo 4) utilizado como control de crecimiento. ITC, itraconazol; VRC, voriconazol; POS, posaconazol.

Dado que las placas comerciales no están disponibles en muchos países, es necesario prepararlas de forma casera. En los estudios realizados se utilizaban placas de plástico multipocillo de la marca Nunc® y quedaría por resolver si sería posible utilizar otros tipos de placas de plástico multipocillo que abaratasen los costes.

El patrón de resistencia se deduce fácilmente del crecimiento en los pocillos de agar con azoles (Figura 9). Las sustituciones TR_{34}-L98H pueden sospecharse en cepas que crecen en los pocillos de agar que contienen itraconazol y voriconazol, las sustituciones G54 pueden sospecharse en cepas que crecen en los pocillos que contienen posaconazol e itraconazol, y las sustituciones TR_{46}-Y121F-T289A pueden sospecharse en cepas que crecen exclusivamente en los pocillos de agar que contienen voriconazol. Un crecimiento vigoroso sobre el agar con azoles sugiere firmemente la presencia de cepas resistentes, mientras que un crecimiento poco prominente refleja con alta probabilidad una falsa resistencia. Sin embargo, se recomiendan métodos de microdilución confirmatorios en las cepas que crezcan en placas que contienen azoles, independientemente del grado de crecimiento fúngico.

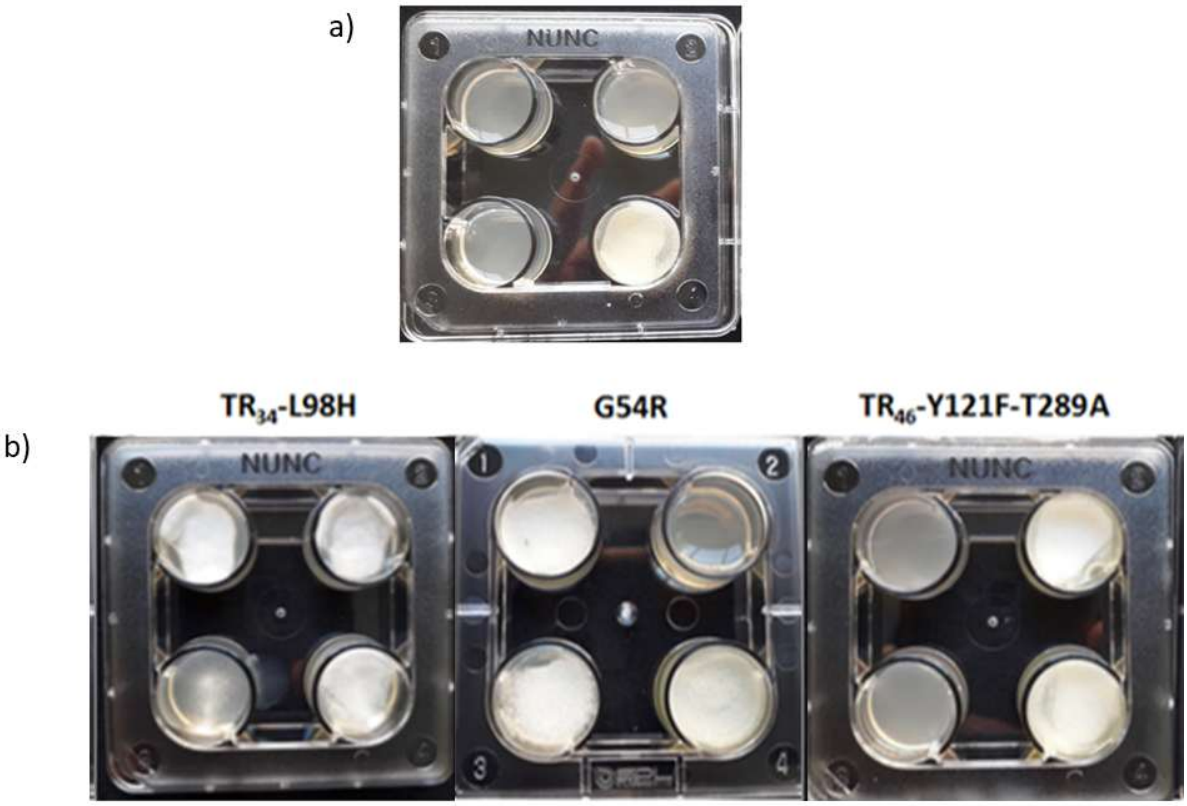

Figura 9. Patrones de crecimiento en las placas de agar con azoles de cepas de *A. fumigatus sensu stricto* sensibles (a) y resistentes (b) a azoles.

1.9.3. Tiras de difusión en gradiente

Otra alternativa para la determinación de la resistencia son las tiras de difusión de gradiente, ampliamente utilizadas en la práctica de los laboratorios de Microbiología Clínica. Este procedimiento tiene el inconveniente de que la interpretación de las CMIs sigue siendo controvertida y no está estandarizada.

Las tiras de difusión de gradiente son tiras de plástico con la escala de CMIs en µg/mL indicada por un lado y un gradiente de antifúngico predefinido por el otro. Cuando la tira se aplica a la superficie de agar previamente sembrado, el gradiente de antifúngico se transfiere inmediatamente al medio. El crecimiento fúngico se hace visible durante la incubación y aparece una elipse de inhibición simétrica centrada a lo largo de la tira. El valor de CMI se lee en la zona de inhibición completa del crecimiento fúngico, donde el extremo en punta de la elipse corta la tira (Figura 10).

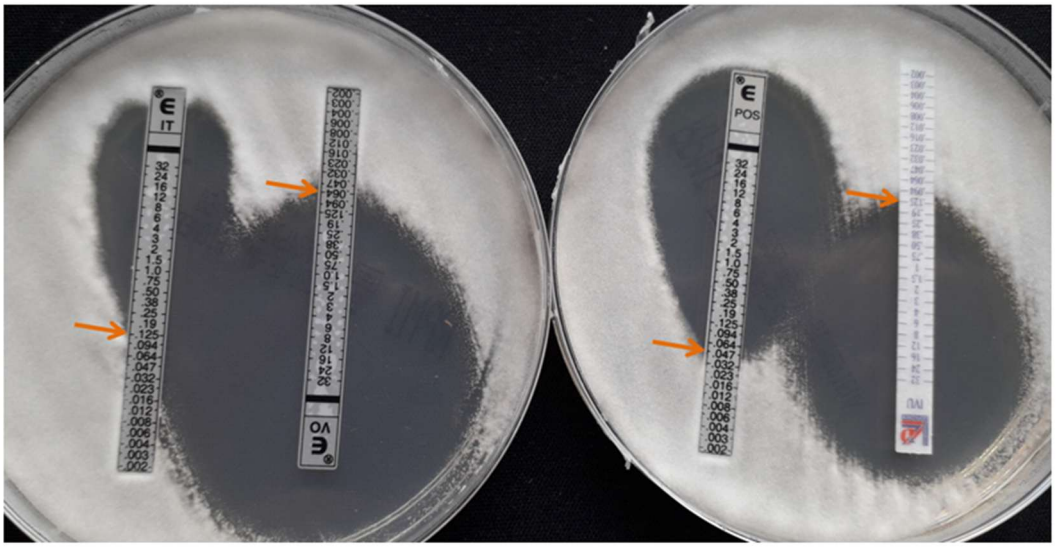

Figura 10. Patrón de crecimiento en placas de agar con tiras de difusión en gradiente de una cepa de *A. fumigatus sensu stricto* sensible a azoles; se indica con una flecha naranja la CMI de itraconazol (IT), voriconazol (VO), posaconazol (POS), e isavuconazol (IVU).

Estudios previos que comparan el procedimiento de microdilución en caldo E.Def 9.4 con las tiras de difusión de gradiente, incluyendo cepas de *A. fumigatus* de tipo salvaje y con mutaciones en el gen *cyp51A* (TR_{34}-L98H, TR_{46}-Y121F-T289A, M220 y G54), han reportado acuerdos categóricos altos, aunque se encuentran limitados por el bajo número de cepas resistentes, los agentes antifúngicos seleccionados, y la ausencia en las evaluaciones de especies crípticas (Idelevich et al., 2018; Arendrup et al., 2017b).

Espinel-Ingroff, utilizando el método estándar del CLSI, propuso valores de corte epidemiológicos frente *A. fumigatus sensu stricto* para itraconazol (>1 mg/L), voriconazol (>0,25 mg/L) y posaconazol (>0,125 mg/L) (Espinel-Ingroff et al., 2019; Espinel-Ingroff et al., 2018; Espinel-Ingroff et al., 2017). Mercier y colaboradores, propusieron puntos de corte epidemiológicos frente a *A. fumigatus sensu lato* para isavuconazol de 0,5 mg/L (Mercier et al., 2023). Sin embargo, estos puntos de corte requieren ser validados por laboratorios independientes y con un mayor número de cepas resistentes.

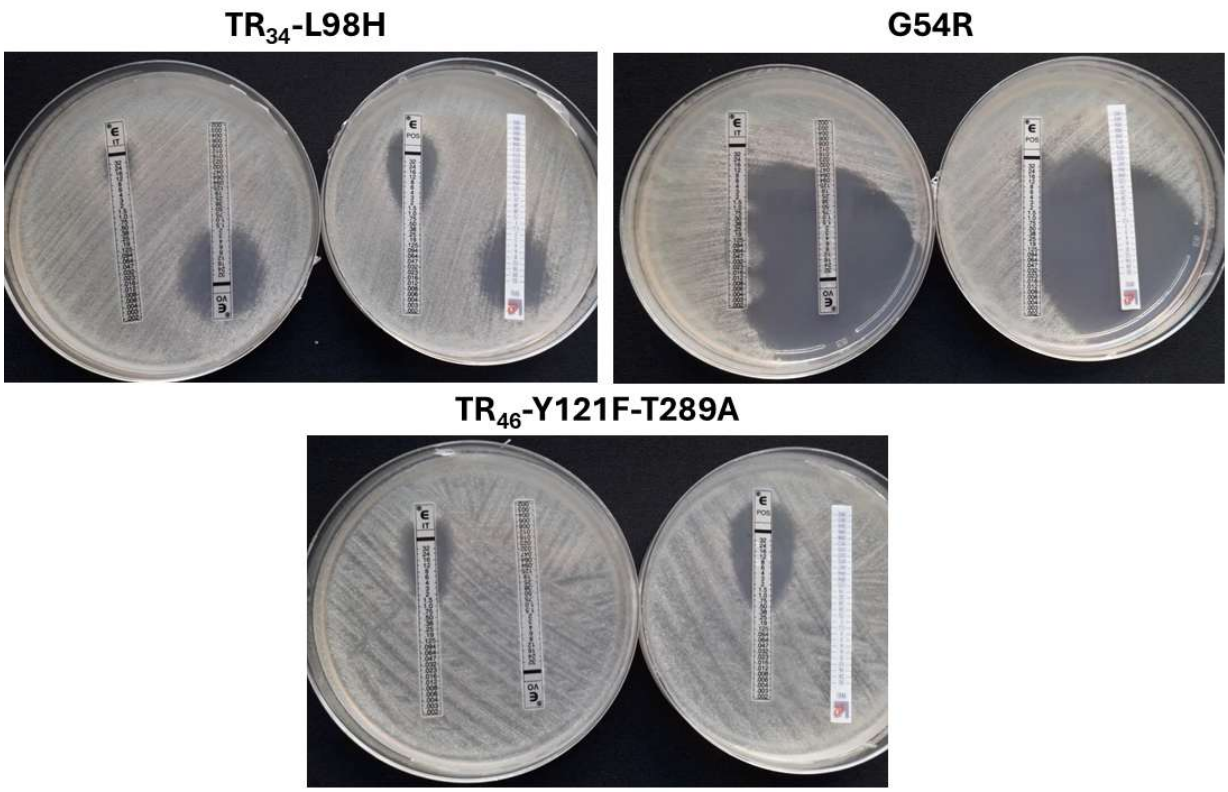

Figura 11. Patrón de crecimiento en placas de agar con tiras de difusión en gradiente de cepas de *A. fumigatus sensu stricto* resistentes a azoles con diferentes mutaciones en el gen *cyp51A*.

2. JUSTIFICACIÓN

La Organización Mundial de la Salud incluyó a *A. fumigatus* dentro de la lista de patógenos fúngicos prioritarios (WHO FPPL) en 2022. Este patógeno ha sido considerado de prioridad crítica dada su ubicuidad, su capacidad de producir enfermedades invasivas, y los niveles crecientes de resistencia a los azoles.

Los azoles son la primera línea del tratamiento de la aspergilosis invasiva y las infecciones producidas por cepas resistentes se asocian a un peor pronóstico, en muchas ocasiones por el retraso en su detección y un tratamiento inadecuado de inicio.

Las tasas de resistencia a azoles en España han sido tradicionalmente bajas, sin embargo, en el año 2019, el estudio *ASPE*IN I (*Aspergillus* in Spain) realizado con cepas de *A. fumigatus sensu lato* procedentes de muestras clínicas de pacientes atendidos en 29 hospitales de España, mostró que el 6,6% de los pacientes eran portadores de cepas resistentes a azoles. Las modificaciones TR_{34}-L98H fue la sustitución dominante, aunque su presencia no estaba muy extendida. En el año 2022, el estudio *ASPE*IN II, el cual se amplió a 81 hospitales españoles, mostró unos datos muy similares de resistencia a azoles, con un predominio también de cepas con las modificaciones TR_{34}-L98H, pero con una distribución mucho mayor.

En este contexto, se hace necesaria la detección de la resistencia a los azoles en cepas clínicas de *A. fumigatus* por parte de los laboratorios de Microbiología Clínica. Entre los métodos disponibles se encuentran: la microdilución en caldo, las placas de agares con azoles y las tiras de difusión en gradiente, pero todos ellos tienen limitaciones. El método estandarizado por EUCAST de la microdilución en caldo resulta laborioso, es necesario el conocimiento exhaustivo de su preparación e interpretación y conlleva mucho tiempo. Las placas de agares con azoles, son un método de cribado estandarizado por EUCAST, pero la preparación casera de las placas y la preparación del inóculo de las cepas lleva tiempo y las placas de plástico recomendadas por el procedimiento de EUCAST tienen un elevado coste. Y las tiras de difusión en gradiente, aunque se utilizan de una forma muy extendida en España, no tienen puntos de corte establecidos y la preparación de los inóculos y la interpretación de los resultados, no están estandarizados.

En consecuencia, los estudios de sensibilidad antifúngica en hongos filamentosos raramente se realizan, o bien se llevan a cabo con tiras de difusión en gradiente sin unos puntos de corte establecidos o se retrasa, debido a la derivación de esta prueba a un laboratorio de referencia.

En este escenario, en la presente tesis se pretenden evaluar los métodos citados para la detección de las resistencias a azoles en *A. fumigatus* y evaluar distintas estrategias para hacerlos más sencillos, objetivos, baratos, y accesibles para el laboratorio de Microbiología Clínica.

3. OBJETIVOS

La presente tesis pretende abordar los siguientes objetivos:

1. Comparación de las lecturas visuales y espectrofotométricas de las CMIs de azoles y anfotericina B frente a cepas de *A. fumigatus* obtenidas mediante el método de microdilución EUCAST.

1.1. Comparación de las lecturas visuales y espectrofotométricas con un *endpoint* de inhibición del crecimiento fúngico ≥ 95%.

1.2. Comparación de las lecturas visuales y espectrofotométricas con un *endpoint* de inhibición del crecimiento fúngico tanto ≥ 95% como ≥ 90%.

2. Evaluación y simplificación del método de placas de agar con azoles (EUCAST E.Def 10) para la detección de cepas de *A. fumigatus* resistentes a azoles.

2.1. Evaluación del impacto de los distintos tipos de plásticos de diferentes placas multipocillo en la preparación de placas de agar con azoles.

2.2. Evaluación del impacto de omitir la fase de filtrado en el proceso de preparación de los inóculos.

2.3. Evaluación del impacto de omitir el ajuste de la concentración del inóculo a 0,5 McFarland en el proceso de preparación de los inóculos.

3. Evaluación y simplificación del método de las tiras de difusión por gradiente para la detección de cepas de *A. fumigatus* resistentes a azoles.

3.1. Establecer puntos de corte para diferenciar la población salvaje de la resistente en *A. fumigatus sensu lato.*

3.2. Evaluación del impacto de omitir las fases de filtrado y ajuste de la concentración del inóculo a 0,5 McFarland en el proceso de preparación de los inóculos.

4. DESARROLLO

CAPÍTULO I: Comparación de las lecturas visuales y espectrofotométricas de las CMIs de azoles y anfotericina B frente a cepas de *A. fumigatus*.

Artículo 1: Azole and Amphotericin B MIC Values against *Aspergillus fumigatus*: High Agreement between Spectrophotometric and Visual Readings Using the EUCAST E. Def 9.3.2 Procedure

El procedimiento de microdilución en caldo de EUCAST E.Def 9.3.2 recomendaba la determinación visual de las CMIs para los azoles y la anfotericina B, sin embargo, esto puede resultar poco objetivo debido a las variaciones entre usuarios. En este estudio se obtuvieron lecturas espectrofotométricas de las CMIs de azoles y anfotericina B frente a cepas de *A. fumigatus* y se compararon con el método visual para superar la subjetividad.

Se incluyeron 847 cepas clínicas de *A. fumigatus sensu lato* (*A. fumigatus sensu stricto* [n=828] y especies crípticas [n=19]) recogidas en 30 hospitales durante un estudio multicéntrico realizado en España. Se estudió la sensibilidad antifúngica según el procedimiento EUCAST E.Def 9.3.2 para anfotericina B, itraconazol, voriconazol, posaconazol e isavuconazol y se realizaron lecturas visuales y espectrofotométricas para obtener las CMIs. Se identificaron las mutaciones *cyp51A* en las cepas resistentes. Se compararon las CMIs establecidas visualmente con aquellas obtenidas espectrofotométricamente (reducción del crecimiento fúngico >95% en comparación con el control y leído a 540 nm). Se calcularon las concordancias esenciales (±1 dilución doble) y categóricas.

En general, se encontraron elevadas concordancias esenciales (97,1%) y categóricas (99,6%). Las concordancias categóricas para anfotericina B, itraconazol y posaconazol fueron del 100%, y consecuentemente, no se encontraron errores. Las concordancias categóricas para voriconazol e isavuconazol fueron del 98,7% y 99,3%, respectivamente. La mayoría de las clasificaciones erróneas para voriconazol e isavuconazol se encontraron en CMIs en el "área de incertidumbre técnica" o solo una dilución por encima del punto de corte. La tasa de resistencia fue ligeramente menor cuando las CMIs

se obtuvieron mediante lectura espectrofotométrica, pero todos los mutantes *cyp51A* relevantes se clasificaron correctamente como resistentes.

En conclusión, las CMIs obtenidas por lectura espectrofotométrica presentaron una elevada concordancia con la lectura visual. Por tanto, la lectura espectrofotométrica de las CMIs de azoles y anfotericina B en cepas de *A. fumigatus sensu lato* podría ser una alternativa a la lectura visual.

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SUSCEPTIBILITY

Azole and Amphotericin B MIC Values against *Aspergillus fumigatus*: High Agreement between Spectrophotometric and Visual Readings Using the EUCAST EDef 9.3.2 Procedure

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ABSTRACT The EUCAST EDef 9.3.2 procedure recommends visual readings of azole and amphotericin B MICs against *Aspergillus* spp. Visual determination of MICs may be challenging. In this work, we aim to obtain and compare visual and spectrophotometric MIC readings of azoles and amphotericin B against *Aspergillus fumigatus sensu lato* isolates. A total of 847 *A. fumigatus sensu lato* isolates (*A. fumigatus sensu stricto* [$n = 828$] and cryptic species [$n = 19$]) were tested against amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole using the EUCAST EDef 9.3.2 procedure. Isolates were classified as susceptible or resistant/non-wild type according to the 2020 updated breakpoints. The area of technical uncertainty for the azoles was defined in the updated breakpoints. Visual and spectrophotometric (fungal growth reduction of >95% compared to the control, read at 540 nm) MICs were compared. Essential (±1 2-fold dilution) and categorical agreements were calculated. Overall, high essential (97.1%) and categorical (99.6%) agreements were found. We obtained 100% categorical agreements for amphotericin B, itraconazole, and posaconazole, and consequently, no errors were found. Categorical agreements were 98.7 and 99.3% for voriconazole and isavuconazole, respectively. Most of the misclassifications for voriconazole and isavuconazole were found to be associated with MIC results falling either in the area of technical uncertainty or within one 2-fold dilution above the breakpoint. The resistance rate was slightly lower when the MICs were obtained by spectrophotometric readings. However, all relevant *cyp51A* mutants were correctly classified as resistant. Spectrophotometric determination of azole and amphotericin B MICs against *A. fumigatus sensu lato* isolates may be a convenient alternative to visual endpoint readings.

KEYWORDS *Aspergillus fumigatus*, EUCAST, azoles, amphotericin B, spectrophotometric

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Azoles are the backbone of treatment and prevention of diseases associated with *Aspergillus* spp. and are to date the only available anti-*Aspergillus* oral drugs. The European Society of Clinical Microbiology and Infectious Diseases guidelines recommend itraconazole for the management of patients with chronic pulmonary aspergillosis and allergic bronchopulmonary aspergillosis. Voriconazole and isavuconazole are indicated as the first-line treatment of pulmonary invasive aspergillosis. Voriconazole is also recommended for primary therapy in patients with central nervous system involvement and chronic pulmonary forms of the infection. Posaconazole is recommended for antifungal prophylaxis during prolonged neutropenia in high-risk patients or as salvage therapy in intolerant or nonresponding individuals. Finally, liposomal amphotericin B is recommended in settings in which azoles are contraindicated—resistant isolates—and as salvage therapy (1). Some *Aspergillus* species are intrinsically resistant to polyenes (*A. terreus*, *A. nidulans*, and *A. flavus*) or azoles (*A. ustus*) (2). Members of *Aspergillus fumigatus sensu lato*, the main etiological agents of aspergillosis, include *A. fumigatus sensu stricto* and cryptic species. Cryptic species commonly show intrinsic resistance to amphotericin B and azoles (3). In contrast, *A. fumigatus sensu stricto* isolates may acquire resistance following exposure to azoles, particularly with environmental azole fungicides (4). Azole resistance in *A. fumigatus sensu stricto* isolates has been increasingly reported worldwide (5–7).

Patients infected by azole-resistant *A. fumigatus sensu lato* isolates show higher mortality than those with azole-susceptible infections (8, 9). Thus, to improve patient care, detection of resistance is of paramount importance. The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) proposed standard methods for the study of azole and amphotericin B susceptibility of *Aspergillus* isolates. The EUCAST EDef 9.3.2 procedure includes clinical breakpoints to classify isolates either as susceptible or resistant and recommends visual determination of MICs (10). Visual inspection may be challenging, and spectrophotometric readings may facilitate MIC determination and overcome subjectivity. However, there is a limited number of studies using the EUCAST methodology in which azole MICs against *A. fumigatus sensu lato* obtained by visual and spectrophotometric readings are compared; furthermore, the available studies are thwarted by a low number of isolates and antifungal drugs tested (11–14).

We recently conducted a Spanish multicenter study of azole resistance in which 847 *A. fumigatus sensu lato* clinical isolates were collected between 15 February and 14 May 2019 (15). Taking advantage of the large number of isolates, the objective in this work is to report and compare azole and amphotericin B MICs using visual and spectrophotometric readings following the EUCAST EDef 9.3.2 procedure.

RESULTS

Isolates classified as resistant/non-wild type according to the updated 2020 EUCAST breakpoints are listed in Table 1, as described in more detail in Materials and Methods. Tables 2 to 6 show MIC distributions of amphotericin B, itraconazole, posaconazole, voriconazole, and isavuconazole against the 847 isolates by regular/stringent visual and spectrophotometric readings. MICs against quality control (QC) strains were within the acceptable limit.

Agreement between MICs by regular visual and spectrophotometric readings. Overall, both MIC endpoints showed high essential (97.1%) and categorical (99.6%) agreements. Essential agreements for individual drugs were as follows: amphotericin B, 98.8%; itraconazole, 94.8%; posaconazole, 97.3%; voriconazole, 98.3%; and isavuconazole, 96.1% (Table 7).

Categorical agreements for amphotericin B, itraconazole, and posaconazole were 100%, and consequently, resistance rates for both MIC endpoints were identical. Categorical agreement for voriconazole was 98.7%, and the rate of resistance was slightly lower when spectrophotometric readings were used for MIC determination. Very major errors (0.7% [$n = 6$]) and major errors (0.4% [$n = 3$]) for voriconazole occurred in *A. fumigatus sensu stricto* isolates with MIC results falling in the area of

TABLE 1 Azole and amphotericin B breakpoints and ECOFFs chosen to classify *Aspergillus fumigatus sensu lato* isolates as susceptible, resistant, or non-wild type[a]

Drug	ECOFF for WT (mg/liter)	Clinical breakpoint (mg/liter)		
		S	R	ATU[b]
Amphotericin B	≤1	≤1	≥2	ND
Itraconazole	≤1	≤1	≥2	2
Posaconazole	≤0.25	≤0.125	≥0.25	0.25
Voriconazole	≤1	≤1	≥2	2
Isavuconazole	≤2	≤1	≥4	2

[a]For details, see reference 17. ECOFF, epidemiological cutoff value; WT, wild type; S, susceptible; R, resistant; ATU, area of technical uncertainty; ND, not defined.
[b]Isolates with itraconazole and voriconazole MIC results that fall in the ATU were always considered resistant; isolates with isavuconazole MICs and posaconazole MIC results that fall in the ATU were considered resistant when voriconazole resistant or itraconazole resistant, respectively.

technical uncertainty (ATU) (MIC = 2 mg/liter). In cryptic species, very major errors occurred in two *Neosartorya udagawae* isolates (10.5%), one of them with MIC results falling in the ATU. Categorical agreement for isavuconazole was 99.3%, and the rate of resistance was slightly lower when spectrophotometric readings were used for MIC determination. Very major errors in isavuconazole occurred in three *A. fumigatus sensu stricto* isolates and in three cryptic species isolates (two of *N. udagawae* and one of *A. fumigatiaffinis*). With the exception of the *A. fumigatiaffinis* isolate, very major errors for isavuconazole ($n = 5$) were detected in isolates with MIC results in the ATU, which also revealed very major errors for voriconazole (see Table S1 in the supplemental material). None of the six isolates for which very major errors were detected in the azole categorical classification harbored relevant *cyp51A* mutations (Table S1).

Agreement between MICs obtained by regular/stringent visual readings. Overall, both visual MIC endpoints showed high essential (97.7%) and categorical (96.7%) agreements. Essential agreements for individual drugs were above 98% (itraconazole, 98.9%; posaconazole, 98.7%; and isavuconazole, 98.6%), with the exception of voriconazole (94.4%) (Table 7).

Categorical agreements for itraconazole and posaconazole were 99.4% (Table 7). Resistance rates obtained by both MIC endpoints were identical in *A. fumigatus sensu stricto*, but slightly higher with stringent visual readings in cryptic species. This led to major errors for both drugs in five isolates (three of *A. lentulus*, one of *A. novofumigatus*, and one of *A. fumigatiaffinis*). Although posaconazole MICs by both visual readings were identical (MIC = 0.25 mg/liter [ATU]), the categorical classification differed due to

TABLE 2 MIC distributions of amphotericin B against 847 *A. fumigatus sensu lato* isolates[a]

MIC reading procedure	MIC distribution by no. of isolates for MIC (mg/liter) of:												No. (%) of resistant isolates[b]
	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥16	
A. fumigatus sensu lato ($n = 847$)													
Regular visual readings	0	0	1	5	70	407	306	45	**7**	**5**	**1**	**0**	13 (1.5)
Spectrophotometric readings	0	0	0	7	96	452	248	31	**6**	**5**	**0**	**2**	13 (1.5)
A. fumigatus sensu stricto ($n = 828$)													
Regular visual readings	0	0	1	5	68	407	305	42	**0**	**0**	**0**	**0**	0 (0)
Spectrophotometric readings	0	0	0	7	94	452	246	29	**0**	**0**	**0**	**0**	0 (0)
Cryptic species ($n = 19$)													
Regular visual readings	0	0	0	0	2	0	1	3	**7**	**5**	**1**	**0**	13 (68.4)
Spectrophotometric readings	0	0	0	0	2	0	2	2	**6**	**5**	**0**	**2**	13 (68.4)
Isolates with tandem repeats ($n = 25$)													
Regular visual readings	0	0	0	0	2	10	13	0	**0**	**0**	**0**	**0**	0 (0)
Spectrophotometric readings	0	0	0	0	3	10	12	0	**0**	**0**	**0**	**0**	0 (0)

[a]MIC distributions by regular visual readings and their correspondent rates of resistance are reported elsewhere (15). Underlined values indicate non-wild-type isolates according to tentative ECOFFs, and values in boldface indicate resistant isolates (EUCAST breakpoint table v. 10.0, 2020 [10]).
[b]Identical numbers of resistant isolates and non-wild-type isolates were obtained.

TABLE 3 MIC distributions of itraconazole against 847 *A. fumigatus sensu lato* isolates[a]

MIC reading procedure	MIC distribution by no. of isolates for MIC (mg/liter) of:												No. (%) of resistant isolates[b]
	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥16	
A. fumigatus sensu lato (n = 847)													
Regular visual readings	0	0	0	0	26	427	328	21	2	**2**	**2**	**39**	45 (5.3)
Stringent visual readings	0	0	0	0	10	269	459	59	2	**2**	**2**	**44**	50 (5.9)
Spectrophotometric readings	0	0	15	22	49	412	287	17	3	**1**	**1**	**40**	45 (5.3)
A. fumigatus sensu stricto (n = 828)													
Regular visual readings	0	0	0	0	26	426	326	15	2	**1**	**1**	**31**	35 (4.2)
Stringent visual readings	0	0	0	0	10	269	457	57	2	**1**	**1**	**31**	35 (4.2)
Spectrophotometric readings	0	0	15	22	49	410	282	15	3	**0**	**1**	**31**	35 (4.2)
Cryptic species (n = 19)													
Regular visual readings	0	0	0	0	0	1	2	6	0	**1**	**1**	**8**	10 (52.6)
Stringent visual readings	0	0	0	0	0	0	2	2	0	**1**	**1**	**13**	15 (78.9)
Spectrophotometric readings	0	0	0	0	0	2	5	2	0	**1**	**0**	**9**	10 (52.6)
Isolates with tandem repeats (n = 25)													
Regular visual readings	0	0	0	0	0	0	1	0	0	**0**	**0**	**24**	24 (96)
Stringent visual readings	0	0	0	0	0	0	0	1	0	**0**	**0**	**24**	24 (96)
Spectrophotometric readings	0	0	0	0	0	0	1	0	0	**0**	**0**	**24**	24 (96)

[a]MIC distributions by regular visual readings and their correspondent rates of resistance are reported elsewhere (15). Values shaded in gray indicate MICs in the area of technical uncertainty (ATU) and were classified as resistant isolates. Underlined values indicate non-wild-type isolates according to tentative ECOFFs, and values in boldface indicate resistant isolates (EUCAST breakpoint table v. 10.0, 2020 [10]).
[b]Identical numbers of resistant isolates and non-wild-type isolates were obtained.

the MICs of itraconazole in four out of the five isolates (see Table S2 in the supplemental material). The percentage of voriconazole resistance was overestimated with the stringent visual endpoint (6.6% versus 15.8%). Categorical agreement was 90.8%. Major errors were found exclusively in *A. fumigatus sensu stricto* isolates (n = 78), in MIC results falling in the ATU. Likewise, the rate of isavuconazole resistance was overestimated when the stringent visual endpoint was used, although to a lesser extent than in the case of voriconazole (4.1% versus 4.4%). Categorical agreement was 97.2%. Major errors were found in *A. fumigatus sensu stricto* isolates (n = 22) and in two isolates of cryptic species (*N. tsurutae* and *A. fumigatiaffinis* [Table S2]). Similarly, most misclassi-

TABLE 4 MIC distributions of posaconazole against 847 *A. fumigatus sensu lato* isolates[a]

MIC reading procedure	MIC distribution by no. of isolates for MIC (mg/liter) of:												No. (%) of isolates	
	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥16	Resistant	Non-wild type
A. fumigatus sensu lato (n = 847)														
Regular visual readings	0	2	46	441	279	42	**27**	**3**	**0**	**1**	**0**	**6**	46 (5.4)	37 (4.4)
Stringent visual readings	0	1	18	268	399	119	**23**	**12**	**0**	**1**	**0**	**6**	51 (6)	42 (5)
Spectrophotometric readings	1	1	70	476	225	36	**28**	**4**	**1**	**1**	**0**	**4**	47 (5.5)	38 (4.5)
A. fumigatus sensu stricto (n = 828)														
Regular visual readings	0	2	46	440	278	32	**20**	**3**	**0**	**1**	**0**	**6**	34 (4.1)	30 (3.6)
Stringent visual readings	0	1	18	268	398	111	**13**	**12**	**0**	**1**	**0**	**6**	34 (4.1)	32 (3.9)
Spectrophotometric readings	1	1	70	475	222	27	**22**	**4**	**1**	**1**	**0**	**4**	34 (4.1)	32 (3.9)
Cryptic species (n = 19)														
Regular visual readings	0	0	0	1	1	10	**7**	**0**	**0**	**0**	**0**	**0**	12 (63.1)	7 (36.8)
Stringent visual readings	0	0	0	0	1	8	**10**	**0**	**0**	**0**	**0**	**0**	17 (89.5)	10 (52.6)
Spectrophotometric readings	0	0	0	1	3	9	**6**	**0**	**0**	**0**	**0**	**0**	12 (63.1)	6 (31.6)
Isolates with tandem repeats (n = 25)														
Regular visual readings	0	0	0	0	0	3	**18**	**2**	**0**	**0**	**0**	**2**	24 (96)	22 (88)
Stringent visual readings	0	0	0	0	0	1	**11**	**11**	**0**	**0**	**0**	**2**	24 (96)	24 (96)
Spectrophotometric readings	0	0	0	0	0	1	**19**	**4**	**1**	**0**	**0**	**0**	24 (96)	24 (96)

[a]MIC distributions by regular visual readings and their correspondent rates of resistance or non-wild-type isolates are reported elsewhere (15). Values shaded in gray indicate MICs in the area of technical uncertainty (ATU). MIC results against *A. fumigatus sensu lato* falling in the ATU were translated to resistant as follows: regular visual readings (n = 9/42), stringent visual readings (n = 9/119), and spectrophotometric readings (n = 9/36). Underlined values indicate non-wild-type isolates according to tentative ECOFFs, and values in boldface indicate resistant isolates (EUCAST Breakpoint table v. 10.0, 2020 [10]).

TABLE 5 MIC distributions of voriconazole against 847 *A. fumigatus sensu lato* isolates[a]

MIC reading procedure	MIC distribution by no. of isolates for MIC (mg/liter) of:												No. (%) of resistant isolates[b]
	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥16	
A. fumigatus sensu lato (n = 847)													
Visual reading	0	0	0	0	3	82	529	177	**19**	**27**	**7**	**3**	56 (6.6)
Stringent visual readings	0	0	0	0	1	17	302	394	**87**	**31**	**11**	**4**	133 (15.7)
Spectrophotometric readings	0	0	0	0	4	138	500	154	**18**	**24**	**6**	**3**	51 (6)
A. fumigatus sensu stricto (n = 828)													
Visual reading	0	0	0	0	3	82	529	176	**13**	**19**	**3**	**3**	38 (4.6)
Stringent visual readings	0	0	0	0	1	17	302	393	**86**	**18**	**7**	**4**	115 (13.9)
Spectrophotometric readings	0	0	0	0	4	138	498	153	**11**	**18**	**4**	**2**	35 (4.2)
Cryptic species (n = 19)													
Visual reading	0	0	0	0	0	0	0	1	**6**	**8**	**4**	**0**	18 (94.7)
Stringent visual readings	0	0	0	0	0	0	0	1	**1**	**13**	**4**	**0**	18 (94.7)
Spectrophotometric readings	0	0	0	0	0	0	2	1	**7**	**6**	**2**	**1**	16 (84.2)
Isolates with tandem repeats (n = 25)													
Visual reading	0	0	0	0	0	0	0	0	**5**	**15**	**2**	**3**	25 (100)
Stringent visual readings	0	0	0	0	0	0	0	0	**1**	**14**	**7**	**3**	25 (100)
Spectrophotometric readings	0	0	0	0	0	0	0	0	**6**	**14**	**3**	**2**	25 (100)

[a]MIC distributions by regular visual readings and their correspondent rates of resistance are reported elsewhere (15). Values shaded in gray indicate MICs in the area of technical uncertainty (ATU) and were classified as resistant isolates. Underlined values indicate non-wild-type isolates according to tentative ECOFFs, and values in boldface indicate resistant isolates (EUCAST breakpoint table v. 10.0, 2020 [10]).
[b]The numbers of resistant isolates and non-wild-type isolates were identical.

fications (23/24 isolates) were associated with MIC results falling in the ATU and mostly affected isolates in which major errors for voriconazole were detected (21/24 isolates). Since stringent visual readings shifted azole MICs to higher values, no very major errors were found.

DISCUSSION

In this study, we show that MICs of azoles and amphotericin B against *A. fumigatus* obtained either by spectrophotometric or regular visual readings have very high essential and categorical agreement.

TABLE 6 MIC distributions of isavuconazole against 847 *A. fumigatus sensu lato* isolates[a]

MIC reading procedure	MIC distribution by no. of isolates for MIC (mg/liter) of:												No. of isolates (%)	
	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥16	Resistant	Non-wild type
A. fumigatus sensu lato (n = 847)														
Regular visual readings	0	0	0	0	0	13	440	333	26	**14**	**17**	**4**	48 (5.6)	35 (4.1)
Stringent visual readings	0	0	0	0	0	2	146	572	90	**11**	**21**	**5**	72 (8.5)	37 (4.4)
Spectrophotometric readings	0	0	0	17	4	14	434	314	31	**12**	**18**	**3**	42 (5)	33 (3.9)
A. fumigatus sensu stricto (n = 828)														
Regular visual readings	0	0	0	0	0	13	440	327	18	**10**	**16**	**4**	35 (4.2)	30 (3.6)
Stringent visual readings	0	0	0	0	0	2	146	568	80	**7**	**20**	**5**	57 (6.9)	32 (3.9)
Spectrophotometric readings	0	0	0	17	4	14	433	306	24	**9**	**18**	**3**	32 (3.9)	30 (3.6)
Cryptic species (n = 19)														
Regular visual readings	0	0	0	0	0	0	0	6	8	**4**	**1**	**0**	13 (68.4)	5 (26.3)
Stringent visual readings	0	0	0	0	0	0	0	4	10	**4**	**1**	**0**	15 (78.9)	5 (26.3)
Spectrophotometric readings	0	0	0	0	0	0	1	8	7	**3**	**0**	**0**	10 (52.6)	3 (15.8)
Isolates with tandem repeats (n = 25)														
Regular visual readings	0	0	0	0	0	0	0	0	1	**6**	**14**	**4**	25 (100)	24 (96)
Stringent visual readings	0	0	0	0	0	0	0	0	0	**2**	**18**	**5**	25 (100)	25 (100)
Spectrophotometric readings	0	0	0	0	0	0	0	0	0	**7**	**15**	**3**	25 (100)	25 (100)

[a]MIC distributions by regular visual readings and their correspondent rates of resistance/non-wild-type isolates were reported elsewhere (15). Values shaded in gray indicate MICs in the area of technical uncertainty (ATU). MIC results against *A. fumigatus sensu lato* falling in the ATU were translated to resistant as follows: regular visual readings (n = 13/26), stringent visual readings (n = 35/90), and spectrophotometric readings (n = 9/31). Underlined values indicate non-wild-type isolates according to tentative ECOFFs, and values in boldface indicate resistant isolates (EUCAST breakpoint table v. 10.0, 2020 [10]).

TABLE 7 Essential and categorical agreement between MICs by visual (regular and stringent) and spectrophotometric readings[a]

MIC reading comparison	*A. fumigatus sensu lato* (%)				*A. fumigatus sensu stricto* (%)				Cryptic species (%)			
	Essential agreement	Categorical agreement	VME	ME	Essential agreement	Categorical agreement	VME	ME	Essential agreement	Categorical agreement	VME	ME
Amphotericin B												
Regular visual vs spectrophotometric readings	98.8	100	0	0	99	100	0	0	89.5	100	0	0
Itraconazole												
Regular visual vs spectrophotometric readings	94.8	100	0	0	95.1	100	0	0	78.9	100	0	0
Regular visual vs stringent readings	98.9	99.4	0	0.6	99.6	100	0	0	68.4	73.7	0	26.3
Posaconazole												
Regular visual vs spectrophotometric readings	97.3	100	0	0	97.1	100	0	0	100	100	0	0
Regular visual vs stringent readings	98.7	99.4	0	0.6	98.7	100	0	0	100	73.7	0	26.3
Voriconazole												
Regular visual vs spectrophotometric readings	98.3	98.7	0.9	0.3	98.4	98.9	0.7	0.4	94.7	89.5	10.5	0
Regular visual vs stringent readings	94.4	90.8	0	9.2	94.3	90.6	0	9.4	100	100	0	0
Isavuconazole												
Regular visual vs spectrophotometric readings	96.1	99.3	0.7	0	96.3	99.6	0.4	0	89.5	84.2	15.8	0
Regular visual vs stringent readings	98.6	97.2	0	2.8	98.5	97.3	0	2.7	100	89.5	0	10.5

[a]Regular visual endpoint MICs were assumed as the gold standards and compared against MICs obtained by other endpoints. MICs (percentages) within ±1 2-fold dilution were considered to be in essential agreement. Isolates were classified as resistant/non-wild type according to the updated 2020 EUCAST breakpoints/ECOFFs. The endpoints were in categorical agreement when the results were in the same susceptibility category (regardless of the MIC). VME, very major error (false susceptibility); ME, major error (false resistance).

The increase in resistant *A. fumigatus* isolates worldwide has promoted antifungal susceptibility testing (5). Azole resistance in *A. fumigatus* may occur during azole therapy or exposure to azole fungicides in the environment (4). Furthermore, cryptic species commonly show intrinsic resistance to amphotericin B and azoles (3). Although the EUCAST EDef 9.3.2 procedure recommends visual inspection for azole and amphotericin B MIC settings against *Aspergillus* species, spectrophotometric readings may offer objectivity, quick automated readings, and overall better performance. Previous studies comparing spectrophotometric and visual readings showed excellent essential (92 to 97%) and categorical (93 to 99%) agreements (11–14). Some of the studies used the CLSI methodology and were undermined by the limited number of *A. fumigatus sensu stricto* isolates tested (up to 133 isolates), the absence of both cryptic species isolates and *cyp51A* mutants, and a low number of studied antifungal drugs (amphotericin B and itraconazole) (12–14). One of the studies, in which the EUCAST method was used, included the four antimold triazoles (itraconazole, posaconazole, voriconazole, and isavuconazole) and a low number of *A. fumigatus sensu stricto* isolates (n = 88). The work did not assess cryptic species, although 15 isolates with *cyp51A* mutations, including isolates with the dominant substitutions TR_{34} L98H, G54, and M220, among others, were examined. Furthermore, since EUCAST has recently changed azole breakpoints against *Aspergillus fumigatus sensu lato*, a validation of spectrophotometric readings, including a large number of isolates classified according to the updated EUCAST breakpoints, is needed.

We recently conducted a survey of azole resistance in *A. fumigatus sensu lato* isolates collected in Spain in 2019 (15). Taking advantage of the large number of isolates collected (n = 847), we obtained and compared MICs using visual and spectrophotometric readings. Nineteen strains were identified as cryptic species, and 45 *A. fumigatus sensu stricto* isolates proved to be azole resistant, being TR_{34} L98H, the dominant mechanism of resistance. Both MIC endpoints show high essential/categorical agreements for amphotericin B (98.8%/100%), itraconazole (94.8%/100%), posaconazole

(97.3%/100%), voriconazole (98.3%/98.7%), and isavuconazole (96.1%/99.3%). No errors were found in amphotericin B, itraconazole, and posaconazole. Most misclassifications for voriconazole and isavuconazole are linked with MIC results falling either in the ATU (10/12 isolates) or in just one 2-fold dilution above the breakpoint (2/12 isolates; MIC = 4 mg/liter). Cross-resistance between voriconazole and isavuconazole is the norm in *A. fumigatus senso stricto* (16). Using voriconazole as a surrogate marker, spectrophotometric readings resulted in misdetection of voriconazole resistance in six *A. fumigatus senso stricto* isolates with either a wild-type *cyp51A* gene or genetic polymorphisms of dubious clinical implications (Table S1).

The EUCAST has recently reviewed the antifungal breakpoints against *A. fumigatus sensu lato*. Breakpoints for amphotericin B, itraconazole, voriconazole, and posaconazole were lowered, while the breakpoint for isavuconazole was increased (17). Based on the updated breakpoints, spectrophotometric MIC readings led to correct classification of all isolates with relevant *cyp51A* mutations as resistant. Interpretation uncertainties regarding MIC values may arise in the ATU, a newly introduced term, where the breakpoints of wild-type isolates and mutant isolates converge (17). Isolates with posaconazole and isavuconazole MICs of 0.25 mg/liter and 2 mg/liter, respectively, cannot be automatically reported as susceptible or resistant. MIC determinations using spectrophotometric readings frequently led to the underestimation of resistance for MIC values falling in the ATU. Here, we were able to easily clarify misclassifications by visually inspecting the tray. False resistance was detected in four *A. fumigatus sensu stricto* isolates for which a voriconazole MIC of 2 mg/liter was determined by spectrophotometric readings.

A higher mortality rate is observed in patients infected with azole-resistant *A. fumigatus sensu lato* isolates. Resistance is frequently caused by mutations in the *cyp51A* gene, some of which are associated with a pan-triazole-resistant phenotype (high-level resistance) (8). Some phenotypes only affect the activity of a single azole or several triazoles with similar molecular structure, and the MIC is close to the clinical breakpoint, resulting in low-level resistance (18). Previous studies have shown that patients infected with low-level voriconazole-resistant *A. fumigatus* (MIC = 2 mg) and low-level isavuconazole-resistant *A. fumigatus* (MIC = 2 mg) may be treated with voriconazole or isavuconazole, respectively, provided that higher doses are administered (19, 20). In cryptic species, very major errors in voriconazole and isavuconazole were detected (Table S1).

Visual MIC readings may be challenging, and taking small colonies into account (stringent visual readings) may result in overestimation of resistance rates and increase the MIC of the isolates one or two 2-fold dilutions, particularly for voriconazole. Thus, major errors in voriconazole and isavuconazole (MIC results falling in the ATU) against *A. fumigatus sensu stricto* may be detected. Correct classification of relevant *cyp51A* gene mutants was achieved by stringent visual readings.

We conclude that spectrophotometric determination is a useful alternative to visual inspection of azole and amphotericin MICs against *A. fumigatus sensu stricto*. Both endpoints show high essential and categorical agreements. Future studies that include more isolates from cryptic species and *A. fumigatus sensu stricto* with other kinds of *cyp51A* mutations are warranted.

MATERIALS AND METHODS

Samples. A total of 847 *A. fumigatus sensu lato* clinical isolates, identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), were collected in a 30-hospital survey conducted in Spain (15). Azole-resistant isolates (*A. fumigatus sensu stricto*, $n = 45$; cryptic species, $n = 19$) were molecularly identified. The distribution of isolates as per species identification was as follows: *A. fumigatus sensu stricto*, $n = 828$; *A. lentulus*, $n = 6$; *A. fumigatiaffinis*, $n = 5$; *Neosartorya tsurutae*, $n = 3$; *Neosartorya udagawae*, $n = 2$; *A. novofumigatus*, $n = 2$; and *A. thermomutatus*, $n = 1$.

The *cyp51A* gene sequence from 45 azole-resistant *A. fumigatus sensu stricto* isolates carried the mutations TR_{34} L98H ($n = 24$), G54R ($n = 5$), TR_{46} Y121F T289A ($n = 1$), F46Y M172V N248T D255E E427K ($n = 2$), F46Y M172V N248T D255E E416Q E427K ($n = 1$), F165L ($n = 1$), and S496L ($n = 1$), as well as the wild-type *cyp51A* gene ($n = 10$).

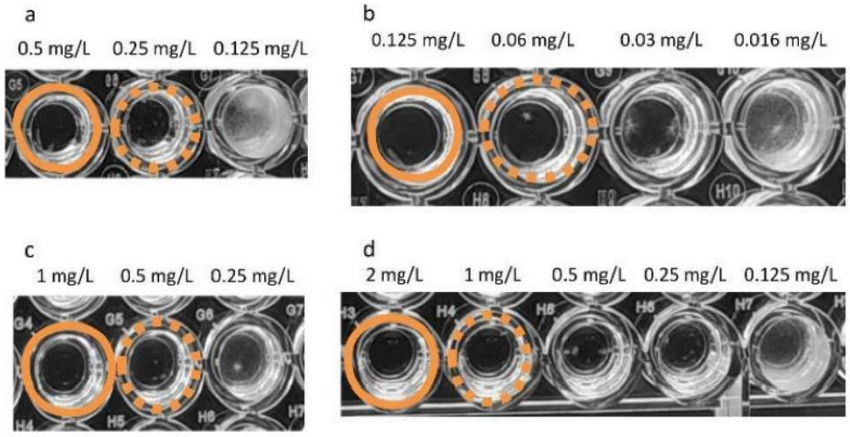

FIG 1 Example of fungal growth of an *A. fumigatus sensu stricto* isolate in the presence of itraconazole (a), posaconazole (b), voriconazole (c), and isavuconazole (d). Two MIC endpoints were used: the regular endpoint (gold standard), where tiny small colonies were disregarded (wells surrounded by rings of dashed lines), and the stringent endpoint, where the tiny colonies were taken into account (wells surrounded by rings of solid lines).

EUCAST antifungal susceptibility testing. All isolates were subcultured on potato dextrose agar or Sabouraud dextrose agar and incubated at 35°C for 2 to 5 days. Isolates from cryptic species were incubated long enough to ensure filtered conidial suspensions reached a sufficient inoculum (equivalent to a McFarland standard of 0.5 using a spectrophotometer). The isolates' antifungal susceptibilities to amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole were determined following the EUCAST EDef 9.3.2 procedure (21). The inoculated trays were incubated for 48 h at 35°C, and MICs were obtained using a visual endpoint (defined as the concentration that completely inhibits fungal growth) and a spectrophotometric endpoint (≥95% inhibition of fungal growth compared to the drug-free control and read at 540 nm, as described elsewhere) (11). Although the EUCAST EDef 9.3.2 procedure recommends ignoring single colonies on the surface, sometimes it is difficult to discern real growth from small colonies. Thus, we interpreted visual MICs using two endpoints—the regular endpoint (very tiny growth was disregarded) or the stringent endpoint (a totally clear well)—as exemplified in Fig. 1. Quality control (QC) was ensured by testing the *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 strains (amphotericin B, itraconazole, voriconazole, and posaconazole), as well as *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 (isavuconazole).

Data analysis. Regular visual endpoint MICs were assumed as the "gold standards" and compared against MICs obtained by other endpoints; MICs (percentage) within ±1 2-fold dilution were considered to be in essential agreement. Isolates were classified as resistant/non-wild type according to the updated 2020 EUCAST breakpoints (Table 1); the intermediate category for amphotericin B and azoles and the category of "susceptible increased exposure" are no longer available, and the term "area of technical uncertainty" (ATU) for the four azoles has been defined (10). The ATU is a warning to laboratories on an uncertainty needing attention before reporting the results and represents an area of confluence of both wild-type and mutant isolates, particularly for voriconazole, posaconazole, and isavuconazole. MIC results in the ATU were interpreted as follows: itraconazole and voriconazole, always resistant; posaconazole, resistant only if the isolate was also resistant to itraconazole; and isavuconazole, resistant only if the isolate was also resistant to voriconazole. Categorical agreement between the three endpoints was assessed. The endpoints were in categorical agreement when the results were in the same susceptibility category (regardless of the MIC). Errors were defined as very major errors (false susceptibility) when the gold standard endpoint classified an isolate as resistant and the other endpoints as susceptible and as major errors (false resistance) when the gold standard endpoint classified an isolate as susceptible and the other endpoints as resistant (22).

Ethical considerations. This study was approved by the Ethics Committee of Hospital Gregorio Marañón (CEIm; study no. 22/19).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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Artículo 2: Spectrophotometric azole and amphotericin B MIC readings against *Aspergillus fumigatus sensu lato* using the EUCAST 9.3.2 methodology. Are ≥90 and ≥95% fungal growth inhibition endpoints equally suitable?

Para obtener más información sobre la idoneidad de la espectrofotometría como alternativa a las lecturas visuales de CMI, en este estudio se obtuvieron las CMI de azoles y anfotericina B utilizando la lectura espectrofotométrica con un *endpoint* de inhibición del crecimiento fúngico ≥ 90% frente al mismo conjunto de cepas del estudio anterior. Las CMIs obtenidas se compararon con las publicadas anteriormente utilizando la lectura espectrofotométrica con un *endpoint* de inhibición del crecimiento fúngico ≥ 95% y con las obtenidas visualmente. Se calcularon las concordancias esenciales (±1 dilución doble) y categóricas.

Los resultados de este estudio indicaron que un *endpoint* de inhibición del 90% también mostró elevados acuerdos esenciales (96,6%) y categóricos (99,5%) para azoles y anfotericina B, siendo más altos para *A. fumigatus sensu stricto* que para especies crípticas. A pesar de una ligera disminución en la tasa de resistencia con el *endpoint* del 90%, no se detectaron errores importantes en cepas con mutaciones relevantes en el gen *cyp51A.*

En conclusión, los acuerdos entre lecturas espectrofotométricas y visuales siguiendo la metodología de EUCAST son elevados independientemente del parámetro de inhibición del crecimiento fúngico elegido (90% o 95%) frente a *A. fumigatus sensu stricto* y fueron menores en especies crípticas.

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Brief Report

Brief Report

Spectrophotometric azole and amphotericin B MIC readings against *Aspergillus fumigatus sensu lato* using the EUCAST 9.3.2 methodology. Are ≥90 and ≥95% fungal growth inhibition endpoints equally suitable?

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Abstract

We recently reported high essential (97.1%) and categorical (99.6%) agreements between azole and amphotericin B MICs against *Aspergillus fumigatus sensu lato* obtained by visual and spectrophotometric readings using a ≥ 95% fungal growth endpoint and following the EUCAST methodology (doi: 10.1128/AAC.01693-20). Here, we compared the aforementioned MICs against spectrophotometric MIC readings obtained using a ≥ 90% inhibition endpoint. Spectrophotometric readings using either ≥ 90% or ≥ 95% fungal growth inhibition resulted in high categorical (>99.9%) agreements with visual MIC readings against *A. fumigatus sensu stricto*. In contrast, agreements with visual MICs against cryptic species were higher with the use of a ≥ 95% fungal growth inhibition endpoint.

Lay Summary

Spectrophotometrically obtained MIC readings using either ≥ 90% or ≥ 95% fungal growth inhibition endpoints and following the EUCAST methodology are suitable against *A. fumigatus sensu stricto*. However, the ≥ 95% fungal growth inhibition endpoint is preferred against cryptic species.

Key words: *Aspergillus fumigatus*, EUCAST, azoles, amphotericin B, spectrophotometry.

Azole resistance in *Aspergillus fumigatus* is a cause for concern in many countries, associated to a poorer prognosis in the affected patients.[1–5] Consequently, clinical microbiology laboratories should be prompted to detect *A. fumigatus* azole-resistant isolates. EUCAST E.Def 9.3.2 microdilution procedure allows detecting azole resistance in *A. fumigatus* isolates, however, a wider implementation of the procedure in clinical settings is hindered by technical complexities, including subjectivity in the determination of antifungal minimum inhibitory concentrations (MICs).[6]

Spectrophotometric readings may overcome the subjectivity of visual MIC inspection. Spectrophotometric MIC readings lie on the measure of fungal growth inhibition in presence or absence of antifungals; antifungal concentrations resulting

in ≥ 90% and ≥ 95% fungal growth inhibition (compared to antifungal-free wells) are the most commonly used endpoints.[7,8] A stringent endpoint (≥95%) may lead to misclassify isolates as falsely resistant; conversely, a less stringent endpoint (≥90%) may lead to miss truly-resistant isolates, but may help disregard artifacts otherwise interpreted as fungal growth (e.g., yellow-colored RPMI medium in wells growing *Aspergillus niger*, precipitation of insoluble antifungal drugs, etc.).[9]

Spectrophotometric MIC readings may become an alternative to visual MIC readings in EUCAST methodology.[6] Works comparing azole and amphotericin B MICs using spectrophotometric (≥90% or ≥ 95% fungal growth inhibition endpoints)[7] and visual readings have provided excellent essential and categorical agreements against *A. fumigatus sensu stricto*.[7,8,10,11] However, the number of studies and isolates tested are low and exclusively focused on *A. fumigatus sensu stricto*, whereas *Fumigati* cryptic species isolates have not been tested at all.

We recently reported high essential and categorical agreements between azole and amphotericin B MICs obtained by visual and spectrophotometric readings (≥95% fungal growth endpoint) against a large number of *A. fumigatus sensu lato* clinical isolates.[12] To gain further insight on the suitability of spectrophotometry as an alternative to visual MIC readings, here we obtained azole and amphotericin B MICs using a ≥ 90% fungal growth inhibition spectrophotometric reading endpoint against the same set of isolates. The obtained MICs were compared with those previously reported using the spectrophotometric ≥ 95% fungal growth inhibition endpoint and with those visually obtained.[12]

We studied *A. fumigatus sensu lato* isolates (n = 847) collected from a recent survey conducted in Spain comprising *A. fumigatus sensu stricto* (azole-resistant isolates [n = 44] and azole-susceptible isolates [n = 784]) and cryptic species (azole-resistant isolates [n = 18] and azole-susceptible isolates [n = 1]).[5] *A. fumigatus sensu stricto* azole-resistant isolates had the following *cyp51A* gene sequence: TR_{34}-L98H (n = 24), G54R (n = 5), TR_{46}/Y121F/T289A (n = 1), F46Y/M172V/N248T/D255E/E427K (n = 2), F46Y/M172V/N248T/D255E/E416Q/E427K (n = 1), F165L (n = 1), and wild-type *cyp*51A gene (n = 10).[5] Antifungal susceptibility of isolates to amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole were obtained according to EUCAST E.Def 9.3.2 procedure.[6] MIC values obtained by spectrophotometric ≥ 90% inhibition endpoint (inhibition of fungal growth compared to the drug-free control, read at 540 nm) were compared against values previously obtained by spectrophotometric ≥ 95% inhibition endpoint and visual readings; both spectrophotometric readings were obtained simultaneously.[12,5] MICs within two-fold dilutions (±1) were considered to be in essential agreement. Isolates were classified as resistant according to updated EUCAST breakpoints (2020) to calculate categorical agreement.[13] Errors were defined as very major (false susceptibility) or major (false resistance) considering visual reading as the gold standard.

Overall, visual MIC reading and ≥ 90% endpoint spectrophotometric MIC readings of azoles and amphotericin B showed high essential (96.6%) and categorical (99.5%) agreements, in both cases higher against *A. fumigatus sensu stricto* than against cryptic species. The following essential agreements for individual drugs were obtained: amphotericin B, 99.1%, isavuconazole, 97.5%, voriconazole, 97%, posaconazole, 95.9%, and itraconazole, 93.3% (Table 1). Visual inspection and the two tested spectrophotometric endpoints led to equal modal MICs (Supplementary Table 1).[12]

None of the isolates for which very major errors were detected harbored relevant *cyp51A* mutations. As expected, the resistance rate was slightly lower when MICs were obtained using the ≥ 90% inhibition endpoint spectrophotometric reading. Categorical agreements for amphotericin B and itraconazole were 99.9%; very major errors (0.1% [n = 1]) occurred in a *N. udagawae* isolate for both drugs (Table 2). Categorical agreement for posaconazole was 99.4%; very major errors (0.6% [n = 5]) occurred in five cryptic species isolates (*N. tsurutae*, n = 2; *N. udawagae*, n = 1; *A. lentulus*, n = 1; *A. fumigatiaffinis*, n = 1) (Table 2). Categorical agreement for voriconazole was 98.8%; very major errors (0.9% [n = 8]) were found in *A. fumigatus sensu stricto* isolates (n = 6) and in two *N. udagawae* isolates, and major errors (0.2% [n = 2]) occurred in two *A. fumigatus sensu stricto* isolates (Table 2). Most voriconazole misclassifications (9/10) were associated with MIC results falling in the area of technical uncertainty (ATU) (MIC = 2 mg/liter). Categorical agreement for isavuconazole was 99.3%; very major errors (0.7% [n = 6]) occurred in three *A. fumigatus sensu stricto* isolates with MIC results falling in the ATU and in three cryptic species isolates (two *N. udagawae* and one *A. fumigatiaffinis*), two of them with MIC results falling in the ATU (Table 2).

When comparing spectrophotometric MICs obtained using either of both fungal growth inhibition endpoints (≥90% and ≥ 95%), categorical agreement was the same for *A. fumigatus sensu stricto*; there was only disagreement in one isolate that showed a major error in voriconazole with the ≥ 95% inhibition endpoint, and was correctly classified as susceptible using the ≥ 90% inhibition endpoint. In contrast, the use of the ≥ 90% inhibition endpoint resulted in lower categorical agreements against cryptic species and led to the following very major errors: one in amphotericin B (*N. udagawae*), one in itraconazole (*N. udagawae*), and five in posaconazole (one *N. udagawae*, two *N. tsurutae*, one *A. lentulus*, and one *A. fumigatiaffinis*). No errors with voriconazole and isavuconazole against cryptic species were found (Table 1).

We have expanded our previous observations on the suitability of spectrophotometric readings to obtain azole and amphotericin B MICs following EUCAST methodology.[5] Overall, ≥90% and ≥ 95% inhibition endpoints showed high agreements with visual readings against *A. fumigatus sensu stricto* isolates; few very major errors (false susceptibility) were found in isolates with voriconazole MIC values falling in the ATU, none of them

Table 1. Essential and categorical agreements between minimum inhibitory concentrations (MICs) obtained by visual and spectrophotometric readings using ≥ 90% and ≥ 95% fungal inhibition growth endpoints for calculations.

	A. fumigatus sensu lato (%)				*A. fumigatus sensu stricto* (%)				Cryptic species (%)*			
Comparisons for MIC readings	Essential agreement	Categorical agreement	VME	ME	Essential agreement	Categorical agreement	VME	ME	Essential agreement	Categorical agreement	VME	ME
Amphotericin B												
Spectrophotometric reading (≥95% vs. ≥ 90%)	99.5	99.9			99.8	100			89.5	94.7		
Visual vs. spectrophotometric reading (≥90%)	99.1	99.9	0.1	0	99	100	0	0	100	94.7	5.3	0
Itraconazole												
Spectrophotometric reading (≥95% vs. ≥ 90%)	98.3	99.9			98.6	100			89.5	94.7		
Visual vs. spectrophotometric reading (≥90%)	93.3	99.9	0.1	0	93.7	100	0	0	73.7	94.7	5.3	0
Posaconazole												
Spectrophotometric reading (≥95% vs. ≥ 90%)	99.1	99.3			99.2	100			94.7	73.7		
Visual vs. spectrophotometric reading (≥90%)	95.9	99.4	0.6	0	96.1	100	0	0	84.2	73.7	26.3	0
Voriconazole												
Spectrophotometric reading (≥95% vs. ≥ 90%)	98.7	99.9			98.7	99.9			100	100		
Visual vs. spectrophotometric reading (≥90%)	97	98.8	0.9	0.2	97.2	99	0.7	0.2	89.5	89.5	10.5	0
Isavuconazole												
Spectrophotometric reading (≥95% vs. ≥ 90%)	98.8	100			98.8	100			100	100		
Visual vs. spectrophotometric reading (≥90%)	97.5	99.3	0.7	0	97.8	99.6	0.4	0	84.2	84.2	15.8	0

*Distribution of cryptic species is as follows: *A. lentulus*, n = 6; *A. fumigatiaffinis*, n = 5; *Neosartorya tsurutae*, n = 3; *Neosartorya udagawae*, n = 2; *A. novofumigatus*, n = 2; and *A. thermomutans*, n = 1. VME: very major error; ME: major errors; MIC = minimum inhibitory concentration.

Table 2. Very major errors (false susceptibility) and major errors (false resistance) found in *Aspergillus fumigatus sensu stricto* and cryptic species isolates. Minimum inhibitory concentrations (MICs) of the drugs for which errors were found are indicated in parenthesis.

			E.Def 9.3.2 Visual reading (MIC, in mg/liter)/Spectrophotometric reading ≥ 90%/spectrophotometric reading ≥ 95%				
Isolate code	Species	*Cyp51A* gene sequence	Amphotericin B	Itraconazole	Posaconazole	Voriconazole	Isavuconazole
6763	*A. fumigatus sensu stricto*	Polymorphism*	S/S/S	S/S/S	S/S/S	R(2)/S(1)/S(1)	R(2)/S(1)/S(1)
7022	*A. fumigatus sensu stricto*	Wild-type	S/S/S	S/S/S	S/S/S	R(2)/S(1)/S(1)	R(2)/S(0.5)/S(0.5)
6461	*A. fumigatus sensu stricto*	Wild-type	S/S/S	S/S/S	S/S/S	R(2)/S(1)/S(1)	R(2)/S(1)/S(1)
7055	*A. fumigatus sensu stricto*	Wild-type	S/S/S	S/S/S	S/S/S	R(2)/S(0.5)/S(0.5)	S/S/S
7043	*A. fumigatus sensu stricto*	Wild-type	S/S/S	S/S/S	S/S/S	R(2)/S(0.5)/S(1)	S/S/S
7042	*A. fumigatus sensu stricto*	Wild-type	S/S/S	S/S/S	S/S/S	R(2)/S(1)/S(1)	S/S/S
6556	*A. fumigatus sensu stricto*	Wild-type	S/S/S	S/S/S	S/S/S	S(1)/R(2)/R(2)	S/S/S
6527	*A. fumigatus sensu stricto*	Wild-type	S/S/S	S/S/S	S/S/S	S(1)/R(2)/R(2)	S/S/S
6469	*A. fumigatus sensu stricto*	Wild-type	S/S/S	S/S/S	S/S/S	S(0.5)/S(0.5)/R(2)	S/S/S
6044	*N. udagawae*	NA	R(2)/S(1)/R(2)	S/S/S	S/S/S	R(2)/S(0.5)/S(1)	R(2)/S(0,5)/S(1)
6659	*N. udagawae*	NA	R/R/R	R(16)/S(1)/R(4)	R(0.25)/S(0.12)/R(0.25)	R(4)/S(0.5)/S(0.5)	R(2)/S(1)/S(1)
6788	*N. tsurutae*	NA	S/S/S	S/S/S	R(0.5)/S(0.25)/R(0.5)	R/R/R	S/S/S
6206	*N. tsurutae*	NA	S/S/S	S/S/S	R(0.5)/S(0.25)/R(0.5)	R/R/R	S/S/S
6112	*A. lentulus*	NA	R/R/R	R/R/R	R(0.25)/S(0.125)/R(0.25)	R/R/R	R/R/R
6062	*A. fumigatiaffinis*	NA	R/R/R	R/R/R	R(0.5)/S(0.125)/R(0.25)	R/R/R	R(4)/S(0.1)/S(0.1)

*F46Y-M172V-N248T-D255E- E416Q-E427K.

NA, not applicable. Bold 'R' letters in red and 'S' letters in green indicate, respectively, resistance or susceptibility according to the microdilution method. MIC = minimum inhibitory concentration.

harboring relevant *cyp51A* gene mutations. As expected, a less stringent endpoint led to a decrease in the number of voriconazole major errors (false resistance) compared to the ≥ 95% inhibition endpoint.[5] In contrast, we observed an increase (amphotericin B, itraconazole, posaconazole) or similar (voriconazole and isavuconazole) number of very major errors for the ≥ 90% inhibition endpoint against cryptic species isolates. Spectrophotometric readings can be used to screen for the presence of azole-resistant *A. fumigatus sensu lato* isolates that should be inspected visually to rule in (or out) resistance.

In conclusion, and in light of our results, either ≥ 90% or ≥ 95% inhibition endpoints are equally suitable for azoles and amphotericin B spectrophotometric MIC readings against *A. fumigatus sensu stricto* and pose an alternative to EUCAST visually obtained MICs. However, the ≥ 95% inhibition endpoint should be recommended against cryptic species.

Supplementary material

Supplementary material is available at *MMYCOL* online.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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CAPÍTULO II: Evaluación y simplificación del método de placas de agar con azoles (EUCAST E. Def 10.1) para la detección de cepas de *A. fumigatus* resistentes a azoles.

Artículo 3: Azole-Resistant *Aspergillus fumigatus* Clinical Isolate Screening in Azole-Containing Agar Plates (EUCAST E.Def 10.1): Low Impact of Plastic Trays Used and Poor Performance in Cryptic Species

Alternativamente a la microdilución en caldo, las placas de agar que contienen azoles pueden utilizarse para detectar de forma rutinaria la resistencia en *A. fumigatus*. Las placas de agar con azoles disponibles en el mercado (VIPcheck©, BovenLeeuwen, Países Bajos) han demostrado ser útiles para detectar la presencia de cepas de *A. fumigatus* resistentes a azoles. Sin embargo, en muchos países no se dispone de placas comerciales y es necesario preparar placas caseras para aplicar este procedimiento. Hasta la fecha, no se ha evaluado el impacto del tipo de placas utilizadas en el rendimiento del método de cribado en agar de la resistencia a los azoles y no se han analizado las especies crípticas.

El objetivo de este artículo fue evaluar el impacto del tipo de plástico utilizado para preparar placas de agar con azoles en el rendimiento del procedimiento EUCAST E.Def 10.1.

Se incluyeron 91 cepas de *A. fumigatus sensu stricto* (n=36 resistentes a azoles) y 52 especies crípticas (n=47 resistentes a azoles), clasificadas como sensibles o resistentes, según el procedimiento EUCAST E. Def 9.3.2 y los puntos de corte clínicos v10.

Se prepararon placas de agar que contenían azoles siguiendo el procedimiento EUCAST E. Def 10.1 en tres tipos de placas multipocillo: placas tratadas (marcas Nunc® y Labclinics®), y placas no tratadas (marca Nunc®). Se evaluó la sensibilidad, la especificidad, y los valores de acuerdo categórico de las placas de agar para detectar resistencia a azoles.

En general, para *A. fumigatus sensu stricto*, los valores de sensibilidad y especificidad del método de detección con agar fueron del 100% y 93,3%,

respectivamente. El tipo de placa utilizada no afectó a estos valores. Todas las cepas con las sustituciones TR_{34}-L98H fueron clasificadas como resistentes a itraconazol y voriconazol por el método de agar; sin embargo, la falsa sensibilidad a posaconazol no fue infrecuente y ocurrió en cepas con CMI de posaconazol de 0,25 mg/L. Las cepas con sustituciones G54R y TR_{46}-Y121F-T289A fueron correctamente clasificadas por el método de agar como resistentes a itraconazol/posaconazol y voriconazol, respectivamente. Se detectó falsa resistencia en cepas que mostraban un crecimiento mínimo. Por el contrario, en las especies crípticas, la sensibilidad y la especificidad global fue mucho menor, del 82,2% y 53,3%, respectivamente.

En conclusión, las placas de agar que contienen azoles demostraron ser un método fiable y fácil de usar para la búsqueda de resistencia a azoles en *A. fumigatus sensu stricto*; el tipo de plástico de las placas utilizado afectó mínimamente al método. Por el contrario, la realización del método en especies crípticas no mostró buenos resultados.

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SUSCEPTIBILITY

Azole-Resistant *Aspergillus fumigatus* Clinical Isolate Screening in Azole-Containing Agar Plates (EUCAST E.Def 10.1): Low Impact of Plastic Trays Used and Poor Performance in Cryptic Species

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ABSTRACT Azole-containing agar is used in routine *Aspergillus fumigatus* azole resistance screening. We evaluated the impact of the type of plastic used to prepare in-house agar plates on the procedure's performance against *A. fumigatus sensu stricto* and cryptic species. *A. fumigatus sensu stricto* ($n = 91$) and cryptic species ($n = 52$) were classified as susceptible or resistant (EUCAST E.Def 9.3.2; clinical breakpoints v10). In-house azole-containing agar plates were prepared following EUCAST E.Def 10.1 on three types of multidish plates. We assessed the sensitivity, specificity, and agreement values of the agar plates to screen for azole resistance. Overall, sensitivity and specificity values of the agar screening method were 100% and 93.3%, respectively. The type of tray used did not affect these values. All isolates harboring TR_{34}-L98H substitutions were classified as resistant to itraconazole and voriconazole by the agar method; however, false susceptibility (very major error) to posaconazole was not uncommon and happened in isolates with posaconazole MICs of 0.25 mg/liter. Isolates harboring G54R and TR_{46}-Y121F-T289A substitutions were correctly classified by the agar method as itraconazole/posaconazole resistant and voriconazole resistant, respectively. False resistance (major error) occurred in isolates showing tiny fungal growth. Finally, agreements between both procedures against cryptic species were much lower. Azole-containing agar plates are a convenient and reliable tool to screen for resistance in *A. fumigatus sensu stricto*; the type of plastic tray used minimally affects the method. On the contrary, the performance against cryptic species is rather poor.

KEYWORDS *Aspergillus fumigatus*, EUCAST E.Def 10.1, agar plates, azoles, resistance

Azole resistance in *Aspergillus fumigatus* is an emerging global problem (1–4). Patients infected by azole-resistant *A. fumigatus* isolates are likely to experience a poor outcome (5, 6). Azole resistance detection may allow for better care to patients with aspergillosis; therefore, clinical microbiology laboratories should conduct antifungal susceptibility testing against *A. fumigatus* on a routine basis (7). Broth microdilution methods are the gold standard for antifungal susceptibility testing. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has developed a method (E.Def 9.3.2) to assess the MIC of antifungals against *A. fumigatus*; MIC interpretation can be done through clinical breakpoints (8). Unfortunately, microdilution procedures are time consuming and technically demanding, which may hamper antifungal susceptibility testing from being widely available in clinical microbiology laboratories.

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Alternatively, azole-containing agar plates can be used to routinely detect azole resistance in *A. fumigatus* in clinical mycology laboratories. The procedure is based on the ability of azole-resistant isolates to grow on azole-containing (itraconazole, voriconazole, posaconazole, and a drug-free well) agar plates. Commercially available azole-containing agar plates (VIPcheck, BovenLeeuwen, The Netherlands) have proven useful in screening for the presence of environmental and clinical azole-resistant *A. fumigatus* isolates (1, 9–14). A multicenter validation of the method using VIPcheck and in-house plates allowed EUCAST to develop a definitive method (E.Def 10.1; termed "agar screening method" from now on) and implement the procedure for routine use (11, 12, 15). Since commercial plates are not available in many countries, preparation of in-house plates is necessary to apply this procedure. The type of plastic used to prepare plates proved to have an impact on the obtained MICs against *Candida* using microdilution procedures, probably as a consequence of drug binding on the surface of the plastic tray (16, 17). To the best of our knowledge, the impact of the trays used on the performance of the azole resistance agar screening method has not yet been assessed. Moreover, the limited number of studies evaluating the performance of the agar screening method were mostly carried out in laboratories located in Northern Europe. Finally, *A. fumigatus* cryptic species were not tested. The objective of this work is to evaluate the performance of the E.Def 10.1 agar screening method to detect azole resistance in clinical *A. fumigatus sensu stricto* and cryptic species isolates collected in Spain in a laboratory in Madrid, Spain. Moreover, we aim to assess the impact of using different types of plastic trays to prepare the azole-containing agar plates.

RESULTS

Overall agreement between microdilution and the agar screening method. Drug resistance, as per the microdilution method and using the EUCAST breakpoints, was 39.2% for itraconazole, 54.5% for voriconazole, and 41.3% for posaconazole. MIC distributions of the three azoles against the isolates studied are shown in Table 1S in the supplemental material. The following categorical agreements between microdilution and the agar screening method were obtained for the three different types of plastic plates and pooling the results from the three azoles: for *A. fumigatus sensu stricto* (Nunc tissue-treated plates, 89.4%; Nunc nontissue-treated plates, 89.7%; Labclinics tissue-treated plates, 89%) and for the cryptic species (Nunc tissue-treated plates, 67.9%; Nunc nontissue-treated plates, 67.3%; Labclinics tissue-treated plates, 68.6%). In general, the three types of trays showed very similar performances. Growth patterns of the studied isolates on azole-containing wells are presented in Table 1. Overall, sensitivity (number of resistant isolates able to grow in any azole-containing well/all resistant isolates) and specificity (number of susceptible isolates unable to grow in all azole-containing wells/all susceptible isolates) values of the agar screening method testing the three different types of plastics were the following for *A. fumigatus sensu stricto*: Nunc tissue-treated plates, 100% and 94.5%; Nunc nontissue-treated plates, 100% and 96.4%; and Labclinics tissue-treated plates, 100% and 89.1%. For the cryptic species, the following values were obtained: Nunc tissue-treated plates, 80.8% and 60%; Nunc nontissue-treated plates, 80.8% and 60%; and Labclinics tissue-treated plates, 85.1% and 40%. Table 2 shows the categorical agreement between the E.Def 9.3.2 and E.Def 10.1 methods and sensitivity and specificity values of the agar screening method obtained for each type of plastic tray.

Agreement between microdilution and the agar screening method for itraconazole. Categorical agreement (95.6% to 98.9%), sensitivity (100%), and specificity (93.1% to 98.3%) values of the agar plates to screen for itraconazole resistance were invariably high against *A. fumigatus sensu stricto* (Table 2). The classification of *A. fumigatus sensu stricto* isolates as per the microdilution method and the ability or failure to grow on itraconazole agar-containing plates are shown in Figure 1a to c. Itraconazole resistance was detected with the agar screening method in all resistant isolates tested, including those harboring TR_{34}-L98H and G54R substitutions, and most showed prominent fungal growth (scoring of $\geq$2); all isolates with an itraconazole MIC of $\geq$4 mg/liter

TABLE 1 Number of *Aspergillus fumigatus sensu stricto* isolates with different growth patterns on azole-containing plates

	Growth on azole-containing agar plates			Azole-resistant isolates					
Type of plate	Itraconazole	Voriconazole	Posaconazole	TR_{34}-L98H ($n=24$)	G54R ($n=5$)	TR_{46}-Y121F-T289A ($n=1$)	Other *cyp51A* mutations ($n=2$)	Wild-type *cyp51A* gene ($n=4$)	Azole susceptible isolates ($n=55$)
Nunc tissue-treated plates	No	No	No	0	0	0	0	0	52
	Yes	No	No	0	0	0	1	0	2[b]
	Yes	**Yes**	No	20	0	0	1	2	0
	Yes	No	**Yes**	0	4	0	0	0	0
	Yes	**Yes**	**Yes**	4	1[a]	0	0	0	0
	No	**Yes**	No	0	0	1	0	2	1[b]
	No	**Yes**	**Yes**	0	0	0	0	0	0
Nunc nontissue-treated plates	No	No	No	0	0	0	0	0	53
	Yes	No	No	0	0	0	1	0	0
	Yes	**Yes**	No	19	0	0	1	2	1[b]
	Yes	No	**Yes**	0	4	0	0	0	0
	Yes	**Yes**	**Yes**	5	1[a]	0	0	0	0
	No	**Yes**	No	0	0	1	0	2	1[b]
	No	**Yes**	**Yes**	0	0	0	0	0	0
Labclinics tissue-treated plates	No	No	No	0	0	0	0	0	49
	Yes	No	No	0	0	0	1	0	4[b]
	Yes	**Yes**	No	17	0	0	1	2	0
	Yes	No	**Yes**	0	4	0	0	0	0
	Yes	**Yes**	**Yes**	7	1[a]	0	0	0	0
	No	**Yes**	No	0	0	1	0	2	1[b]
	No	**Yes**	**Yes**	0	0	0	0	0	1[b]

[a]Isolate harboring a G54R substitution that grew on voriconazole-containing agar.
[b]Azole-susceptible isolates that grew on azole-containing agars.

showed a growth score of ≥1 (Table 3). The few major errors found ($n=6$, 1.1% to 4.4%) occurred in isolates with MICs between 0.25 and 0.5 mg/liter that showed fungal growth with a scoring of 0.5 (Fig. 2a, Table 3, and Table 3S).

Categorical agreement (67.3% to 69.2%), sensitivity (73.9% to 78.3%), and specificity (62.1% to 65.5%) values of the agar plates to screen for itraconazole resistance were much lower against cryptic species. Very major errors (9.6% to 11.5%) were not necessarily restricted to a given species (*A. lentulus* [$n=2$], *A. felis* [$n=2$], *A. novofumigatus* [$n=1$], and *A. fumigatiaffinis* [$n=2$]) (Table 2S). Major errors (19.2% to 21.1%) were

TABLE 2 Categorical agreements between the agar screening and microdilution methods

	A. fumigatus sensu stricto % (no.)					Cryptic species % (no.)				
Drug and type of plate	Categorical agreement[a]	VME[b]	ME	S	Sp	Categorical agreement	VME	ME	S	Sp
Itraconazole										
Nunc tissue-treated plates	97.8	0	2.2 ($n=2$)	100	96.6	69.2	11.5 ($n=6$)	19.2 ($n=10$)	73.9	65.5
Nunc nontissue-treated plates	98.9	0	1.1 ($n=1$)	100	98.3	69.2	9.6 ($n=5$)	21.1 ($n=11$)	78.3	62.1
Labclinics tissue-treated plates	95.6	0	4.4 ($n=4$)	100	93.1	67.3	11.5 ($n=6$)	21.1 ($n=11$)	73.9	62.1
Voriconazole										
Nunc tissue-treated plates	96.7	1.1 ($n=1$)	2.2 ($n=2$)	96.8	96.7	78.8	19.2 ($n=10$)	1.9 ($n=1$)	78.7	80
Nunc nontissue-treated plates	95.6	1.1 ($n=1$)	3.3 ($n=3$)	96.8	95	71.1	25 ($n=13$)	3.8 ($n=2$)	72.3	60
Labclinics tissue-treated plates	95.6	1.1 ($n=1$)	3.3 ($n=3$)	96.8	95	78.8	17.3 ($n=9$)	3.8 ($n=2$)	80.9	60
Posaconazole										
Nunc tissue-treated plates	73.6	26.4 ($n=24$)	0	27.3	100	55.8	36.5 ($n=19$)	7.7 ($n=4$)	26.9	84.6
Nunc nontissue-treated	74.7	25.3 ($n=23$)	0	30.3	100	61.5	32.7 ($n=17$)	5.8 ($n=3$)	34.6	88.5
Labclinics tissue-treated plates	75.8	23.1 ($n=21$)	1.1 ($n=1$)	36.4	98.3	59.6	30.8 ($n=16$)	9.6 ($n=5$)	38.5	80.8

[a]Isolates were classified as resistant as per the updated 2020 EUCAST breakpoints. The endpoints were in categorical agreement when the results were in the same susceptibility category.
[b]ME, major error (false resistance); S, sensitivity; Sp, specificity; VME, very major error (false susceptibility).

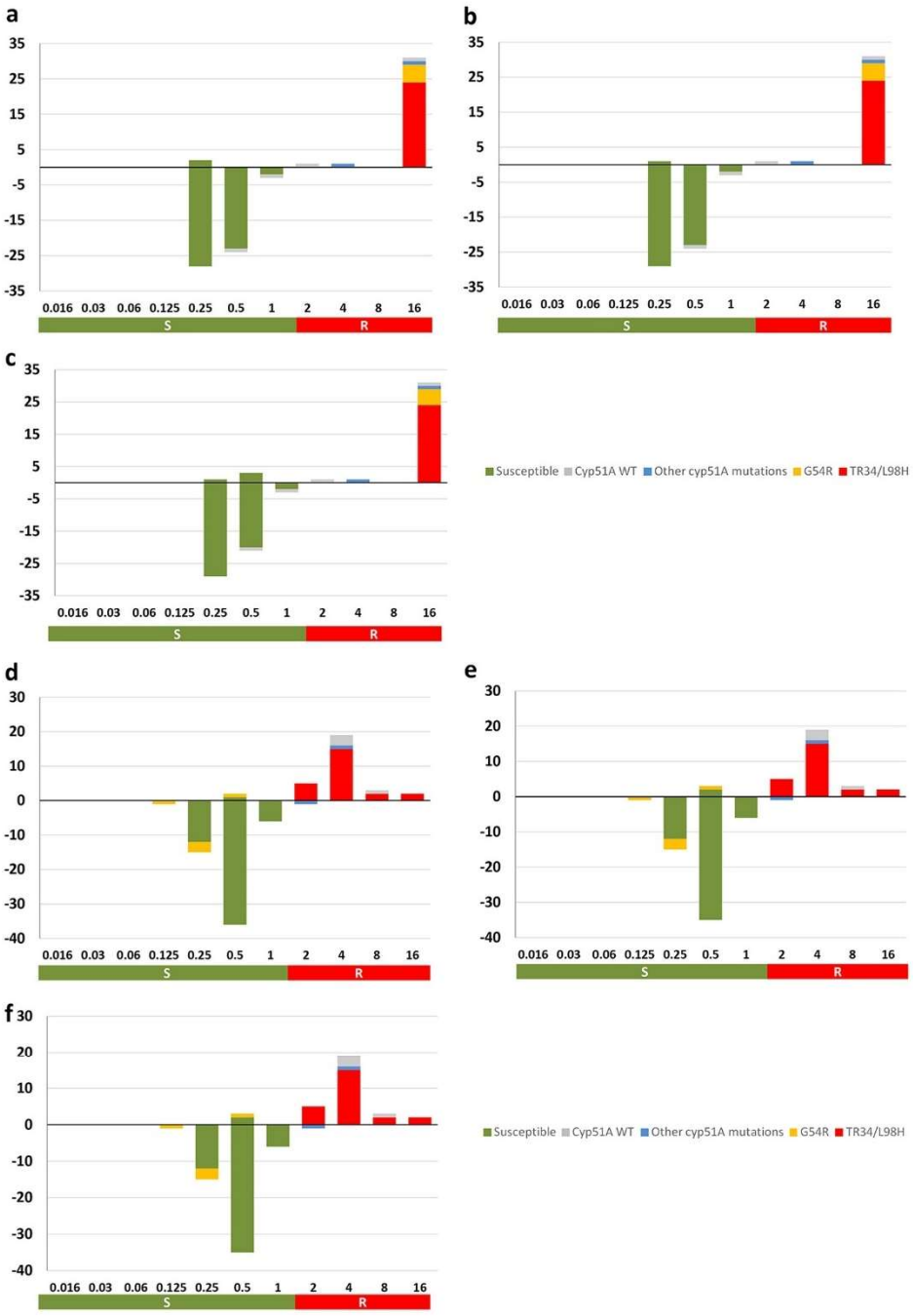

FIG 1 Number of *Aspergillus fumigatus sensu stricto* isolates falling in each MIC value according to the E.Def 9.3.2 method with (bars above the *x* axis) or without (bars below the *x* axis) fungal growth on agar plates containing itraconazole (a to c), voriconazole (d to f), and posaconazole (g to i). Susceptible isolates are depicted in green, and resistant isolates are depicted in other colors depending on the *cyp51A* gene mutation. Samples were obtained from isolates inoculated on Nunc tissue-treated plates (a, d, and g), Nunc nontissue-treated plates (b, e, and h), and Labclinics tissue-treated plates (c, f, and i).

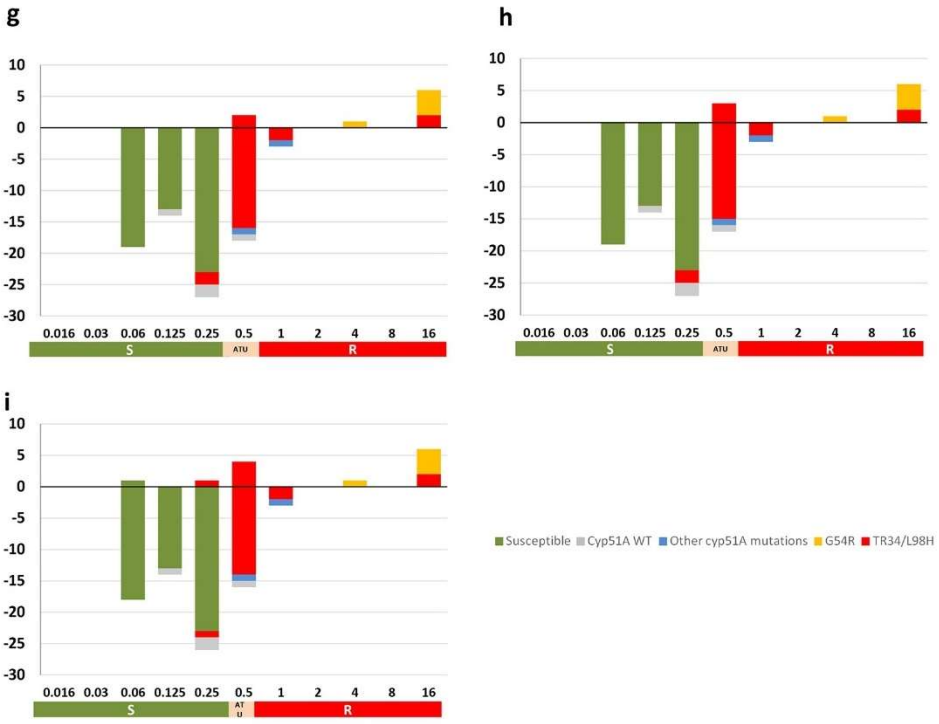

FIG 1 Continued

found in 12 isolates with MICs between 0.25 and 1 mg/liter (Fig. 2b and Table 3S). All isolates showed fungal growth with scorings of ≤1, regardless of their itraconazole MIC (Fig. 3a to c).

Agreement between microdilution and the agar-containing plate method for voriconazole. Categorical agreement (95.6% to 96.7%), sensitivity (96.8%), and specificity (95% to 96.7%) values of the agar plates to screen for voriconazole resistance were high against *A. fumigatus sensu stricto* (Table 2). Figure 1d to f show the classification of *A. fumigatus sensu stricto* isolates as per the microdilution method and the ability or failure to grow on voriconazole agar-containing plates. All isolates harboring TR_{34}-L98H and TR_{46}-Y121F-T289A substitutions were correctly classified as voriconazole resistant by the agar screening method, and most showed fungal growth with scorings between 1 and 2; most isolates with a voriconazole MIC of ≥2 mg/liter showed fungal growth with scorings of ≥1 (Fig. 2b and Table 3). Very major error (1.1%) occurred exclusively in an isolate with a MIC of 2 mg/liter harboring an F165L mutation with dubious correlation with azole resistance (Table 2S). Major errors (2.2% to 3.3%) occurred in four isolates harboring a G54 mutation, the F46Y-M172V-E427K mutations, or the *cyp51A* wild-type gene ($n = 2$), with a MIC of 0.5 mg/liter and with fungal growth scores between 0.5 and 1 (Fig. 2c and Table 3S).

Categorical agreement (71.1% to 78.8%), sensitivity (72.3% to 80.9%), and specificity (60% to 80%) values of the agar plates to screen for itraconazole resistance were much lower against cryptic species. Very major errors were not restricted to a given cryptic species (*A. lentulus* [$n = 6$], *A. fumigatiaffinis* [$n = 2$], *A. thermomutatus* [$n = 1$], *Neosartorya tsurutae* [$n = 1$], *N. udagawae* [$n = 2$], and *N. fischeri* [$n = 3$]) (Table 2S). Few major errors were found (1.9% to 3.8%) and occurred in isolates with voriconazole MICs between 0.25 mg/liter and 1 mg/liter that showed fungal growth scores from 0.5 to 1 (Fig. 3d to f and Table 3S); the remainder of the isolates showed fungal growth scores of ≤2.

TABLE 3 Fungal growth scoring of phenotypically azole-resistant *Aspergillus fumigatus sensu stricto* isolates on wells containing itraconazole, voriconazole, and posaconazole and assorted by the type of *cyp51A* mutation[a]

Drug and type of mutation	No. of isolates		No. of isolates for each score															
			Nunc tissue-treated plates					Nunc nontissue-treated plates					Labclinics tissue-treatedplates					
	Overall	Phenotypically resistant	0	0.5	1	2	3	0	0.5	1	2	3	0	0.5	1	2	3
Itraconazole																		
TR_{34}-L98H	24	24	0	0	4	19	1	0	0	2	21	1	0	0	2	21	1	
G54R	5	5	0	0	0	2	3	0	0	0	2	3	0	0	0	2	3	
TR_{46}-Y121F-T289A	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	
F46Y-M172V-N248T-D255E-E427K	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	
F165L	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	
Wild-type *cyp51A* gene	4	2	2	2	0	0	0	2	2	0	0	0	2	2	0	0	0	
Voriconazole																		
TR_{34}-L98H	24	24	0	0	18	5	1	0	0	19	4	1	0	0	18	5	1	
G54R	5	0	4	1	0	0	0	4	1	0	0	0	4	1	0	0	0	
TR_{46}-Y121F-T289A	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	
F46Y-M172V-N248T-D255E-E427K	1	1	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	
F165L	1	1	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	
Wild-type *cyp51A* gene	4	4	0	2	1	1	0	0	2	1	1	0	0	2	1	1	0	
Posaconazole																		
TR_{34}-L98H	24	24	20	2	1	1	0	19	3	1	1	0	17	5	1	1	0	
G54R	5	5	0	0	0	2	3	0	0	0	2	3	0	0	0	2	3	
TR_{46}-Y121F-T289A	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	
F46Y-M172V-N248T-D255E-E427K	1	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	
F165L	1	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	
Wild-type *cyp51A* gene	4	2	4	0	0	0	0	4	0	0	0	0	4	0	0	0	0	

[a]Differences among the trays used are shown.

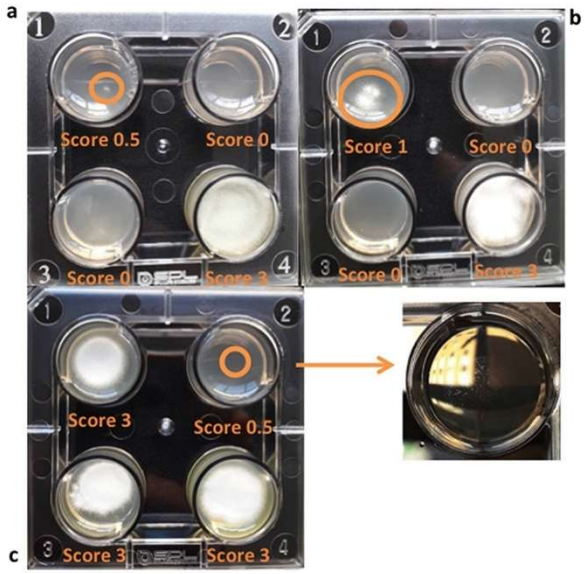

FIG 2 Example of major errors (false resistance) detected for itraconazole-susceptible (a; isolate code 6142) and voriconazole-susceptible (c; isolate code 6430) *Aspergillus fumigatus sensu stricto* isolates and for an itraconazole-susceptible *Neosartorya udagawae* isolate (b; isolate code 6158). Fungal growth was observed for all isolates on agar wells with a scoring of 0.5 or 1. Wells in the plates contained itraconazole (4 mg/liter; well 1), voriconazole (2 mg/liter; well 2), posaconazole (0.5 mg/liter; well 3), and no azole (growth control; well 4). The *A. fumigatus sensu stricto* isolate 6430 harbored the G54R mutation. Images of the isolates inoculated on Labclinics tissue-treated trays were obtained.

Agreement between microdilution and the agar-containing plate method for posaconazole. Posaconazole-containing agar plates showed the lowest categorical agreement (73.6% to 75.8%) and sensitivity (27.3% to 36.4%) but the highest specificity values (98.3% to 100%) to screen for resistance against *A. fumigatus sensu stricto* (Table 2). Figure 1g to i show the classification of *A. fumigatus sensu stricto* isolates as per the microdilution method and the ability or failure to grow on posaconazole agar-containing plates. Fungal growth patterns in each well are shown in Figure 4. All isolates harboring G54R substitutions were correctly classified as posaconazole resistant and showed prominent fungal growth (scoring of 2 to 3) (Fig. 4c and Table 3). All isolates with fungal growth scorings of $\geq$1 had a posaconazole MIC of $\geq$4 mg/liter. A large number of very major errors (23.1% to 26.4%) were found ($n = 3$, MIC of 0.25 mg/liter; $n = 19$, MIC of 0.5 mg/liter; $n = 3$, MIC of 1 mg/liter), and many ($n = 21/24$) were observed in isolates harboring TR_{34}-L98H substitutions (Table 2S). A major error was identified in an isolate with a posaconazole MIC of 0.06 mg/liter that showed fungal growth with a scoring of 0.5 (Table 3S).

Categorical agreement (55.8% to 61.5%), sensitivity (26.9% to 38.5%), and specificity (80.8% to 88.5%) values of the agar plates to screen for posaconazole resistance were lower against cryptic species. All isolates showed fungal growth with scorings of $\leq$1 (Fig. 3g to i). Very major errors were not restricted to a given species (*A. lentulus*, $n = 4$; *A. felis*, $n = 2$; *A. novofumigatus*, $n = 7$; *A. fumigatiaffinis*, $n = 3$; *N. tsurutae*, $n = 2$; and *N. udagawae*, $n = 1$) and occurred in isolates with posaconazole MICs of 0.25 mg/liter ($n = 7$), 0.5 mg/liter ($n = 6$), or 1 mg/liter ($n = 6$) (Table 2S). All major errors (5.8% to 9.6%) occurred in isolates with posaconazole MICs between 0.06 mg/liter and 0.25 mg/liter that showed fungal growth with scores between 0.5 and 1 (Table 3S).

DISCUSSION

There are high agreements between broth microdilution and the agar screening method for itraconazole and voriconazole against *A. fumigatus sensu stricto* isolates.

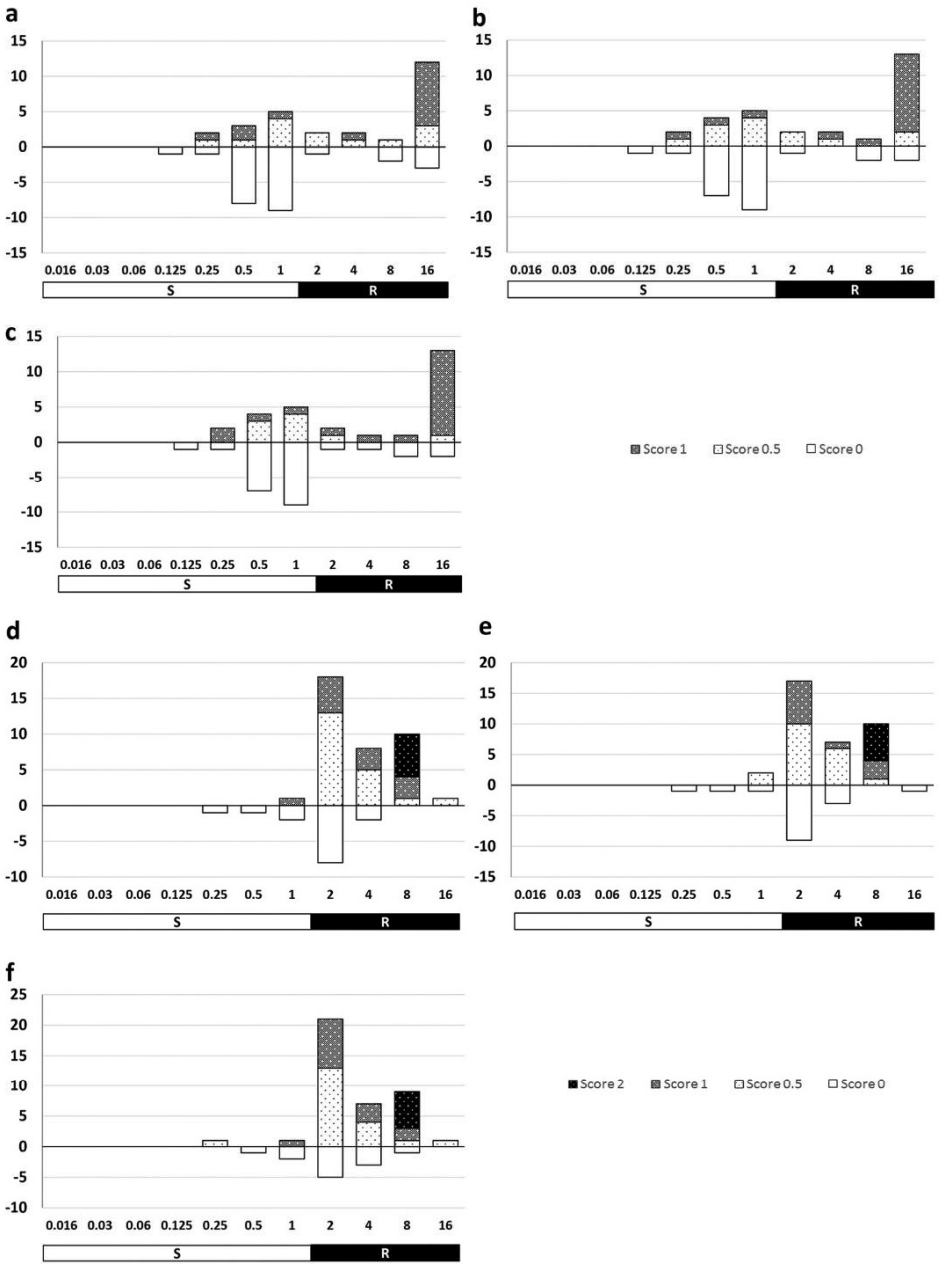

FIG 3 Number of cryptic species isolates falling in each MIC value according to the E.Def 9.3.2 method and showing fungal growth with scorings of 0, 0.5, 1, 2, or 3 on agar plates containing itraconazole (a to c), voriconazole (d to f), and posaconazole (g to i). Isolates with or without fungal growth on the agar plates are represented as bars above and below the *x* axis, respectively. Scores obtained from Nunc tissue-treated plates (a, d, and g), Nunc nontissue-treated plates (b, e, and h), and Labclinics tissue-treated plates (c, f, and i) are shown.

The type of plastic hardly affects the results. By contrast, agreements between the tested procedures against cryptic species are much lower.

A. fumigatus resistance is an emerging problem worldwide, and countries are affected to variable degrees (6). More specifically, azole resistance rates are on the rise according to a recent Spanish survey (4). Mortality rates in patients infected by azole-

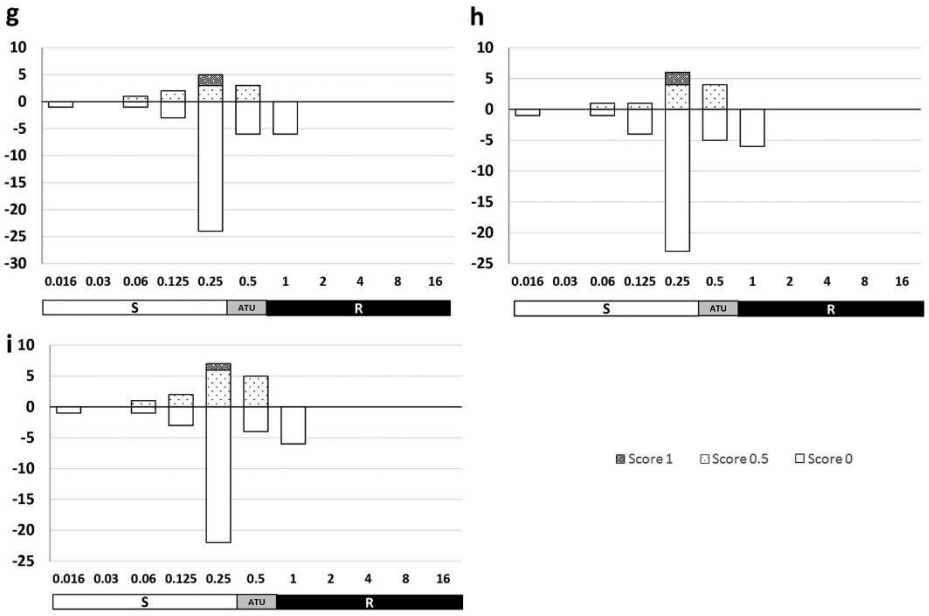

FIG 3 Continued

resistant *A. fumigatus* are significantly higher than in subjects infected by azole-susceptible isolates (5, 6).

In this scenario, strategies to enable early detection of azole resistance in *A. fumigatus* clinical isolates are key. The microdilution gold standard EUCAST E.Def 9.3.2 procedure is technically demanding, and its routine use in many microbiology laboratories may not be possible. We recently showed that spectrophotometric azole MIC readings were rather similar to visual inspection, which may minimize variability in terms of MIC settings (18). However, this is insufficient to bring the EUCAST microdilution methodology to the doorstep of clinical microbiology laboratories. To date, commercial methods for antifungal susceptibility testing against *Aspergillus* spp., to be used on a daily basis in a clinical microbiology laboratory, have not been officially well validated. Previous evaluations of Sensititre YeastOne and Etest against *A. fumigatus* showed variable results. Moreover, studies reporting the highest categorical agreements with broth microdilution reference methods examined a low number of mutant isolates and excluded cryptic species (19–22). Thus, antifungal susceptibility testing against molds is either not performed or outsourced to referral laboratories with the consequent delay in result reporting.

Azole-containing agar plates are a convenient tool that may help overcome the above-discussed limitations. Two previous studies report excellent performance of azole-containing plates to spot resistant isolates and with high correlation with the EUCAST microdilution method (11, 12). In both studies, commercially available VIPcheck plates (plus in-house plates in one study) were tested. The performance of the procedure to screen for azole resistance in cryptic species has not yet been studied. Furthermore, the impact of using different trays to prepare in-house plates has not been assessed. EUCAST has recently updated azole breakpoints against *A. fumigatus sensu lato* to accommodate the concept of the area of technical uncertainty (ATU) and the new category of "Susceptible, increased exposure" (8).

Here, we confirm the good performance of azole-containing plates to screen for the presence of azole resistance in *A. fumigatus sensu stricto* in a clinical microbiology department of a hospital located in the South of Europe. The resistance pattern is easily inferred from the growth on azole-containing agar wells (Table 1). Azole-resistant *A.*

FIG 4 Growth pattern and score of azole-resistant *A. fumigatus sensu stricto* isolates harboring the following *cyp51A* gene substitutions: F165L (a), TR_{46}-Y121F-T289A (b), G54R (c), and TR_{34}-L98H (d). Wells in the plates contained itraconazole (4 mg/liter; well 1), voriconazole (2 mg/liter; well 2), posaconazole (0.5 mg/liter; well 3), and no azole (growth control; well 4).

fumigatus sensu stricto isolates are, in all cases, correctly classified as such by using itraconazole-containing agar. According to our results, TR_{34}-L98H substitutions can be suspected in isolates growing on itraconazole- and voriconazole-containing agars, G54 mutations can be suspected in isolates growing on posaconazole- and itraconazole-containing agars, and TR_{46}-Y121F-T289A substitutions can be suspected in isolates growing exclusively on voriconazole-containing agar plates. Finally, the type of plastic trays used does not affect sensitivity and specificity values. Very major errors occur just with voriconazole (1.1%) in an isolate with an MIC falling in the ATU (2 mg/liter) and that does not harbor a mutation that clearly correlates to azole resistance. The ATU corresponds to a MIC value at which the categorization is doubtful due to an overlap between wild-type isolates and mutant isolates, as recently shown (4). By contrast, the number of very major errors with posaconazole is high and may be due to the concentration of the drug in the agar plate (0.5 mg/liter), higher than the revised EUCAST posaconazole breakpoint (0.25 mg/liter, coinciding with the ATU). The E.Def 10.1 method was developed using concentrations of drugs according to the breakpoints in use at that moment. However, such high concentration of drug in the well seems to be too high, which is confirmed by the high number of isolates with TR_{34}-L98H substitutions showing low levels of posaconazole resistance unable to grow on posaconazole-containing plates. Lower posaconazole concentration in agar plates (0.25 mg/liter) may increase major errors but may also reduce very major errors in terms of detection of low-level posaconazole-resistant TR_{34}-L98H isolates. The posaconazole agar well may be skipped since posaconazole-resistant isolates were able to grow on itraconazole-containing plates; however, the growth pattern on posaconazole and itraconazole agar-containing plates is helpful to suspect the presence of an isolate harboring the G54 *cyp51A* gene substitution.

Major errors with itraconazole and voriconazole only occur in a few isolates (1.1% to 4.4%) that show tiny fungal growth (scoring of 0.5 to 1) on azole-containing agars. By contrast, all isolates harboring relevant substitutions (TR_{34}-L98H, G54R, and TR_{46}-Y121F-T289A) grow vigorously on itraconazole- and voriconazole-containing plates, in line with their susceptibility profile as per the microdilution method. Vigorous growth

must raise the alarm about the presence of truly resistant isolates, whereas tiny fungal growth may imply false resistance. We recommend confirmatory microdilution methods in isolates that grow on azole-containing plates, regardless of the extent of fungal growth.

Performance of azole-containing agar plates is much lower against cryptic species isolates; voriconazole-containing agar wells show the highest values of sensitivity against these infrequent species. Weak growth (scoring of 0.5 to 1), more frequently seen on itraconazole-containing plates, was observed for azole-susceptible cryptic species.

The present study is limited by the fact that only one of the studied isolates harbored the TR_{46}-Y121F-T289A substitutions, and other mutations were not tested. Lower concentrations of posaconazole may improve the performance of this drug in agar plates.

We conclude that the azole-containing agar plate method, regardless of the type of plastic tray, is a convenient screening method to differentiate between azole-susceptible and azole-resistant clinical *A. fumigatus sensu stricto* isolates. By contrast, for cryptic species isolates, the azole susceptibility pattern is poorly inferred from their growth on agar-containing plates. Further studies, including resistant isolates with a wider range of mutations and testing lower concentrations of posaconazole in the agars, are warranted.

MATERIALS AND METHODS

Isolates studied. *A. fumigatus sensu lato* isolates ($n = 143$) comprising *A. fumigatus sensu stricto* (azole-resistant isolates [$n = 36$] and azole-susceptible isolates [$n = 55$]) and cryptic species (azole-resistant isolates [$n = 47$] and azole-susceptible isolates [$n = 5$]) from a survey recently conducted in Spain and from patients cared for at Gregorio Marañón hospital were studied (Table 1S) (4). Identification of isolates was carried out using a Microflex LT matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) spectrometer (Bruker Daltonics, Bremen, Germany); molecular testing was additionally used to identify cryptic species ($n = 52$) and azole-resistant *A. fumigatus sensu stricto* isolates ($n = 36$) (4, 23). Azole-resistant *A. fumigatus sensu stricto* had the following *cyp51A* gene mutations: TR_{34}-L98H ($n = 24$), G54R ($n = 5$), TR_{46}-Y121F-T289A ($n = 1$), F46Y-M172V-N248T-D255E-E427K ($n = 1$), F165L ($n = 1$), and wild-type *cyp51A* gene ($n = 4$). Cryptic species isolates included *Aspergillus felis* ($n = 2$), *Aspergillus lentulus* ($n = 13$), *Aspergillus fumigatiaffinis* ($n = 7$), *Neosartorya tsurutae* ($n = 3$), *Neosartorya udagawae* ($n = 7$), *Aspergillus novofumigatus* ($n = 8$), *Aspergillus viridinutans* ($n = 2$), *Neosartorya fischeri* ($n = 8$), *Neosartorya hiratsukae* ($n = 1$), and *Aspergillus thermomutatus* ($n = 1$).

EUCAST antifungal susceptibility testing, broth microdilution (E.Def 9.3.2), and agar screening method (E.Def 10.1). Antifungal susceptibility of the isolates to itraconazole, voriconazole, and posaconazole (Sigma-Aldrich, Madrid, Spain) was determined following the EUCAST E.Def 9.3.2 procedure and using tissue-treated trays (CELLSTAR, 655 180, Greiner Bio-One, Frickenhausen, Germany) (24). The inoculated trays were incubated at 35°C, and MICs were obtained after 48 h with a visual endpoint determination. Isolates were classified as susceptible or resistant according to EUCAST breakpoints (v10) (8). Interpretation of MICs falling in the area of technical uncertainty (ATU) for the three azoles, defined in the updated breakpoints, was done as follows: itraconazole and voriconazole (MIC = 2 mg/liter, always considered resistant) and posaconazole (MIC = 0.25 mg/liter, considered resistant only if the isolate was also itraconazole resistant).

The agar screening method was conducted according to EUCAST E.Def 10.1 (15). Three different multidish plate types were used: tissue-treated plates (Nunc Z688746, Sigma-Aldrich, Madrid, Spain; Labclinics, PLC30004, Labclinics, SA, Barcelona, Spain) and nontissue-treated plates (Nunc Z688754, Sigma-Aldrich, Madrid, Spain). The inoculated plates were incubated at 35°C, and visual inspection of fungal growth was assessed after 48 h. Isolates were classified as susceptible (when fungal growth was only observed on the antifungal-free well) or as "potentially" azole resistant (when growth was observed on the antifungal-free well and in one or more azole-containing wells). Patterns of fungal growth on each well are shown in Figure 4; fungal growth was scored as 0.5 (1 to 5 tiny colonies), 1 (more than 5 tiny colonies or weak growth where the agar was inoculated), 2 (clearly visible growth but less prominent than the control well), or 3 (prominent growth similar to that in the control well).

Quality control was ensured by testing *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 isolates (E.Def 9.3.2) and the isolates from the culture collection of the University of Gothenburg *A. fumigatus* CCUG 74258/SSI-4524 (TR_{34}-L98H) and *A. fumigatus* CCUG 74259/SSI-5586 (G54W) (E.Def 10.1) (8). For the quality control isolates, MICs were within acceptable ranges and grew well (scoring of 3) on the antifungal-free well of the agar procedure.

Data analysis. The E.Def. 9.3.2 broth microdilution procedure was considered the gold standard, and categorical agreement with the agar screening method was carried out for each of the three types of plastic plates assessed in this study. Errors were defined as very major (false susceptibility) when the

gold standard method classified an isolate as resistant, and the agar screening method classified an isolate as susceptible. Errors were defined as major (false resistance) when the gold standard method categorized an isolate as susceptible, and the agar screening method classified an isolate as resistant. *A. fumigatus sensu stricto* isolates leading to very major errors with itraconazole and/or voriconazole were retested, and the *cyp51A* gene of isolates leading to major errors was sequenced. Finally, the overall performance of the three types of plastic plates was evaluated by calculating sensitivity/specificity based on the classification of the isolates as resistant/susceptible as per the EUCAST breakpoints (v10) for each of the azoles (8). Sensitivity was defined as the probability that a resistant isolate grew in one or more azole-containing wells; specificity was defined as the probability that a susceptible isolate did not grow in any azole-containing well.

Ethical considerations. This study was approved by the Ethics Committee of Hospital Gregorio Marañón (CEIm; study number 22/19).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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Artículo 4: Screening of azole resistance in *Aspergillus fumigatus* using the EUCAST E.Def 10.2 azole-containing agar method: a single study suggests that filtration of conidial suspensions prior to inoculum preparation may not be needed

El filtrado de las suspensiones de conidias es un paso indispensable para la preparación del inóculo propuesto en el procedimiento EUCAST E. Def 10.2. En este estudio, se evaluó si omitir este paso antes del ajuste del inóculo influía negativamente en el rendimiento del método de agar con azoles para la detección de resistencia a azoles en *A. fumigatus.*

Se incluyeron 92 cepas de *A. fumigatus sensu stricto*, clasificados como sensibles o resistentes a azoles según el método de microdilución E.Def 9.4 de EUCAST y los puntos de corte clínicos v10. Las cepas resistentes a azoles tenían la secuencia del gen *cyp51A* de tipo salvaje (n=3) o presentaron las siguientes substituciones: TR_{34}-L98H (n=26), G54R (n=5), TR_{46}-Y121F-T289A (n=1), F46Y-M172V-N248T-D255E-E427K (n=1), F165L (n=1), y G448S (n=1). Se prepararon placas de agar caseras con azoles según el procedimiento EUCAST E.Def 10.2. Las suspensiones de conidias se obtuvieron añadiendo agua destilada con Tween 20 al 0,1%. Posteriormente se utilizaron dos tipos de suspensiones antes de ajustar el inóculo a 0,5 McFarland, filtradas o sin filtrar. Se evaluó la concordancia, sensibilidad y especificidad de las placas de agar inoculadas con los dos tipos de suspensiones utilizando la microdilución como método de referencia.

La concordancia para el método de detección en agar con suspensiones de conidias filtradas y no filtradas fue del 100% para itraconazol y voriconazol, y del 97,8% para posaconazol. La sensibilidad y la especificidad del procedimiento para confirmar o descartar resistencia cuando se usaron suspensiones no filtradas también fueron muy elevadas, 100% y 98,2%, respectivamente. Las cepas que albergaban las sustituciones TR_{34}-L98H, G54R y TR_{46}-Y121F-T289A fueron detectadas con el método modificado.

En conclusión, omitir la filtración de las suspensiones de conidias no influyó negativamente en el rendimiento del método E.Def 10.2 para la detección de la resistencia a azoles en *A. fumigatus sensu stricto*.

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Screening of azole resistance in *Aspergillus fumigatus* using the EUCAST E.Def 10.2 azole-containing agar method: A single study suggests that filtration of conidial suspensions prior to inoculum preparation may not be needed

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Abstract

Background: Azole resistance screening in *Aspergillus fumigatus* isolates can be routinely carried out by using azole-containing plates (E.Def 10.2 method), that requires filtering conidial suspensions prior inoculum adjustment.

Objectives: We evaluated whether skipping the filtration step of conidial suspensions negatively influences the performance of the E.Def 10.2.

Patients/Methods: *A. fumigatus* sensu stricto isolates (n = 92), classified as azole-susceptible or azole-resistant according to the EUCAST microdilution E.Def 9.4 method, were studied. Azole-resistant isolates had either wild type *cyp51A* gene sequence (n = 3) or the TR_{34}-L98H (n = 26), G54R (n = 5), TR_{46}-Y121F-T289A (n = 1), F46Y-M172V-N248T-D255E-E427K (n = 1), F165L (n = 1) or G448S (n = 1) *cyp51A* gene substitutions. In-house azole-containing agar plates were prepared according to the EUCAST E.Def 10.2 procedure. Conidial suspensions were obtained by adding distilled water (Tween 20 0.1%). Subsequently, the suspensions were either filtered or left unfiltered prior to inoculum adjustment to 0.5 McFarland. Using microdilution as the gold standard, agreement, sensitivity and specificity of the agar plates inoculated with two inoculums were assessed.

Results: Agreements for the agar screening method with either unfiltered or filtered conidial suspensions were high for itraconazole (100%), voriconazole (100%) and posaconazole (97.8%). Sensitivity (100%) and specificity (98.2%) of the procedure to rule in or out resistance when unfiltered suspensions were used were also high. Isolates harbouring the TR_{34}-L98H, G54R and TR_{46}-Y121F-T289A substitutions were detected with the modified method.

Conclusions: Unfiltered conidial suspensions does not negatively influence the performance of the E.Def 10.2 method when screening for *A. fumigatus* sensu stricto.

KEYWORDS
Aspergillus fumigatus, azole-containing plates, conidial suspensions, *cyp51A* gene EUCAST, E. Def 10.2, resistance

See Acknowledgements for the ASPEIN Study Group†.

1 | INTRODUCTION

Azole resistance in *Aspergillus fumigatus*, an emerging global problem spreading through many countries, is associated to a poor prognosis.[1–4] Consequently, screening for azole-resistant isolates should be routinely done in clinical microbiology laboratories. Azole-containing (itraconazole, voriconazole or posaconazole) agar plates are non-MIC based procedures to screen for azole resistance in *A. fumigatus*. The method lies on the ability of azole-resistant isolates to grow on azole-containing agar plates.[5–7] EUCAST has developed a procedure (E.Def 10.2) to prepare in-house azole-containing plates.

E.Def 10.2 requires filtering conidial suspensions prior inoculum adjustment, which takes time and increases laboratory workload.[7] The objective of this study is to evaluate whether skipping conidial suspension filtration step affects the E.Def 10.2 procedure performance.

2 | MATERIALS AND METHODS

2.1 | Isolates studied and antifungal susceptibility testing using the broth microdilution method (E.Def 9.4)

We selected a panel of *A. fumigatus* sensu stricto isolates ($n = 92$) classified as azole-susceptible or azole-resistant. Isolates were either collected in a survey recently conducted in Spain or were stored in the Gregorio Marañón hospital clinical isolate collection.[4] Briefly, antifungal susceptibility of the isolates to itraconazole, voriconazole or posaconazole (Sigma-Aldrich, Madrid, Spain) had been previously determined following the EUCAST E.Def 9.4 procedure using tissue-treated trays (CELLSTAR® Ref. 655,180, Greiner bio-one).[8] Isolates were classified as azole-susceptible or azole-resistant according to EUCAST breakpoints (v10).[9] Interpretation of MICs falling in the area of technical uncertainty (ATU) for the three azoles was done as follows: itraconazole and voriconazole (MIC = 2 mg/L, always considered resistant), posaconazole (MIC = 0.25 mg/L, considered resistant only if the isolate was also itraconazole-resistant). Quality control was ensured by testing *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305. Azole-resistant isolates had either wild type *cyp51A* gene sequence ($n = 3$) or the TR_{34}-L98H ($n = 26$), G54R ($n = 5$), TR_{46}-Y121F-T289A ($n = 1$), F46Y-M172V-N248T-D255E-E427K ($n = 1$), F165L ($n = 1$) or G448S ($n = 1$) *cyp51A* gene substitutions. Azole-resistant isolates were retested.

2.2 | Azole-containing agar plate method (E.Def 10.2) using different conidial suspensions

The agar screening method was conducted according to EUCAST E.Def 10.2 procedure using home-made multi-dish plates (Labclinics® tissue-treated plates).[7] Conidial suspensions were prepared in distilled water (Tween 20 0.1%) and in parallel filtered (Merck Millipore®, Nylon Net Filter 11.0 μm pore size) or left unfiltered prior to inoculum adjustment to 0.5 McFarland. The inoculated plates were incubated at 35°C and visual inspection of fungal growth was assessed after 48 h. Isolates were classified as susceptible (when fungal growth was only observed in the antifungal-free well) or as "potentially" azole-resistant (when growth was observed in the antifungal-free well and one or more azole-containing wells). Quality control was ensured by testing the isolates from the culture collection of the University of Gothenburg *A. fumigatus* CCUG 74258/SSI-4524 (TR_{34}-L98H) and *A. fumigatus* CCUG 74259/SSI-5586 (G54W).[9]

2.3 | Data analysis

The E.Def 9.4 broth microdilution procedure was considered the gold standard. Comparisons of results obtained by using the gold standard with those obtained using either unfiltered and filtered conidial suspensions were made for the agar screening method against individual azoles (categorical agreement). Errors were defined as very major (false susceptibility) when the microdilution procedure classified an isolate as resistant and the agar screening method as susceptible, and as major (false resistance) when the microdilution procedure categorised an isolate as susceptible and the agar screening method as resistant. Moreover, overall performance of the agar plates inoculated using either unfiltered or filtered conidial suspensions to detect azole resistance (to any of the three azoles tested) or rule out azole resistance (to the three azoles tested) was evaluated by calculating the sensitivity (probability that a resistant isolate grew in one or more azole-containing wells) and specificity (probability that a susceptible isolate did not grow in any azole-containing well).[9] The agar screening procedure was re-conducted for isolates with categorical disagreements (very major and major errors against itraconazole or voriconazole), or when discrepant results between the conidial suspensions were found.

2.4 | ETHICS STATEMENT

This study was approved by the Ethics Committee of Hospital Gregorio Marañón (CEIm; study no. 22/19).

3 | RESULTS

3.1 | Categorical agreement between E.Def 9.4 and E.Def 10.2. Sensitivity and specificity of the E.Def 10.2 method using different conidial suspensions

Azole resistance screening on agar plates inoculated with unfiltered vs. filtered suspensions yielded very high categorical agreement for itraconazole (98.6% vs. 98.6%), voriconazole (97.8% vs. 97.8%) and posaconazole (77.2% vs. 75%) (Table 1). Comparisons using

unfiltered and filtered suspensions for itraconazole, voriconazole and posaconazole were 100%, 100% and 97.8%. Sensitivity and specificity values were 100% and 98.2% regardless the filtration of the conidial suspension. All isolates harbouring TR_{34}-L98H substitutions or G54R and TR_{46}-Y121F-T289A substitutions were correctly classified by the agar screening method for unfiltered and filtered suspensions.

3.2 | Very major and major errors, and disagreements between unfiltered and filtered conidial suspensions

Few very major errors were identified with itraconazole (1.1%; one isolate with an MIC of 2 mg/L harbouring a G448S mutation found in wells inoculated with either conidial suspension) and voriconazole

TABLE 1 Categorical agreements between the studied screening method on azole-containing agar plates using filtered and unfiltered conidia suspensions and compared to the E.Def 9.4 method

Drug	Conidia suspension preparation	Categorical agreement	Errors	
			Very major (false susceptibility)	Major (false resistance)
Itraconazole	Unfiltered	98.9%	1.1% ($n = 1$)	0
	Filtered	98.9%	1.1% ($n = 1$)	0
Voriconazole	Unfiltered	97.8%	1.1% ($n = 1$)	1.1% ($n = 1$)
	Filtered	97.8%	1.1% ($n = 1$)	1.1% ($n = 1$)
Posaconazole	Unfiltered	77.2%	22.8% ($n = 21$)	0
	Filtered	75%	25% ($n = 23$)	0

TABLE 2 Very major errors (false susceptibility) and major errors (false resistance) found in the isolates tested using filtered or unfiltered conidia suspensions

Isolate code	*cyp51A* gene substitutions	Errors	E.Def 9.4 visual reading (MIC, mg/L)/unfiltered inoculum agar plates/filtered inoculum agar plates		
			Itraconazole	Voriconazole	Posaconazole
3917	G448S	VME	R(2)/S/S	R/R/R	R(0.25)/S/S
6433	F165L	VME	R/R/R	R(2)/S/S	R(0.5)/S/S
4728	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6118	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6293	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6308	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6317	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6319	TR_{34}-L98H	VME	R/R/R	R/R/R	R(1)/S/S
6371	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6374	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6390	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6393	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6402	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6407	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6414	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6422	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.25)/S/S
6432	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/R/S
6444	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/R/S
6503	TR_{34}-L98H	VME	R/R/R	R/R/R	R(1)/S/S
6639	TR_{34}-L98H	VME	R/R/R	R/R/R	R(1)/S/S
6878	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6884	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6964	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
7057	F46Y5E-E427K	ME	S/S/S	S(0.5)/R/R	S/S/S

Abbreviations: ME, major error; R, resistant; S, susceptible; VME, very major error.

(1.1%; one isolate with an MIC of 2 mg/L harbouring a F165L mutation found in wells inoculated with either conidial suspension). In contrast, a large number of very major errors (n = 21, 22.8% and n = 23, 25%) were found with posaconazole when conidial suspensions were unfiltered or filtered, respectively. Very major errors were found with posaconazole for unfiltered conidial suspensions in isolates with MICs of 0.25 mg/L (n = 2), 0.5 mg/L (n = 16), or 1 mg/L (n = 3). Most isolates (n = 19) harboured the TR_{34}-L98H substitution and the remaining isolates the F165L and G448S mutations (Table 2). Major errors were anecdotic and only occurred with voriconazole (1.1%); one isolate harbouring the F46Y-M172V-N248T-D255E-E427K polymorphism (MIC of 0.5 mg/L) grew slightly on plates inoculated with unfiltered and filtered conidial suspensions (Table 2). Due to this major error, 100% specificity of the method was not achieved.

Discrepancies were only noted with unfiltered and filtered conidial suspensions for posaconazole and when the filtered conidial suspension was used with two isolates harbouring the TR_{34}-L98H substitution.

4 | DISCUSSION

Performance of the azole-containing agar method for screening azole resistance in *A. fumigatus* was not negatively influenced by skipping conidial suspension filtration prior to inoculum adjustment.

The azole-containing agar plate method is a useful procedure that may help routinely detect azole resistance in *A. fumigatus* in clinical mycology laboratories.[5,6,10] However, the procedure takes time and its day-to-day implementation slows down the laboratory workload. We recently reported that the type of plastic tray used minimally affects the performance of the E.Def 10.2 agar screening method.[10] Here, and in order to alleviate the laboratory workload, we studied whether filtering conidial suspensions prior to inoculum preparation may be skipped. Overall, we observe that sensitivity (100%) and specificity (98.2%) of the agar screening method are high and remain unaffected when plates are inoculated with unfiltered conidial suspensions, that is in line with a previous study in which plates were inoculated with unfiltered conidia suspensions and also reported high sensitivity and specificity values (98% and 93%, respectively).[6]

We note few discrepancies when unfiltered conidial suspensions are used for posaconazole and none for itraconazole and voriconazole. Furthermore, skipping conidial suspension filtering does not lead to errors in the classification of isolates with TR_{34}-L98H substitutions (growing on itraconazole- and voriconazole-containing agars), isolates with G54R mutations (growing on itraconazole- and posaconazole-containing agars), or isolates with TR_{46}-Y121F-T289A substitutions (growing exclusively on voriconazole-containing agar plates).

We conclude that skipping the filtration step of conidial suspensions prior inoculum preparation does not negatively influence the performance of the azole-containing agar plate method when screening for the presence of azole-resistant clinical *A. fumigatus* sensu stricto isolates.

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CONFLICT OF INTEREST

All authors report no conflicts of interest relevant to this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Artículo 5: Azole resistance screening in *Aspergillus fumigatus sensu stricto* using the azole-containing agar method (EUCAST E. Def 10.2): conidial suspension filtration and inoculum adjustment before inoculum preparation may not be needed

El ajuste del inóculo es otro paso indispensable del procedimiento EUCAST E.Def 10.2 En este estudio se evaluó si omitir tanto el paso de filtración como el de ajuste del inóculo influía negativamente en el rendimiento del procedimiento E.Def 10.2 para la detección de resistencia a azoles en *A. fumigatus.*

Se estudiaron 98 cepas de *A. fumigatus sensu stricto*, clasificadas como sensibles o resistentes a azoles según el método de microdilución E.Def 9.4 de EUCAST y los puntos de corte clínicos v10. Las cepas resistentes a azoles tenían la secuencia del gen *cyp51A* de tipo salvaje (n=1), la sustitución TR_{34}-L98H (n=41), G54R (n=5), TR_{46}-Y121F-T289A (n=1) o la sustitución G448S (n=1). Se prepararon placas de agar con azoles según el procedimiento EUCAST E.Def 10.2. Las suspensiones de conidias se obtuvieron añadiendo agua destilada con Tween 20 al 0,1%. Posteriormente, las suspensiones se filtraron y ajustaron al 0,5 McFarland, o se dejaron sin filtrar ni ajustar. Se evaluó la concordancia, sensibilidad y especificidad de las placas de agar inoculadas con los dos inóculos utilizando la microdilución como método de referencia.

La concordancia entre el método de detección en agar con suspensiones de conidias filtradas y ajustadas y no filtradas ni ajustadas fue del 99% para itraconazol, 100% para voriconazol, y del 94,9% para posaconazol. La sensibilidad y la especificidad del procedimiento para confirmar o descartar resistencia cuando se usaron suspensiones sin filtrar ni ajustar fue del 100%. Las cepas que albergaban las sustituciones TR_{34}-L98H, G54R y TR_{46}-Y121F-T289A fueron detectadas con el método modificado.

En conclusión, omitir la filtración y el ajuste del inóculo de las suspensiones de conidias no influyó negativamente en el rendimiento del método E.Def 10.2 para la detección de la resistencia a azoles en *A. fumigatus sensu stricto*.

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Azole resistance screening in *Aspergillus fumigatus sensu stricto* using the azole-containing agar method (EUCAST E.Def 10.2): conidial suspension filtration and inoculum adjustment before inoculum preparation may not be needed

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ABSTRACT Azole resistance screening in *Aspergillus fumigatus sensu stricto* can be routinely carried out by using azole-containing agar plates (E.Def 10.2 procedure); however, conidial suspension filtering and inoculum adjustment before inoculum preparation are time-consuming. We evaluated whether skipping the filtration and inoculum adjustment steps negatively influenced the performance of the E.Def 10.2 procedure. *A. fumigatus sensu stricto* isolates (n = 98), previously classified as azole susceptible or azole resistant (E.Def 9.4 method), were studied. Azole-resistant isolates had either the wild-type *cyp51A* gene sequence (n = 1) or the following *cyp51A* gene substitutions: TR_{34}-L98H (n = 41), G54R (n = 5), TR_{46}-Y121F-T289A (n = 1), or G448S (n = 1). In-house azole-containing agar plates were prepared according to the EUCAST E.Def 10.2 procedure. Conidial suspensions obtained by adding distilled water (Tween 20 0.1%) were either filtered and the inocula adjusted to 0.5 McFarland or left unfiltered and unadjusted. Agreements between the agar screening methods using inocula prepared by each procedure were high for itraconazole (99%), voriconazole (100%), and posaconazole (94.9%). Sensitivity and specificity (considering the susceptibility category as per the microdilution E.Def 9.4 method as the gold standard) of E.Def 10.2 were 100% to rule in or rule out resistance when unfiltered and unadjusted suspensions were used; the resistance phenotype of isolates harboring the TR_{34}-L98H, G54R, or TR_{46}-Y121F-T289A substitutions was correctly detected. Unfiltered and unadjusted conidial suspensions do not negatively influence the performance of the E.Def 10.2 method when screening for azole resistance in *A. fumigatus sensu stricto*.

IMPORTANCE Azole resistance screening in *Aspergillus fumigatus sensu stricto* can be routinely carried out by using azole-containing plates (E.Def 10.2 procedure); however, conidial suspension filtering and inoculum adjustment before inoculation of plates are time-consuming. We, here, showed that unfiltered and unadjusted conidial suspensions do not negatively influence the performance of the E.Def 10.2 method when screening for azole resistance in *A. fumigatus sensu stricto*.

KEYWORDS *Aspergillus fumigatus*, azole-containing plates, azole resistance, EUCAST, detection, E.Def 10.2

Azole resistance in *Aspergillus fumigatus*, an emerging global problem spreading across many countries, is associated with a poor prognosis (1–4). Consequently, screening for azole-resistant isolates should be routinely performed in clinical microbiology laboratories. However, carrying out antifungal susceptibility testing in the

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clinical microbiology laboratory is hindered by the lack of feasible and easy-to-perform procedures. Microdilution-based procedures are the gold standard for conducting antifungal susceptibility testing in the clinical microbiology laboratory; however, they are cumbersome and require expertise. In contrast, azole-containing (itraconazole, voriconazole, or posaconazole) agar plates are non-MIC-based procedures to screen for azole resistance in *A. fumigatus* that can fit into the routine clinical microbiology laboratory. This procedure relies on the ability of azole-resistant isolates to grow on azole-containing agar plates (5–7).

EUCAST has developed a procedure (E.Def 10.2) to prepare in-house azole-containing plates and carry out the whole method in the clinical microbiology laboratory. E.Def 10.2 requires filtering and inoculum adjustment of conidial suspensions before agar plate inoculation, which takes time and increases the laboratory workload (7). We recently reported that using unfiltered conidial suspensions for inoculum adjustment does not negatively influence the performance of the E.Def 10.2 method (8). This study aimed to evaluate whether skipping the inoculum adjustment step also affects the performance of the E.Def 10.2 procedure.

MATERIALS AND METHODS

Isolates studied and antifungal susceptibility testing using the broth microdilution method (E.Def 9.4)

We selected a set of *A. fumigatus sensu stricto* isolates (n = 98) previously classified as azole susceptible or azole resistant. Isolates were either collected in a survey recently conducted in Spain or stored in the Gregorio Marañón Hospital's clinical isolate collection (4). Briefly, antifungal susceptibility of the isolates to itraconazole, voriconazole, or posaconazole (Sigma-Aldrich, Madrid, Spain) was determined following the EUCAST E.Def 9.4 procedure using tissue-treated trays (Cellstar Ref. 655180, Greiner bio-one, Frickenhausen, Germany) (9). Half the isolates were classified as azole resistant (n = 49) according to EUCAST breakpoints (v10). The interpretation of MICs falling within the area of technical uncertainty (ATU) for the three azoles was conducted as follows: itraconazole and voriconazole (MIC = 2 mg/L, always considered resistant), posaconazole (MIC = 0.25 mg/L, considered resistant only if the isolate was also itraconazole resistant). Quality control was ensured by testing *Aspergillus flavus* ATCC 204304 and *A. fumigatus* ATCC 204305. We amplified and sequenced both strands of the *cyp51A* gene, including the promoter region, of those azole-resistant isolates (10). Azole-resistant isolates had either the wild-type *cyp51A* gene sequence (n = 1) or the following *cyp51A* gene substitutions: TR_{34}-L98H (n = 41), G54R (n = 5), TR_{46}-Y121F-T289A (n = 1), or G448S (n = 1). Each isolate showing mutations in the *cyp51A* sequence was fully analyzed twice independently.

Azole-containing agar plate method (E.Def 10.2)

The 98 isolates were retrieved from the freezer to conduct the azole-containing agar evaluation. First, we conducted a pilot study by selecting 6 out of the 98 isolates (three azole-susceptible and three azole-resistant isolates). Conidial suspensions from the six isolates were prepared in 5 mL of Tween 20-containing distilled water by stirring with a wetted cotton swab used to harvest conidia from several colonies. Highly concentrated suspensions were obtained and diluted to 1/10, 1/100, 1/1,000, and 1/10,000 (Fig. 1), and further quantified with a Neubauer Chamber. Table 1 shows the conidial counts of each dilution used. A total of 25 µL of each suspension was inoculated on azole-containing agar plates as such (unfiltered and unadjusted suspensions), which meant to skip the filtration and further adjustment recommended by the E.Def 10.2 procedure. The best performance was achieved when using conidial suspensions diluted to 1/10 and 1/100 as inocula (Table 1). Therefore, conidium suspensions used to conduct further evaluations were prepared by trying to obtain similar visual turbidity to the one found in 1/10 and 1/100 dilutions (Fig. 1).

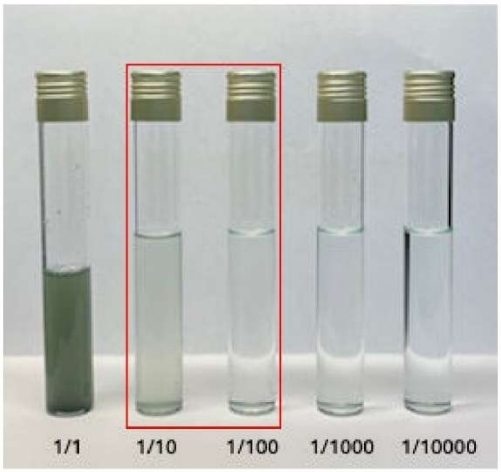

FIG 1 A conidial suspension of isolate 6118 was prepared in distilled water and then serially diluted to 1/10,000. The figure shows the aspect of each dilution of the unfiltered-unadjusted inoculum.

The agar screening method was conducted according to the EUCAST E.Def 10.2 procedure using multi-dish plates (Labclinics tissue-treated plates) (7). The posaconazole concentration in the well was adapted to the updated EUCAST breakpoints (v10) (0.25 mg/L). Conidial suspensions were prepared in distilled water (Tween 20 0.1%), and a volume of 25 µL of inocula was used to inoculate the agar plates, after being vortexed. Two types of inocula were prepared: (i) following the principles of the E.Def 10.2 method [inocula filtered (Merck Millipore, Nylon Net Filter 11.0 µm pore size, Cork, Ireland) and adjusted to 0.5 McFarland, which leads to a conidial count ranging from 2×10^6 CFU/mL to 5×10^6 CFU/mL in the inocula]; (ii) leaving the conidial suspension unfiltered and unadjusted and with a visual turbidity similar to the one found in 1/10 and 1/100 dilutions according to the pilot study (Fig. 1); these suspensions were used to inoculate the azole-containing agar plates and were subsequently quantified with a Neubauer Chamber (Table S1). The time to obtain filtered and adjusted or unfiltered and unadjusted inocula was up to 10 minutes and less than a minute, respectively.

Inoculated plates using either type of inocula from each of the 98 isolates were incubated at 35°C and visually inspected for fungal growth assessment after 48 hours. Isolates were then classified as susceptible (when fungal growth was only observed in the antifungal-free well) or as "potentially" azole resistant (when growth was observed in the antifungal-free well plus one or more azole-containing wells). According to previous studies, the TR_{34}-L98H substitutions can be suspected when the isolate grows on itraconazole-containing and voriconazole-containing agar wells, the G54 mutation can be suspected when the isolate grows on posaconazole-containing and itraconazole-containing wells, and the TR_{46}-Y121F-T289A substitution can be suspected when the isolate grows exclusively on voriconazole-containing agar wells (8, 11).

Quality control was ensured by testing the isolates from the culture collection of the University of Gothenburg, including *A. fumigatus* CCUG 74258/SSI-4524 (TR_{34}-L98H) and *A. fumigatus* CCUG 74259/SSI-5586 (G54W) (12).

Data analysis

The E.Def 9.4 broth microdilution procedure was considered the gold standard. Categorical agreement with the agar screening method was assessed using different conidial suspensions. Errors were defined as very major (false susceptibility) when the microdilution procedure classified an isolate as resistant and the agar screening method as susceptible, and as major (false resistance) when the microdilution procedure categorized an isolate as susceptible and the agar screening method as resistant. Results

TABLE 1 Unfiltered-unadjusted conidial suspensions of each of the six isolates used in the pilot study to assess conidial counts at any of the dilutions studied[a]

Isolate code	*cyp51A* gene substitution	Dilution	Conidia (CFU/mL)	Errors
6118	TR_{34}-L98H	1/1	Uncountable	0
		1/10	15.8×10^6	0
		1/100	1.25×10^6	1 VME (POS)
		1/1,000	0.1×10^6	1 VME (POS)
		1/10,000	0	3 VME (ITC, VOR, POS)
6422	TR_{34}-L98H	1/1	Uncountable	0
		1/10	9.1×10^6	0
		1/100	0.6×10^6	1 VME (POS)
		1/1,000	0	3 VME (ITC, VOR, POS)
		1/10,000	0	3 VME (ITC, VOR, POS)
6429	G54	1/1	Uncountable	0
		1/10	27.5×10^6	0
		1/100	1.3×10^6	0
		1/1,000	0.1×10^6	0
		1/10,000	0	2 VME (ITC, POS)
6110	NA	1/1	Uncountable	2 ME (ITC, VOR)
		1/10	18.4×10^6	0
		1/100	1.8×10^6	0
		1/1,000	0.2×10^6	0
		1/10,000	0	0
6570	NA	1/1	Uncountable	0
		1/10	22.8×10^6	0
		1/100	0.9×10^6	0
		1/1,000	0	0
		1/10,000	0	0
6617	NA	1/1	Uncountable	0
		1/10	8.1×10^6	0
		1/100	0.8×10^6	0
		1/1,000	0	0
		1/10,000	0	0

[a]NA, not applicable (susceptible isolates); VME, very major errors; ME, major errors; ITC, itraconazole; VOR, voriconazole; POS, posaconazole.

obtained using unfiltered-unadjusted and filtered-adjusted conidial suspensions were compared. Finally, the overall performance of agar plates inoculated using conidial suspensions prepared either way was assessed by calculating the sensitivity (probability that a resistant isolate grew in one or more azole-containing wells) and specificity (probability that a susceptible isolate did not grow in any azole-containing well) (12).

RESULTS

The pilot study with the six isolates led to conidial counts for the 1/10 and 1/100 dilutions falling within the expected range when the inoculum was exactly prepared according to the E.Def 10.2 procedure (Table 1). Conidial counts of the unfiltered-unadjusted inocula fell within the range of 1.3×10^6 CFU/mL and 1.5×10^7 CFU/mL; considering the conidial count obtained when exactly following the E.Def 10.2 procedure as a reference (2×10^6 CFU/mL to 5×10^6 CFU/mL), only 1 and 23 isolates yielded conidial counts below or above that range, respectively (Table S1).

The MIC distributions of itraconazole, voriconazole, and posaconazole for the isolates tested are shown in Table 2. Azole resistance screening on agar plates inoculated with unfiltered-unadjusted vs filtered-adjusted suspensions yielded very high categorical agreements for itraconazole (100% vs 99%), voriconazole (100% vs 100%), and posaconazole (96.9% vs 91.8%) (Table 3). Comparisons using unfiltered-unadjusted

TABLE 2 MIC distributions of itraconazole, voriconazole, and posaconazole against the 98 isolates[a]

Antifungal drug	MIC distributions (number of isolates at each MIC, in mg/L)											Resistance	
	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥16	No. of isolates	%
Itraconazole	0	0	0	0	27	21	2	**2**	**0**	**0**	**46**	48	49
Voriconazole	0	0	0	1	13	35	7	**7**	**26**	**5**	**4**	42	42.9
Posaconazole	0	0	17	10	27	**32**	**4**	**0**	**1**	**0**	**7**	48	49

[a]Cells shaded in gray indicate the number of MIC values within the ATU translated as resistant isolates as follows: itraconazole and voriconazole (all isolates in the cell) and posaconazole (*n* = 4). Values in bold indicate resistant isolates (EUCAST Breakpoint table v 10.0).

and filtered-adjusted suspensions for itraconazole, voriconazole, and posaconazole also resulted in very high agreements (99%, 100%, and 94.9%, respectively). Sensitivity and specificity values were 100%, regardless of how conidial suspensions were prepared. All isolates harboring TR_{34}-L98H, G54R, or TR_{46}-Y121F-T289A substitutions were correctly detected after azole resistance phenotype inspection on azole-containing agar plates, regardless of suspension preparation.

Only one very major error was identified with itraconazole-containing agar plates for an isolate with an MIC of 2 mg/L and harboring the G448S mutation. Interestingly, the isolate failed to grow on plates inoculated with filtered-adjusted inocula but grew in agar wells inoculated with unfiltered-unadjusted conidial suspension. No very major errors were found for voriconazole. Finally, a few very major errors were identified in posaconazole-containing agar wells inoculated with unfiltered-unadjusted or filtered-adjusted conidial suspensions, respectively (6.3% and 16.7%). For unfiltered-unadjusted conidial suspensions, very major errors were found in isolates with posaconazole MICs of 0.25 mg/L (*n* = 1) or 0.5 mg/L (*n* = 2), all of which harbored the TR_{34}-L98H substitution. For filtered-adjusted conidial suspensions, very major errors were found in isolates with posaconazole MICs of 0.5 mg/L (*n* = 7), which harbored the TR_{34}-L98H substitution, and an isolate with an MIC of 0.25 mg/L harboring the G448S mutation (Table 4). Three of the isolates harboring TR_{34}-L98H substitutions showed very major errors with posaconazole regardless of the inocula used. No very major errors were found in posaconazole-resistant isolates with an MIC of >0.5 mg/L. In isolates with an MIC of 0.5 mg/L, very major errors were found when conidial suspensions were left unfiltered-unadjusted and filtered-adjusted (*n* = 2/32, 6% and *n* = 6/32, 19%, respectively). Likewise, in isolates with an MIC of 0.25 mg/L, very major errors were found when conidial suspensions were left unfiltered-unadjusted and filtered-adjusted (*n* = 1/4, 25% and *n* = 2/4, 50%, respectively).

No major errors were found, and the lowest number of very major errors was detected when using unfiltered-unadjusted inocula, which was notorious in the case of posaconazole. Errors were not due to the size of conidial counting, since inocula of the single isolate yielding the lowest conidial count and of the isolates yielding the highest conidial counts (*n* = 23) were correctly classified by the azole-agar containing method.

TABLE 3 Categorical agreements between the studied screening method with azole-containing agar plates using unfiltered-unadjusted and filtered-adjusted conidia suspensions and compared to the E.Def 9.4 method

Drug	Conidia suspension preparation	Categorical agreement (%)	Errors		
			Very major (false susceptibility; %: no. of VME/no. of resistant isolates)	Major (false resistance; %: no. of ME/no. of susceptible isolates)	% Errors: no. of errors/no. of total isolates
Itraconazole	Unfiltered-unadjusted	100	0	0	0
(4 mg/L)	Filtered-adjusted	99	2.1% (*n* = 1)	0	1%
Voriconazole	Unfiltered-unadjusted	100	0	0	0
(2 mg/L)	Filtered-adjusted	100	0	0	0
Posaconazole	Unfiltered-unadjusted	96.9	6.3% (*n* = 3)	0	3.1%
(0.25 mg/L)	Filtered-adjusted	91.8	16.7% (*n* = 8)	0	8.2%

TABLE 4 Very major errors (false susceptibility) found in isolates tested using unfiltered-unadjusted or filtered-adjusted conidia inocula[a]

Isolate code	*cyp51A* gene substitutions	E.Def 9.4 visual reading (MIC, mg/L)/E.Def 10.2 unfiltered-unadjusted inoculum/E.Def 10.2 filtered-adjusted inoculum		
		Itraconazole	Voriconazole	Posaconazole
3917	G448S	**R(2)**/R/S	R/R/R	**R(0.25)**/R/S
6407	TR_{34}-L98H	R/R/R	R/R/R	**R(0.5)**/R/S
6422	TR_{34}-L98H	R/R/R	R/R/R	**R(0.25)**/S/S
8636	TR_{34}-L98H	R/R/R	R/R/R	**R(0.5)**/R/S
8682	TR_{34}-L98H	R/R/R	R/R/R	**R(0.5)**/R/S
9098	TR_{34}-L98H	R/R/R	R/R/R	**R(0.5)**/S/S
9244	TR_{34}-L98H	R/R/R	R/R/R	**R(0.5)**/R/S
10007	TR_{34}-L98H	R/R/R	R/R/R	**R(0.5)**/S/S

[a]Bold "R" letters and "S" letters indicate, respectively, resistance or susceptibility according to the microdilution E.Def 9.4 method.

DISCUSSION

The performance of the azole-containing agar E.Def 10.2 method for azole resistance screening in *Aspergillus fumigatus sensu stricto* was not negatively influenced by skipping conidial suspension filtration and further inoculum adjustment.

In light of several recent studies showing increasing rates of azole resistance in *A. fumigatus sensu stricto* isolates and the poor prognosis of affected patients, antifungal susceptibility testing is now recommended in the clinical microbiology laboratory. However, it is rarely conducted following the standard EUCAST microdilution method, thus alternative, feasible methods, such as azole-containing agar plates adapted to the clinical microbiology routine, would be welcome. The E.Def 10.2 azole-containing agar plate screening method is a useful procedure that may help routinely detect azole resistance in *A. fumigatus sensu stricto* in clinical mycology laboratories (5, 6, 11). However, the procedure takes time when it comes to inoculum preparation and its day-to-day implementation slows down the laboratory workload. Inoculum preparation requirements are identical to inoculate microtiter trays following E.Def 9.4 or azole-containing agar wells following E.Def 10.2. However, we hypothesized that the performance of the agar-containing plate procedure might not be hugely impacted by the way inocula are prepared. We have been assessing the performance of E.Def 10.2 when skipping some steps to obtain ready-to-use inocula to alleviate the workload in the clinical microbiology laboratory and letting antifungal susceptibility testing fill in. We recently reported that the type of plastic tray used and that skipping conidial suspension filtration before inoculum adjustment minimally affected the performance of the method (11). Here, we studied whether inoculum adjustment to 0.5 MacFarland may also be skipped. Overall, we observed that the performance of the agar screening method remained unaffected when plates were inoculated with unfiltered-unadjusted conidial suspensions.

We only noted a few discrepancies when unfiltered-unadjusted conidial suspensions were used in posaconazole-containing wells. Furthermore, skipping inoculum adjustment did not lead to errors in the classification of isolates harboring the TR_{34}-L98H substitution (growing in itraconazole-containing and voriconazole-containing agar wells).

We adapted the final posaconazole concentration in the well to fit the updated EUCAST breakpoints (v10) (0.25 mg/L). In previous studies, the number of very major errors in posaconazole-containing agar wells was high (categorical agreement <78%). This occurred with several isolates harboring the TR_{34}-L98H substitution and showing low levels of posaconazole resistance, which were unable to grow on posaconazole-containing plates. This could have been a consequence of the high concentration of the drug in the agar well (0.5 mg/L) since the E.Def 10.1 method was developed adapting drug concentrations to the breakpoints in use at that time (8, 11). Lucio et al. lowered the posaconazole concentration by one twofold dilution (from 0.5 mg/L to 0.25 mg/L)

and obtained good agreements in strains harboring TR_{34}-L98H, TR_{53}, and TR_{46}-Y121F-T289A substitutions (13). Lowering the posaconazole concentration in the agar wells to 0.25 mg/L leads to a reduction in the number of very major errors, particularly in low-level posaconazole-resistant TR_{34}-L98H isolates, without an increase in the number of major errors.

We conclude that skipping the filtration and adjustment of conidial suspensions did not negatively influence the performance of the E.Def 10.2 azole-containing agar plate method when screening for the presence of azole-resistant clinical *A. fumigatus sensu stricto* isolates.

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ETHICS APPROVAL

Given its *in vitro* nature, this study did not require Ethics Committee approval.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Table S1 (JCM00369-24-s0001.docx). Conidial counts found in each of the unfiltered-unadjusted inocula obtained from the 98 isolates used in the study.

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CAPÍTULO III: Evaluación de las tiras de difusión en gradiente para la detección de cepas de *A. fumigatus* resistentes a azoles.

Artículo 6: Gradient diffusion strips for detecting azole resistance in *Aspergillus fumigatus sensu lato*

Las tiras de difusión en gradiente pueden ser una alternativa al método de la microdilución en caldo de EUCAST (E.Def 9.4), aunque la interpretación de las CMIs es controvertida por la ausencia de puntos de corte. Los estudios que comparan ambos procedimientos son escasos, incluyen un bajo número de cepas, y se restringen a agentes antifúngicos seleccionados.

En este artículo, se evaluó el rendimiento de las tiras de difusión en gradiente para detectar resistencia a azoles en *A. fumigatus sensu lato*, incluyendo cepas con mutaciones en el gen *cyp51A* y de especies crípticas.

Se incluyeron 89 cepas de *A. fumigatus sensu stricto* y 52 cepas de especies crípticas, las cuales se clasificaron como sensibles o resistentes a itraconazol, voriconazol, posaconazol e isavuconazol según el método de microdilución EUCAST E.Def 9.4 y los puntos de corte clínicos v10. Las cepas resistentes a azoles de *A. fumigatus sensu stricto* tuvieron las siguientes mutaciones en el gen *cyp51A*: TR_{34}-L98H (n=24), G54R (n=5), TR_{46}-Y121F-T289A (n=1), F46Y-M172V-N248T-D255E-E427K (n=1), F165L (n=1) o el gen *cyp51A* tipo salvaje (n=3). Las CMIs obtenidas con las tiras de difusión en gradiente (ETEST®, Biomerieux, Marcy-l'Etoile, Francia y Liofilchem, Roseto degli Abruzzi, Italia) se obtuvieron siguiendo las pautas del fabricante. Se estudiaron las distribuciones de CMIs obtenidas con las tiras de difusión en gradiente para establecer puntos de corte que separaran las cepas mutantes de los sensibles.

Para *A. fumigatus sensu stricto*, las CMIs de itraconazol >1,5 mg/L, voriconazol >0,38 mg/L, posaconazol >0,75 mg/L e isavuconazol >0,5 mg/L separaron correctamente las cepas resistentes de las sensibles, con dos excepciones: dos cepas resistentes a los azoles con el gen *cyp51A* tipo salvaje. Considerando dichos puntos de corte, los valores de sensibilidad y especificidad para detectar resistencia a azoles fueron de 97% y 100%, respectivamente, para itraconazol, voriconazol y posaconazol y de 93,3% y 100% para

isavuconazol. Para las cepas de especies crípticas, las CMIs de voriconazol >1 mg/L e isavuconazol >0,75 mg/L separaron las cepas resistentes de las sensibles, sin embargo, las excepciones en este caso fueron mucho mayores (voriconazol (n = 15), isavuconazol (n = 27). Considerando dichos puntos de corte, los valores de sensibilidad y especificidad fueron de 68,1% y 100%, respectivamente, para voriconazol y de 25% y 100%, respectivamente, para isavuconazol. Para itraconazol y posaconazol no fue posible establecer puntos de corte.

En conclusión, se logró establecer puntos de corte preliminares para detectar correctamente cepas resistentes de *A. fumigatus sensu stricto* utilizando las tiras de difusión en gradiente. Sin embargo, el rendimiento frente a las especies crípticas fue deficiente.

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ORIGINAL ARTICLE

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Gradient diffusion strips for detecting azole resistance in *Aspergillus fumigatus* sensu lato

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Abstract

Background: Studies comparing gradient diffusion strips (GDSs) and the EUCAST E.Def 9.4 microdilution method are scarce, thwarted by a low number of isolates, and restricted to selected antifungal agents.

Objectives: We evaluated the performance of GDSs to detect azole resistance in *A. fumigatus*, including cryptic species.

Patients/Methods: *A. fumigatus* sensu stricto ($n = 89$) and cryptic species ($n = 52$) were classified as susceptible or resistant to itraconazole, voriconazole, posaconazole and isavuconazole (EUCAST E.Def 9.4; clinical breakpoints v10). *A. fumigatus* sensu stricto azole-resistant isolates had the following *cyp51A* gene mutations: TR_{34}-L98H ($n = 24$), G54R ($n = 5$), TR_{46}-Y121F-T289A ($n = 1$), F46Y-M172V-N248T-D255E-E427K ($n = 1$), F165L ($n = 1$) and *cyp51A* gene wild type ($n = 3$). GDSs (ETEST®, bioMèrieux, Marcy-l'Etoile, France and Liofilchem®, Roseto degli Abruzzi, Italy) MICs were obtained by following the manufacturer's guidelines.

Results: For *A. fumigatus* sensu stricto, itraconazole MICs >1.5 mg/L, voriconazole >0.38 mg/L, posaconazole >0.75 mg/L, and isavuconazole >0.5 mg/L correctly separated resistant from susceptible isolates with two exceptions. Considering the aforementioned cut-off MICs, sensitivity/specificity values of GDSs to detect azole resistance were: itraconazole (97%/100%), voriconazole (97%/100%), posaconazole (97%/100%) and isavuconazole (93.3%/100%). For cryptic species isolates, voriconazole MICs >1 mg/L and isavuconazole >0.75 mg/L separated resistant isolates from susceptible isolates with 15 and 27 exceptions, respectively. Considering the aforementioned cut-off MICs, sensitivity/specificity values were as follows: voriconazole (68.1%/100%) and isavuconazole (25%/100%). For itraconazole and posaconazole, it was not possible to establish cut-off values.

Conclusions: We set tentative cut-off MIC values to correctly spot resistant *Aspergillus fumigatus* sensu stricto isolates using GDSs. The performance against cryptic species was poor.

KEYWORDS
Aspergillus fumigatus, azole resistance, EUCAST, gradient diffusion strips

1 | INTRODUCTION

The spreading of azole resistant *Aspergillus fumigatus*—associated to a poor prognosis—, requires routine screening.[1–4] EUCAST E.Def 9.4 method assesses the minimum inhibitory concentration (MIC) of antifungals against *A. fumigatus*, but it is time-consuming and requires expertise. Gradient diffusion strips (GDSs) may be an alternative, albeit interpretation of MICs remains controversial. The E.Def 9.4 method is needed as gold standard to study the potential of GDSs to detect resistance. Studies comparing both procedures are scarce, thwarted by a low number of isolates, and restricted to selected antifungal agents.[5–6]

We assessed the role of GDSs to detect azole resistance in *A. fumigatus* sensu stricto and cryptic species isolates.

2 | MATERIALS AND METHODS

2.1 | Isolates

We studied *A. fumigatus* sensu lato isolates (n = 141) comprising *A. fumigatus* sensu stricto (azole-resistant isolates [n = 35] and azole-susceptible isolates [n = 54]) and cryptic species (azole-resistant isolates [n = 47] and azole-susceptible isolates [n = 5]) collected from a survey recently conducted in Spain and from patients cared at Gregorio Marañón hospital.[4] Cryptic species and azole-resistant *A. fumigatus* sensu stricto isolates were molecularly identified.[4,7] Azole-resistant *A. fumigatus* sensu stricto had the following *cyp51A* gene substitutions: TR_{34}-L98H (n = 24), G54R (n = 5), TR_{46}-Y121F-T289A (n = 1), F46Y-M172V-N248T-D255E-E427K (n = 1), F165L (n = 1) or the wild type *cyp51A* gene (n = 3). Cryptic species isolates involved *A. lentulus* (n = 13), *A. novofumigatus* (n = 8), *Neosartorya fischeri* (n = 8), *A. fumigatiaffinis* (n = 7), *N. udagawae* (n = 7), *N. tsurutae* (n = 3), *A. viridinutans* (n = 2), *A. felis* (n = 2), *N. hiratsukae* (n = 1) and *A. thermomutatus* (n = 1).

2.2 | Antifungal susceptibility

MICs of itraconazole, voriconazole, posaconazole (Sigma-Aldrich) and isavuconazole (Basilea Pharmaceutica International Ltd.) were determined following EUCAST E.Def 9.4 method; trays (tissue-treated trays, CELLSTAR® Ref. 655,180, Greiner bio-one, Frickenhausen, Germany) were incubated for 48 hours at 35°C without shaking.[8]

Gradient diffusion strips (GDSs) were used to determine antifungal susceptibility to itraconazole, voriconazole, posaconazole (ETEST®; bioMèrieux) and isavuconazole (Liofilchem®). Conidial

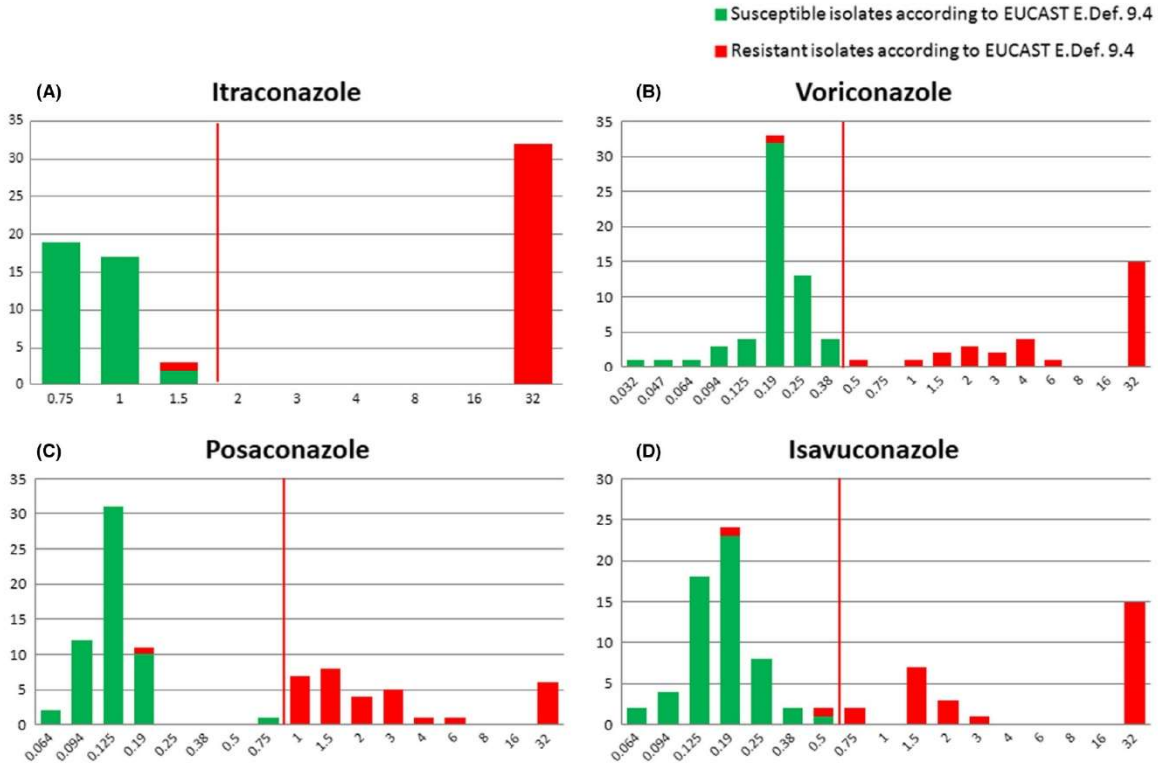

FIGURE 1 MIC distributions of itraconazole, voriconazole, posaconazole and isavuconazole obtained by GDSs against *Aspergillus fumigatus* sensu stricto isolates. Red bar and green bar sections indicate resistant isolates and susceptible isolates, respectively, to the drug in question according to EUCAST's methodology and clinical breakpoints v.10. Red lines indicate the here proposed GDSs MIC cut-off values

suspensions were prepared in distilled water (Tween 20 0.1%) and filtered (Merck Millipore®, Nylon Net Filter 11.0 µm pore size, Cork, Ireland) prior inoculum adjustment to 0.5 McFarland. A dry swab was soaked into the inoculum and the agar RPMI plate was then streaked using the swabs and incubated for 48 hours at 35°C. MICs were obtained following the manufacturer's guidelines and defined as the drug concentration at which the border of the elliptical growth inhibition zone intercepted the scale on the antifungal strip.

2.3 | Data analysis

MICs obtained following the E.Def 9.4 method were considered the gold standard; isolates were classified as azole-susceptible or azole-resistant as per EUCAST clinical breakpoints.[9] MIC distributions of the four azoles obtained by GDSs were studied to set cut-off values able to distinguish resistant from susceptible isolates. All categorical discrepancies between MICs by E.Def 9.4 and GDSs were retested. Using the aforementioned cut-off values, sensitivity was defined as the probability that a resistant isolate had a MIC higher that that value and specificity as the probability that a susceptible isolate had an MIC lower than that value.

2.4 | Ethics Statement

Given the in vitro nature of this study, approval of the Ethics Committee was not required.

3 | RESULTS

Figures 1 and 2 summarise GDS MIC distributions of the four azoles tested against *A. fumigatus* sensu stricto and cryptic species, respectively.

All *A. fumigatus* sensu stricto isolates showing GDS MIC values of itraconazole >1.5 mg/L, voriconazole >0.38 mg/L, posaconazole >0.75 mg/L and isavuconazole >0.5 mg/L were identified as resistant to the azole in question using E.Def 9.4. Using GDS MIC cut-off values, two azole-resistant *cyp51A*-wild-type gene isolates were misclassified as isavuconazole-susceptible, one isolate was also misclassified as voriconazole-susceptible and one was also misclassified as itraconazole- and posaconazole-susceptible (Table 1). GDS sensitivity and specificity values to detect azole resistance considering the aforementioned MIC cut-off values were as follows: itraconazole (97%/100%), voriconazole (97%/100%), posaconazole (97%/100%) and isavuconazole (93.3%/100%). Isolates harbouring the G54,

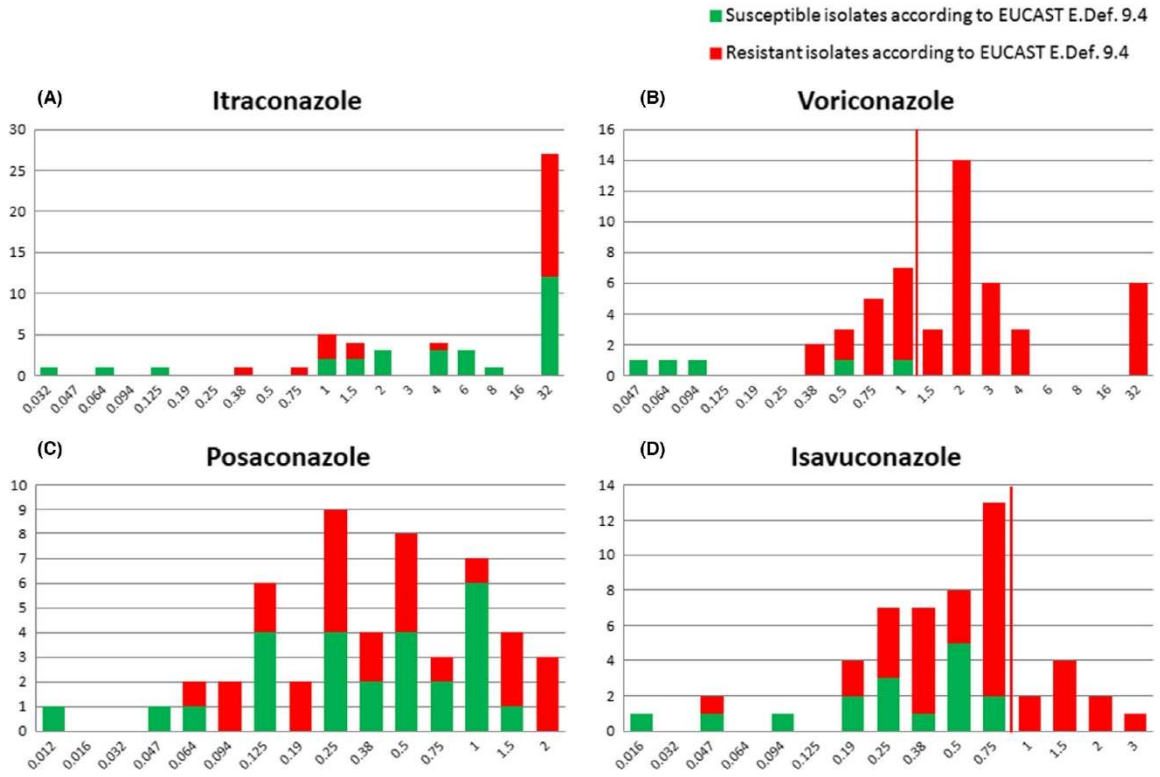

FIGURE 2 MIC distributions of itraconazole, voriconazole, posaconazole and isavuconazole obtained by GDSs against *Aspergillus fumigatus* cryptic species isolates. Red bar and green bar sections indicate resistant isolates and susceptible isolates, respectively, to the drug in question according to EUCAST's methodology and clinical breakpoints v.10. Red lines indicate the here proposed GDSs MIC cut-off values

TABLE 1 *Aspergillus fumigatus* sensu stricto isolates falsely classified as susceptible by GDSs according to the proposed cut-off; both isolates resulted in wild type *cyp51A* gene sequences

	E.Def 9.4 method/GDS (MIC, in mg/L)			
Isolate code	Itraconazole	Voriconazole	Posaconazole	Isavuconazole
6545	S(0.25)/0.38	**R(4)**/0.19	S(0.125)/0.094	**R(8)**/0.19
6567	**R(16)**/1.5	**R(2)/0.5**	**R(0.5)**/0.19	**R(4)**/0.5

Note: Numbers in bold indicate a phenotype of resistance as per the EUCAST methodology.
Abbreviations: R, resistant; S, susceptible.

TABLE 2 Cryptic species isolates falsely classified as susceptible to voriconazole or to isavuconazole by the GDSs according to the proposed cut-off

		E.Def 9.4 method/GDS (MIC, in mg/L)	
Isolate code	Cryptic species	Voriconazole	Isavuconazole
2415	*Aspergillus viridinutans*	**R(2)**/0.38	S(0.5)/0.25
5744	*Neosartorya fischeri*	**R(2)**/0.38	**R(2)**/0.19
5888	*Aspergillus lentulus*	**R(16)**/0.5	**R(2)**/0.25
6789	*Aspergillus novofumigatus*	**R(4)**/0.5	**R(2)**/0.38
6009	*Aspergillus fumigatiaffinis*	**R(4)**/0.75	**R(2)**/0.047
6044	*Neosartorya udagawae*	**R(2)**/0.75	**R(2)**/0.75
6062	*A. fumigatiaffinis*	**R(4)**/0.75	**R(4)**/0.5
6659	*N. udagawae*	**R(4)**/0.75	**R(2)**/0.25
6778	*Neosartorya tsurutae*	**R(2)**/0.75	S (1)/0.19
4089	*N. udagawae*	**R(2)**/1	S (1)/0.5
5514	*A. lentulus*	**R(2)**/1	S (1)/0.38
5953	*A. lentulus*	**R(2)**/1	**R(2)**/0.19
6112	*A. lentulus*	**R(4)**/1	**R(2)**/0.38
6770	*A. novofumigatus*	**R(4)**/1	**R(2)**/0.75
6325–2	*A. fumigatiaffinis*	**R(8)**/1	**R(4)**/0.25
6183	*A. lentulus*	**R(4)/1.5**	**R(2)**/0.38
6113	*A. lentulus*	**R(2)/2**	**R(2)**/0.25
6195–2	*A. lentulus*	**R(8)/2**	**R(2)**/0.38
5869	*A. lentulus*	**R(2)/2**	**R(2)**/0.5
2836	*N. fischeri*	**R(2)/2**	**R(2)**/0.75
2838	*N. fischeri*	**R(2)/2**	**R(2)**/0.75
2839	*N. fischeri*	**R(2)/2**	**R(2)**/0.75
2840	*N. fischeri*	**R(2)/2**	**R(2)**/0.75
2842	*N. fischeri*	**R(2)/2**	**R(2)**/0.75
3055	*N. fischeri*	**R(2)/2**	**R(2)**/0.75
6683	*A. lentulus*	**R(4)/2**	**R(2)**/0.75
5629	*A. lentulus*	**R(2)/2**	**R(2)**/0.38
5465	*N. fischeri*	**R(2)/3**	**R(2)**/0.75
6172	*A. lentulus*	**R(8)/3**	**R(8)**/0.75
6326–2	*A. fumigatiaffinis*	**R(8)/4**	**R(4)**/0.38
5357	*A. fumigatiaffinis*	**R(2)/4**	**R(2)**/0.5

Note: Numbers in bold indicate a phenotype of resistance as per the EUCAST methodology or by using gradient diffusion strips to define MIC cut-off. Cells in grey indicate cases in which false susceptibility was detected using gradient diffusion strips.
Abbreviations: R, resistant; S, susceptible.

TR34-L98H or TR46-Y121F-T289A *cyp51A* gene substitutions were correctly detected.

Cryptic species isolates showing voriconazole or isavuconazole MIC values >1 mg/L or >0.75 mg/L, respectively, were identified as resistant to the azole in question as per the E.Def 9.4 method. It was not possible to establish cut-off values for itraconazole and posaconazole due to extremely irregular MIC distributions. GDS MIC cut-off values >1 mg/L for voriconazole or >0.75 mg/L for isavuconazole

led to false susceptibility in 15 isolates and 27 isolates, respectively (Table 2). Considering the aforementioned cut-off MICs, GDS sensitivity and specificity to detect azole resistance were 68.1%/100% for voriconazole and 25%/100% for isavuconazole.

4 | DISCUSSION

In most cases, our proposed GDS cut-off MIC values distinguish resistant *A. fumigatus* sensu stricto isolates. Sensitivity and specificity values for the four azoles were >93%/100%. Performance against cryptic species was rather poor.

A. fumigatus resistance is a problem worldwide and conducting early antifungal susceptibility testing is essential.[1,4,10] Previous studies comparing E.Def 9.4 with GDSs, including wild type and *cyp51A* mutant *A. fumigatus* isolates (TR_{34}-L98H, TR_{46}-Y121F-T289A, M220 and G54), reported high categorical agreements, although limited by the low number of isolates, the selected antifungal agents, and absence of cryptic species.[5,6]

Espinel-Ingroff, using the Clinical and Laboratory Standards Institute gold standard method, proposed epidemiological cut-off values against *A. fumigatus* sensu stricto for itraconazole (2 mg/L), voriconazole (0.5 mg/L) and posaconazole (0.25 mg/L).[11–13] Our cut-off values are similar and led to errors in only two azole-resistant *cyp51A*-wild type gene isolates. To the best of our knowledge, this is the first time that cryptic species have been tested. We can only propose cut-off MIC values for voriconazole (>1 mg/L) and isavuconazole (>0.75 mg/L) and found a number of isolates resulting in false susceptibility to those drugs. The mediocre performance with cryptic species may be due to poor sporulation to irregular conidia inoculums.

This work is limited by the low number of isolates harbouring the TR_{46}-Y121F-T289A substitutions alongside to other *cyp51A* gene mutations.

In conclusion, we propose GDS MIC cut-off values for itraconazole, voriconazole, posaconazole and isavuconazole against *A. fumigatus* sensu stricto isolates. Further studies including more isolates and a wider range of mutations are warranted.

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CONFLICT OF INTEREST

All authors report no conflicts of interest relevant to this work.

DATA AVAILABILITY STATEMENT

Data will be available upon request to the corresponding author.

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Artículo 7: Effective Detection of Azole Resistance in *Aspergillus fumigatus sensu stricto* Using a Gradient Diffusion Plastic Strip: A Comparison of Filtered adjusted *vs*. Unfiltered unadjusted Inocula

Manuscrito en prensa

En este estudio se evaluó si omitir los pasos de filtración y ajuste del inóculo influía negativamente en el rendimiento de las tiras de difusión en gradiente para la detección de resistencia a azoles en *A. fumigatus.*

Se incluyeron 98 cepas de *A. fumigatus sensu stricto*, procedentes de estudios nacionales sobre resistencia a azoles en España, previamente clasificadas como sensibles o resistentes a itraconazol, voriconazol y posaconazol según el método de microdilución E.Def 9.4 de EUCAST y los puntos de corte clínicos v10. Las cepas resistentes tuvieron las siguientes mutaciones en el gen *cyp51A*: TR_{34}-L98H±S297T-F495I (n=41), G54R (n=5), TR_{46}-Y121F-T289A (n=1), G448S (n=1), y el gen *cyp51A* tipo salvaje (n=1). Las suspensiones de conidias se prepararon siguiendo los principios del método E.Def 9.4, o se dejaron sin filtrar o ajustar. Se evaluaron las distribuciones de CMIs de itraconazol, voriconazol y posaconazol obtenidas con las tiras de difusión en gradiente, y los valores de concordancia obtenidos utilizando ambos tipos de suspensiones.

De acuerdo con las distribuciones de CMI obtenidas con las tiras de difusión en gradiente y utilizando ambos tipos de preparaciones de inóculo, se propusieron los siguientes puntos de corte para clasificar las cepas como resistentes: >1 mg/L para itraconazol, >0,5 mg/L para voriconazol y >0,25 mg/L para posaconazol. Los puntos de corte de itraconazol y voriconazol propuestos en este estudio son similares a los comunicados en el anterior estudio. Sin embargo, el punto de corte de posaconazol propuesto en este estudio fue más bajo, lo que puede deberse al mayor número de cepas resistentes que mostraron CMIs de posaconazol más bajas (0,25 mg/L, 0,38 mg/L y 0,5 mg/L).

Utilizando estos puntos de corte (>1 mg/L para itraconazol, >0,5 mg/L para voriconazol y >0,25 mg/L para posaconazol), todas las cepas se clasificaron correctamente salvo dos (G448S y TR_{46}-Y121F-T289A) en los que se produjeron clasificaciones erróneas al menos para un azol. Se observaron elevados valores de

concordancia esencial entre los métodos, independientemente de la forma de preparación de los inóculos: 95,9% para itraconazol y 98% para voriconazol y posaconazol. En general, los valores de sensibilidad y especificidad de las tiras de difusión en gradiente, utilizando los puntos de corte propuestos para detectar la resistencia a los azoles, apenas se vieron afectados por la preparación del inóculo, siendo estos superiores al 98% en todos los casos.

En conclusión, omitir la filtración y el ajuste del inóculo de las suspensiones de conidias no influyó negativamente en el rendimiento de las tiras de difusión en gradiente. Este es un método útil para el cribado de la resistencia a azoles en cepas de *A. fumigatus sensu stricto* cuando las CMIs obtenidas se interpretan de la siguiente forma: itraconazol >1 mg/L, voriconazol >0,5 mg/L y posaconazol >0,25 mg/L.

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Abstract

Gradient diffusion plastic strips are utilized for azole susceptibility testing against *Aspergillus fumigatus* in some clinical microbiology laboratories, however, they lack proper validation.

We evaluated the performance of this method for detecting azole resistance in *A. fumigatus sensu stricto* and we assessed whether skipping filtration and inoculum adjustment of conidial suspensions negatively influenced its performance.

A total of 98 *A. fumigatus sensu stricto* isolates, previously classified as azole-susceptible or resistant, were studied. Azole-resistant isolates had specific *cyp51A* gene mutations, including TR_{34}-L98H±S297T-F495I, G54R, TR_{46}-Y121F-T289A, and G448S. Conidial suspensions were prepared following the principles of the E.Def 9.4 method or left unfiltered and unadjusted.

Based on minimum inhibitory concentration (MIC) distributions obtained with the gradient diffusion plastic strip using both types of inoculum preparations, cut-off values were proposed to classify isolates as resistant: >1 mg/L for itraconazole and voriconazole and >0.25 mg/L for posaconazole. These cut-offs coincided with EUCAST breakpoints and correctly classified all except one (TR_{46}-Y121F-T289A) isolate. High essential agreement values (95.9%–98%) were obtained, regardless of inoculum preparation.

Overall, sensitivity/specificity values of the gradient diffusion plastic strip using the proposed cut-off values for itraconazole (100%/98%), voriconazole (100%/100%), and posaconazole (100%/98%) to screen for azole resistance were not impacted by the inoculum preparation method.

Thus, the gradient diffusion plastic strip is a reliable method for screening azole resistance in *A. fumigatus* isolates using the proposed MIC cut-offs and inoculum preparation without filtration and adjustment of conidial suspensions did not negatively influence the performance of the method.

Lay summary

Gradient diffusion plastic strip is a reliable method for screening for azole resistance in *A. fumigatus sensu stricto* isolates and skipping filtration and adjustment of conidial suspensions did not negatively influence the performance of the method.

Introduction

Azole-resistant aspergillosis is a matter of concern; mortality in patients who received initial triazole treatment doubles that of cases with triazole-susceptible aspergillosis. In light of the increasing resistance across many countries and the poor prognosis of the patients affected, screening for azole resistance in *Aspergillus fumigatus* should be available in clinical microbiology laboratories, especially when isolates represent invasive, recurrent, or non-responsive infections, and particularly in areas where azole-resistant *A. fumigatus* is prevalent.[1-5]

The microdilution reference EUCAST E.Def 9.4 method affords specific guidance to assess the minimum inhibitory concentration (MIC) of antifungals against *A. fumigatus,* but it is cumbersome, time-consuming, and requires expertise.[6] Methods based on agar drug diffusion, such as the azole-containing agar plate EUCAST E.Def 10.2 method, offer alternatives to microdilution in clinical microbiology laboratories. However, gradient diffusion strips have become popular, being an easy and simple method to apply in hospital clinical microbiology laboratories, albeit the MIC interpretation remains controversial due to the lack of proper validation and method-specific MIC cut-off values.

Plastic strip manufacturer's guidelines recommend allowing the inoculum particles to settle for 15 minutes and adjusting to 0.5 McFarland. By following such standards, we previously proposed MIC cut-off values to detect azole-resistant *A. fumigatus sensu stricto* isolates to itraconazole (>1.5 mg/L), voriconazole (>0.38 mg/L), and posaconazole (>0.75 mg/L).[7] Nevertheless, the preparation of filtered and adjusted inocula for agar diffusion methods may take time. We previously showed that unfiltered and unadjusted conidial suspensions do not negatively influence the performance of the azole-containing agar plate EUCAST method (E.Def 10.2) when screening for azole resistance in *A. fumigatus sensu stricto*.[8] Likewise, we here assessed the performance of gradient diffusion strips for detecting azole resistance against *A. fumigatus sensu stricto* isolates and the effect of skipping filtration and further inoculum adjustment of conidial suspensions.

Materials and methods

Isolates studied. We selected a set of *A. fumigatus sensu stricto* isolates (n=98), collected in Spanish hospitals or stored in the Gregorio Marañón hospital clinical isolate collection, that were classified as azole-susceptible or azole-resistant (n=49) according to the EUCAST microdilution method, as previously reported elsewhere.[8] Azole-resistant isolates had either the wild-type *cyp51A* gene sequence (n = 1) or the following *cyp51A* gene substitutions: TR_{34}-L98H±S297T-F495I (n = 41), G54R (n = 5), TR_{46}-Y121F-T289A (n = 1), or G448S (n=1). MICs and isolate classification as per susceptibility category following the microdilution procedure were chosen as gold standards.

Performance assessment of gradient diffusion plastic strips using different conidial inocula. Gradient diffusion plastic strips (ETEST®, Biomerieux, Marcy-l'Etoile, France) were used to determine antifungal susceptibility to itraconazole, voriconazole, and posaconazole. The isolates were retrieved from the freezer and subcultured on Sabouraud dextrose agar to conduct the gradient diffusion plastic strip evaluation. Conidial suspensions were prepared in distilled water (Tween 20 0.1%) and dry swabs were soaked in the inoculum and the RPMI 1640 agar plates supplemented with 2% glucose (bioMérieux, Marcy-l'Etoile, France) were then streaked. Inocula were prepared by: a) following the principles of the E.Def 9.4 method (inocula filtered [Merck Millipore®, Nylon Net Filter 11.0 µm pore size, Cork, Ireland] and adjusted to 0.5 McFarland); or b) leaving the conidial suspension unfiltered and unadjusted and with a visual turbidity similar to that seen in 1/10 and 1/100 dilutions; we previously demonstrated that highly concentrated conidial suspensions from isolates that were diluted as mentioned, worked well as inocula.[8] Plates were then incubated at 35ºC for 48 hours and MICs were obtained following the manufacturer's guidelines and defined as the drug concentration at which the border of the elliptical growth inhibition zone intercepted the scale on the antifungal strip.

The E.Def 9.4 broth microdilution procedure was considered the gold standard. MIC distributions of the three azoles obtained using gradient diffusion plastic strips were studied to establish cut-off values able to distinguish resistant from susceptible isolates. Using the EUCAST breakpoints v 11.0 (https://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals), sensitivity was defined as the probability that a resistant isolate had a MIC higher than that value, and specificity as the probability that a susceptible isolate had a MIC below that value. The essential agreement of the gradient diffusion plastic strips method was evaluated between the use of unfiltered-unadjusted and filtered-adjusted conidial

suspensions; MICs within ±1 two-fold dilution were considered to be in essential agreement. Categorical agreement between the diffusion plastic strip method using filtered-adjusted and unfiltered-unadjusted conidia suspensions and the E.Def 9.4 method was assessed. Errors were defined as very major (false susceptibility) when the microdilution procedure classified an isolate as resistant and the diffusion plastic strip method as susceptible, and as major (false resistance) when the microdilution procedure categorized an isolate as susceptible and the diffusion plastic strip method as resistant. Discrepancies were retested and the results were confirmed.

Quality control was ensured by testing resistant isolates from the culture collection of the University of Gothenburg: *A. fumigatus* CCUG 74258/SSI-4524 (TR_{34}-L98H) and *A. fumigatus* CCUG 74259/SSI-5586 (G54W).[13]

Ethics Statement. Given its *in vitro* nature, this study did not require Ethics Committee approval.

Results

Given the gradient diffusion plastic strip-obtained MIC distributions with both kinds of inoculum preparations, the following cut-off values were proposed: >1 mg/L for itraconazole and voriconazole, and >0.25 mg/L for posaconazole (Figure 1).

By using these cut-off values, which coincided with the EUCAST breakpoints, all isolates were classified correctly except for one isolate harbouring TR_{46}-Y121F-T289A *cyp51A* gene substitutions, which was misclassified as resistant to itraconazole and posaconazole (EUCAST MIC of 0.5 mg/L and 0.25 mg/L, respectively) independently of the inocula preparation method chosen (Table 1). Considering the EUCAST cut-off values, sensitivity/specificity values of gradient diffusion plastic strips for itraconazole (100%/98%), voriconazole (100%/100%), and posaconazole (100%/98%) to screen for azole resistance, values were unaffected by the inoculum preparation method (Table 2).

High essential agreement values were seen between MICs obtained by plate inoculation using unfiltered-unadjusted and filtered-adjusted conidial suspensions: 95.9% for itraconazole and 98% for voriconazole and posaconazole. Likewise, high categorical agreement values were seen between the gradient diffusion plastic strip and E.Def 9.4 methods for the three azoles (≥98%), and only 2% of major errors were found for itraconazole and posaconazole. One major error was found exclusively in an isolate harbouring the TR_{46}-Y121F-T289A *cyp51A* gene substitution. No very major errors were found (Table 2).

Discussion

The performance of the gradient diffusion plastic strip method for detecting azole resistance in *A. fumigatus sensu stricto* was not compromised by the omission of conidial suspension filtration and subsequent inoculum adjustment. By using the cut-off here proposed, which coincided with EUCAST breakpoint values, this procedure is a good tool for detecting azole resistance in *A. fumigatus sensu stricto*.

Given the numerous studies demonstrating the increasing rates of azole resistance in *A. fumigatus sensu stricto* isolates and the grim outcome for affected patients, performing antifungal susceptibility testing in clinical microbiology laboratories is now recommended.[1, 4] However, the microdilution gold standard EUCAST E.Def 9.4 procedure, which is technically challenging and requires expertise, is seldom performed. Alternative and more feasible methods, such as the gradient diffusion plastic strip method, might offer a more convenient approach for conducting antifungal susceptibility testing against *A. fumigatus*, although the interpretation of MIC values remains controversial.

Previous studies compared the EUCAST broth microdilution method and gradient diffusion plastic strips, including wild-type and *cyp51A* mutant *A. fumigatus* isolates (TR_{34}-L98H, TR_{46}-Y121F-T289A, M220, and G54), reporting high categorical agreements, although constrained by small sample sizes and a limited range of antifungal agents.[9, 10] Espinel-Ingroff, using the CLSI method as the gold standard, proposed epidemiological cut-off values against *A. fumigatus sensu stricto* for itraconazole (>1 mg/L), voriconazole (>0.25 mg/L), and posaconazole (>0.125 mg/L).[11-13]

We had previously evaluated the performance of gradient diffusion plastic strips for detecting azole resistance in *A. fumigatus sensu lato*, including *cyp51A* gene mutant isolates and cryptic species, proposing MIC cut-off values for *A. fumigatus sensu stricto*: itraconazole >1.5 mg/L, voriconazole >0.38 mg/L, and posaconazole >0.75 mg/L.[7] At that time, inoculum preparation implied filtering and adjusting according to the E.Def 9.4 procedure, and two very major errors were found. Among cryptic species, we observed that poor performance may be due to the poor sporulation of isolates, which could lead to irregular conidia inoculums. Therefore, we did not include cryptic species in the current evaluation. The cut-offs put forward herein are slightly different from those previously proposed.[7] The itraconazole cut-off value is similar to that previously reported; however, those for voriconazole and posaconazole are different, probably due to the higher number of resistant isolates here tested. The sensitivity using the previously reported cut-off values was 97% for each drug; however, applying the current cut-off values would have

resulted in sensitivity values ranging from 90% (for voriconazole) and 100% (for itraconazole) in the isolates previously tested.[7] Nevertheless, the sensitivity values reported herein were 100% regardless of inoculum preparation. The differences in performance of the current and previous cut-offs may be due to the fact the isolates tested here and in the previous study were not exactly the same.

By following the here reported cut-off values, we only found a major error in one azole-resistant isolate harbouring TR_{46}-Y121F-T289A *cyp51A* gene substitutions. This isolate was misclassified as itraconazole resistant (EUCAST MIC of 0.5 mg/L), with a gradient diffusion plastic strip MIC of 1.5 mg/L and 2 mg/L, in plates inoculated with filtered-adjusted and unfiltered-unadjusted conidial suspension, respectively; this isolate was also misclassified as resistant to posaconazole (EUCAST MIC of 0.25 mg/L, classified as ATU), with a gradient diffusion plastic strip MIC of 0.75 mg/L, independently the method chosen to prepare the inoculum.

Inoculum preparation requirements to inoculate gradient diffusion plastic strip plates with filtered and adjusted inoculums are identical to those used to inoculate microtiter trays following E.Def 9.4. We here demonstrated that the performance of the gradient diffusion plastic strip method was hardly impacted by how the inoculum was prepared.

Future studies using a higher number of isolates harbouring TR_{46}-Y121F-T289A substitutions alongside other *cyp51A* gene mutations, and also external and multicentre studies, are warranted to validate the current observations.

We conclude that the gradient diffusion plastic strip is a method for screening for azole resistance in *A. fumigatus sensu stricto* isolates when MICs obtained are interpreted using cut-off values of >1 mg/L for itraconazole and voriconazole and >0.25 mg/L for posaconazole. Skipping filtration and adjustment of conidial suspensions did not negatively influence the performance of the method.

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TABLES AND FIGURES

Table 1. *A. fumigatus sensu stricto* isolates misclassified by gradient diffusion plastic strips according to the proposed cut-off

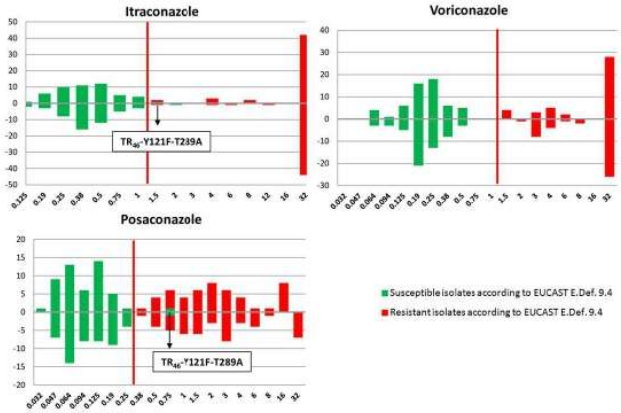
Isolate code	*cyp51A* gene substitutions	E.Def 9.4 method/gradient diffusion plastic strip with filtered and adjusted inocula/ gradient diffusion plastic strip with unfiltered and unadjusted inocula (MIC, in mg/L)		
		Itraconazole	Voriconazole	Posaconazole
6544	TR_{46}-Y121F-T289A	**S(0.5)**/**1.5**/**2**	**R(16)**/32/32	**S(0.25)**/**0.75**/**0.75**

Bold "R" letters in red and "S" letters in green indicate resistance or susceptibility, respectively, according to the microdilution E.Def 9.4 method. MIC, minimum inhibitory concentration.

Table 2. Essential and categorical agreement between the diffusion plastic strip method using filtered-adjusted and unfiltered-unadjusted conidia suspensions and the E.Def 9.4 method. Sensitivity and specificity values of the methods are also shown

Drug	Conidia suspension preparation	Categorical agreement	Very major errors	Major errors	Essential agreement	Sensitivity	Specificity
Itraconazole	Filtered-adjusted	99%	0	2% (n=1)	95.9%	100%	98%
	Unfiltered-unadjusted	99%	0	2% (n=1)		100%	98%
Voriconazole	Filtered-adjusted	100%	0	0	98%	100%	100%
	Unfiltered-unadjusted	100%	0	0		100%	100%
Posaconazole	Filtered-adjusted	99%	0	2% (n=1)	98%	100%	98%
	Unfiltered-unadjusted	99%	0	2% (n=1)		100%	98%

Figure 1. Gradient diffusion plastic strip-obtained MIC distributions against the isolates obtained by using filtered-adjusted (bars above the X-axis) and unfiltered-unadjusted conidial inocula



5. DISCUSIÓN

A. fumigatus ha sido considerado un patógeno de prioridad crítica, dada su ubicuidad, su capacidad de producir enfermedades invasivas, y los niveles crecientes de resistencia a los azoles. De hecho, la Organización Mundial de la Salud lo incluyó dentro de la lista de patógenos fúngicos prioritarios (WHO FPPL) en 2022. La resistencia a azoles, fármacos de elección para el tratamiento de las infecciones causadas por estos hongos, ha aumentado considerablemente a nivel mundial (van der Linden et al., 2015; Snelders et al., 2008; Lestrade et al., 2020).

Las pruebas de sensibilidad antifúngica en *A. fumigatus* han adquirido relevancia no sólo como consecuencia del aumento del número de cepas resistentes, sino también debido a que las tasas de mortalidad en pacientes infectados por cepas resistentes a los azoles son significativamente superiores a las de los sujetos infectados por cepas sensibles (van der Linden et al., 2015). La resistencia a los azoles en *A. fumigatus* puede aparecer durante la terapia o por la exposición a fungicidas azólicos en el medio ambiente (Anderson et al., 2005). Además, las especies crípticas del complejo pueden mostrar resistencia intrínseca a la anfotericina B y a los azoles (Perlin et al., 2017). Las implicaciones de la resistencia ambiental a los azoles en la elección del tratamiento antifúngico primario dependen de la epidemiología local de la resistencia, ya que una elevada prevalencia en el medio ambiente determinará la probabilidad de que un paciente presente una aspergilosis invasiva causada por una cepa resistente.

En este escenario, son fundamentales las estrategias que permitan la detección precoz de la resistencia a los azoles en las cepas clínicas de *A. fumigatus* en los laboratorios de Microbiología Clínica. Actualmente se recomienda realizar pruebas de sensibilidad, especialmente en regiones con tasas de resistencia superiores al 5% (Wiederhold y Verweij, 2020). Las directrices de la ESCMID de 2018 recomiendan identificar todas las cepas clínicamente relevantes del género *Aspergillus* a nivel de especie, por si se tratase de una especie con resistencia intrínseca, y realizar pruebas de sensibilidad antifúngica para su manejo clínico y estudios epidemiológicos. La sensibilidad antifúngica debe evaluarse en pacientes con enfermedad invasiva, excepto en regiones donde los programas de vigilancia no reportan resistencia a azoles; en estos casos, los estudios epidemiológicos se deben realizar de forma periódica en un mínimo de 100 cepas (Ullmann et al., 2018).

Entre las pruebas para estudiar la sensibilidad antifúngica, el método de microdilución en caldo estandarizado por EUCAST presenta una considerable complejidad técnica, ya que requiere un conocimiento detallado tanto para su preparación como para la interpretación de resultados, además de demandar una cantidad significativa de tiempo. Las placas de agar con azoles, aunque constituyen un método de cribado estandarizado por EUCAST, pueden implicar un elevado coste debido a la adquisición del material plástico utilizado, además del tiempo empleado tanto para la preparación manual de las placas como del inóculo de las cepas. En el caso de las tiras de difusión en gradiente, a pesar de su uso ampliamente extendido en España, no cuentan con puntos de corte definidos, y tanto la preparación de los inóculos como la interpretación de los resultados carecen de estandarización.

Como consecuencia de estas limitaciones, los estudios de sensibilidad antifúngica para hongos filamentosos no suelen realizarse, o bien se realizan con tiras de difusión en gradiente con las limitaciones que esto conlleva. Además, en algunos casos los resultados pueden retrasarse hasta dos semanas al tener que ser derivadas las cepas a un laboratorio de referencia.

Este proyecto de tesis se ha enfocado en evaluar y simplificar mediante diversas estrategias estos tres métodos mencionados, con el fin de facilitar la realización de pruebas de sensibilidad a antifúngicos en *A. fumigatus*, logrando que sean más accesibles, objetivas, económicas y prácticas para el laboratorio de Microbiología Clínica.

El primer bloque de trabajos de esta tesis se centró en evaluar la lectura espectrofotométrica de las CMIs de azoles y anfotericina B y compararlas con las visuales.

En 2021, el procedimiento estándar de microdilución de EUCAST vigente en aquel momento (E.Def 9.3.2) recomendaba la inspección visual para la determinación de la CMI de azoles y anfotericina B frente a especies de *Aspergillus*.

La lectura de la CMI de azoles frente a *Aspergillus* por medio de métodos de microdilución puede resultar controvertida y ser uno de los motivos para no desarrollar este tipo de estudios en el entorno hospitalario. Estudios anteriores a los aquí presentados, compararon las lecturas espectrofotométricas y visuales, y mostraron

excelentes acuerdos esenciales (92%-97%) y categóricos (93-99%). No obstante, la mayoría de estos estudios utilizaban la metodología CLSI y se estaban limitados por el número bajo de cepas de *A. fumigatus sensu stricto* analizadas (hasta 133 cepas), la no inclusión de especies crípticas, ni de cepas resistentes con mutaciones en el gen *cyp51A*, así como un escaso número de antifúngicos estudiado (anfotericina B e itraconazol) (Dannaoui et al., 1999; Llop et al., 2000; Meletiadis et al., 2001). Sólo uno de estos estudios utilizó el método EUCAST (Meletiadis et al., 2017). En este caso, se evaluaron los cuatro triazoles (itraconazol, posaconazol, voriconazol e isavuconazol) y 15 cepas con mutaciones en el gen *cyp51A* (TR_{34}-L98H, G54, M220 y otras). Sin embargo, este estudio también estuvo limitado por el bajo número de cepas de *A. fumigatus sensu stricto* (88 cepas) y la ausencia de especies crípticas.

Las lecturas espectrofotométricas de las CMIs se basan en la medida de la inhibición del crecimiento fúngico en presencia, o ausencia, de antifúngicos. Los *endpoints* de inhibición del crecimiento fúngico del 90% y el 95%, en comparación con los pocillos sin antifúngicos, son los criterios de valoración más comúnmente utilizados en los estudios previos (Meletiadis et al., 2017; Meletiadis et al., 2001). Un criterio de valoración estricto (95%) puede llevar a clasificar erróneamente las cepas como falsamente resistentes; por el contrario, un criterio de valoración menos estricto (90%) puede llevar a pasar por alto cepas verdaderamente resistentes, pero puede ayudar a ignorar artefactos que de otro modo se interpretarían como crecimiento fúngico (por ejemplo, medio RPMI de color amarillo en pocillos en los que crece *A. niger*, precipitación de fármacos antifúngicos insolubles, etc.) (Jorgensen et al., 2018).

Por otro lado, dado que EUCAST modificó los puntos de corte de los azoles frente a *A. fumigatus sensu lato* en 2020, era necesario validar las lecturas espectrofotométricas incluyendo un gran número de cepas clasificadas según los puntos de corte actualizados. Los valores de los puntos de corte se redujeron para anfotericina B, itraconazol, voriconazol y posaconazol, y se aumentaron para isavuconazol (Arendrup et al., 2020).

En esta tesis, se aprovechó el gran número de cepas recogidas en un estudio epidemiológico sobre la resistencia a azoles en *A. fumigatus sensu lato* en España (*ASPE*IN) en 2019 procedentes de 30 hospitales (Escribano et al., 2021) y se compararon las CMIs obtenidas tanto por lectura visual como por lectura espectrofotométrica,

utilizando una inhibición del crecimiento fúngico del 95% como definición de la CMI, siguiendo el procedimiento EUCAST E.Def 9.3.2. Se observó que ambas formas de determinación de la CMI mostraban acuerdos esenciales/categóricos muy elevados para la anfotericina B (98,8%/100%), el itraconazol (94,8%/100%), el posaconazol (97,3%/100%), el voriconazol (98,3%/98,7%) y el isavuconazol (96,1%/99,3%).

La lectura espectrofotométrica de la CMI permitió la correcta categorización como resistentes de todas las cepas con mutaciones relevantes en el gen *cyp51A*. No se encontraron errores en anfotericina B, itraconazol y posaconazol. La mayoría de las clasificaciones erróneas para voriconazol e isavuconazol se encontraron en CMIs en el ATU (n = 10/12 cepas) o sólo una dilución por encima del punto de corte (n = 2/12 cepas; CMI = 4 mg/L). La lectura espectrofotométrica dio lugar a una falsa clasificación como resistente a voriconazol en seis cepas de *A. fumigatus sensu stricto* que tenían la secuencia del gen *cyp51A* de tipo salvaje o polimorfismos y en dos cepas de *N. udagawae*. En isavuconazol, la lectura espectrofotométrica dio lugar a una detección errónea de la resistencia a isavuconazol en tres cepas de *A. fumigatus sensu stricto* que tenían la secuencia del gen *cyp51A* de tipo salvaje y en tres cepas crípticas (dos *N. udagawae* y un *A. fumigatiaffinis*).

El ATU, representa a una zona de incertidumbre para interpretar el valor de la CMI cuando el punto de corte se encuentra en un lugar donde se produce una confluencia de cepas de tipo salvaje y cepas mutantes (Arendrup et al., 2020). Las cepas con una CMI de posaconazol e isavuconazol de 0,25 mg/L y 2 mg/L, respectivamente, no pueden informarse como sensibles o resistentes. La mayoría de los errores resultaron de una subestimación de la resistencia en las CMIs obtenidas por espectrofotómetro y resultaron ser valores que coincidían con el ATU. Aunque los pacientes infectados por cepas de *A. fumigatus sensu lato* resistentes a los azoles presentan una mayor mortalidad, la resistencia suele estar asociada a mutaciones en el gen *cyp51A* y algunas de ellas se asocian a un fenotipo panazol resistente (resistencia de alto nivel) (Lestrade et al., 2019a). En algunos fenotipos solo se afecta la actividad de un único azol, o de azoles con estructura molecular similar, y la CMI está cerca del punto de corte clínico, lo que da lugar a una resistencia de bajo nivel (Lewis y Verweij, 2017). Estudios anteriores han demostrado que los pacientes infectados por *A. fumigatus* resistentes a voriconazol

de bajo nivel (CMI = 2 mg/L), y *A. fumigatus* resistentes a isavuconazol de bajo nivel (CMI = 2 mg/L) podrían ser tratados con voriconazol o isavuconazol, respectivamente, siempre que se administren dosis más altas (Seyedmousavi et al., 2014; Buil et al., 2018).

Para evaluar la idoneidad de la espectrofotometría como alternativa a las lecturas visuales de CMIs, era necesario realizar una evaluación similar a la anteriormente comentada, pero usando una inhibición del crecimiento del 90% como definición de la CMI. Para esto se utilizaron las mismas cepas del estudio anterior en el que se estudiaron las CMIs de azoles y anfotericina B utilizando dicha inhibición del 90%. Las CMI obtenidas se compararon con las obtenidas anteriormente utilizando la lectura espectrofotométrica de inhibición del crecimiento fúngico del 95%.

Las lecturas espectrofotométricas, utilizando tanto la inhibición del crecimiento fúngico del 90% como la del 95% y comparadas con las lecturas visuales, tuvieron altos acuerdos esenciales y categóricos. Y por ello, ambos *endpoints* de inhibición fúngica son una buena alternativa a la determinación visual de la CMI. En cuanto a la inhibición del 90%, en *A. fumigatus sensu stricto*, se encontraron pocos errores muy graves (falsa sensibilidad), los cuales fueron en cepas con valores de CMIs de voriconazol en ATU y ninguno de ellos era portador de mutaciones relevantes en el gen *cyp51A*. Hubo una disminución del número de errores graves de voriconazol (falsa resistencia) en comparación con el criterio de inhibición del 95%. En el caso concreto de las especies crípticas, los acuerdos fueron mayores con el *endpoint* de inhibición fúngica del 95%. En las especies crípticas, se observó un aumento (anfotericina B, itraconazol, posaconazol) o un número similar (voriconazol e isavuconazol) de errores muy graves para el *endpoint* de inhibición del 90% y, por el contrario, no se encontraron errores graves.

En marzo de 2022, EUCAST publicó la nueva versión del procedimiento E.Def 9.4, en la que a la luz de la publicación de datos procedentes de diferentes laboratorios – entre los que se encuentran los recogidos en esta tesis – confirmando la correcta clasificación de las cepas como sensibles o resistentes (Meletiadis et al., 2022; Serrano-Lobo et al., 2021; Serrano-Lobo et al., 2020), se incorporaba la lectura espectrofotométrica, con un *endpoint* del 90% para las CMIs de anfotericina B y azoles frente a *A. fumigatus* (Guinea et al., 2022). El estudio de Meletiadis y colaboradores de 2022, realizado con 200 cepas de *A. fumigatus sensu stricto*, reportaba un acuerdo categórico del 100% para ambos

endpoints (90% y 95%), el acuerdo esencial fue mayor para el *endpoint* de inhibición del crecimiento fúngico del 90%, con el cual no se encontraron errores graves, y, sin embargo, con el *endpoint* de inhibición del crecimiento fúngico del 95%, se encontraron 8 errores graves. En esta tesis, aunque en las especies crípticas fue superior el *endpoint* de inhibición del 95%, en *A. fumigatus sensu stricto* ambos *endpoints* fueron prácticamente iguales, con la única excepción de una cepa que mostró un error menor en voriconazol con el *endpoint* de inhibición del 95%, y se clasificó correctamente como sensible utilizando el *endpoint* de inhibición del 90%.

Si bien la lectura espectrofotométrica aporta objetividad y facilita la lectura de las CMIs, sigue siendo metodológicamente complejo implementar estudios de microdilución en caldo en los laboratorios de Microbiología Clínica. Por tanto, hay que recurrir a métodos comerciales o tecnología menos compleja. Hasta la fecha, los métodos comerciales para las pruebas de sensibilidad antifúngica frente a *Aspergillus* spp., para uso diario en un laboratorio de Microbiología Clínica, no han sido bien validados. Las evaluaciones previas de Sensititre YeastOne y Etest® frente *A. fumigatus* mostraron resultados variables y, además, los estudios que mostraron los acuerdos categóricos más altos con los métodos de referencia de microdilución en caldo evaluaron un número bajo de cepas con mutaciones y excluyeron las especies crípticas (Berkow et al., 2020; Idelevich et al., 2018; Espinel-Ingroff et al., 2019).

Las placas de agar con azoles son una alternativa que puede ayudar a superar las limitaciones mencionadas. Diversos estudios, incluyendo una validación multicéntrica del método utilizando tanto placas VIPcheck© como placas elaboradas en los propios laboratorios participantes, demostraron un excelente rendimiento de las placas que contienen azoles para detectar cepas resistentes. Los resultados mostraron una alta correlación con el método de microdilución de EUCAST. Esto permitió que EUCAST desarrollara el método E.Def 10 e implementara el procedimiento para uso rutinario (Arendrup et al., 2018; Buil et al., 2017; Guinea et al., 2019).

Las placas comerciales son una buena alternativa, sin embargo, no están disponibles en todos los países, y su precio es elevado. La preparación de las placas de manera casera comporta decidir qué tipo de material plástico se deben utilizar. Los estudios previos a los realizados en esta tesis no incluyeron especies crípticas, ni habían evaluado el

impacto del uso de diferentes tipos de plásticos para la preparación de las placas de forma casera.

En esta tesis se confirmó el buen rendimiento de las placas que contienen azoles para detectar la resistencia a azoles en *A. fumigatus sensu stricto*. El patrón de resistencia se dedujo fácilmente a partir del crecimiento en pocillos de agar con azoles. Así, las sustituciones TR_{34}-L98H pueden sospecharse en cepas que crecen en agares que contienen itraconazol y voriconazol, las sustituciones G54 pueden sospecharse en cepas que crecen en agares que contienen itraconazol y posaconazol, y las sustituciones TR_{46}-Y121F-T289A pueden sospecharse en cepas que crecen exclusivamente en placas de agar que contienen voriconazol.

Además, el tipo de plástico utilizado no tuvo impacto en los valores de sensibilidad y especificidad del procedimiento, lo cual supone una ventaja económica a la hora de preparar las placas de agar con azoles de forma casera. En itraconazol no hubo errores muy graves. En voriconazol se produjeron errores muy graves (1,1%) solo en una cepa con una CMI en ATU (2 mg/L) y que no albergaba una mutación que se correlacionara claramente con la resistencia a azoles. En posaconazol, el número de errores muy graves sí fue más elevado, lo que podría deberse a la alta concentración del fármaco en la placa de agar (0,5 mg/L), superior al punto de corte de EUCAST para posaconazol (0,25 mg/L, coincidente con el ATU). Los errores graves con itraconazol y voriconazol sólo se produjeron en unas pocas cepas (1,1%-4,4%) que muestran un crecimiento fúngico débil. En cambio, todas las cepas que albergaban sustituciones relevantes (TR_{34}-L98H, G54R y TR_{46}-Y121F-T289A) crecieron vigorosamente en placas que contienen itraconazol y voriconazol, en consonancia con su perfil de sensibilidad según el método de microdilución. En posaconazol los errores graves solo se produjeron en una cepa que mostraba también un crecimiento débil. Un crecimiento vigoroso debe hacer saltar la alarma sobre la presencia de cepas verdaderamente resistentes, mientras que un crecimiento fúngico débil puede implicar una falsa resistencia (Figura 12).

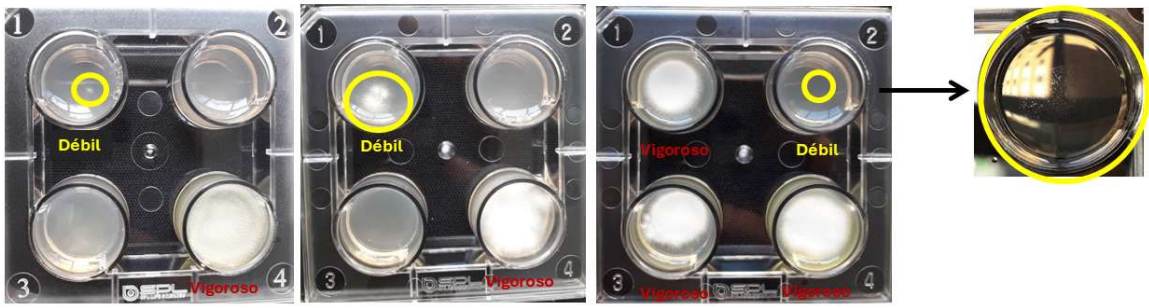


Figura 12. Diferencias entre un crecimiento vigoroso y un crecimiento débil en las placas de agar con azoles.

No obstante, independientemente del grado de crecimiento observado, y aunque este método pueda constituir un recurso valioso para el cribado de resistencia a azoles y la detección de crecimiento fúngico deba considerarse como un indicio importante para ajustar el tratamiento del paciente, se deben confirmar los hallazgos mediante métodos de referencia de microdilución (Ullmann et al., 2018).

En el caso de las especies crípticas, el rendimiento de las placas de agar que contienen azoles fue mucho menor, y los pocillos de agar que contienen voriconazol mostraron la mayor sensibilidad en la detección de resistencia frente a estas especies. Se observó un crecimiento débil (falsa resistencia), especialmente en las placas que contienen itraconazol, en un número alto de especies crípticas sensibles a los azoles. El menor rendimiento observado en el caso de las especies crípticas podría atribuirse a su crecimiento más lento, baja capacidad de esporulación y, por lo tanto, a la menor homogeneidad de las conidias en los inóculos. Futuros estudios deberán enfocarse en estas especies crípticas para determinar si el rendimiento del método mejora al incrementar el tiempo de incubación de las placas de agar que contienen azoles, y/o al emplear inóculos de conidias estandarizados mediante el uso de una cámara de Neubauer.

Con el fin de aliviar la carga de trabajo del laboratorio, se estudió si se podían simplificar los métodos omitiendo el filtrado de las suspensiones de conidias antes de la preparación del inóculo, ya que la forma de preparación del inóculo es la misma que para inocular las placas de microdilución según E.Def 9.4. En general, observamos que la sensibilidad (100%) y la especificidad (98,2%) del método de cribado en agar son elevadas y no se veían afectadas cuando las placas se inoculaban con suspensiones de

conidias sin filtrar. Se observaron pocas discrepancias cuando se utilizaban suspensiones de conidias sin filtrar para el posaconazol y ninguna para el itraconazol y el voriconazol. Además, omitir el filtrado de la suspensión de conidias no condujo a errores en la clasificación de cepas con sustituciones TR_{34}-L98H, G54R o TR_{46}-Y121F-T289A.

Para simplificar todavía más la metodología, se omitió el ajuste del inóculo a 0,5 MacFarland antes de proceder a inocular las placas de agar con azoles. En general, se observó que el rendimiento del método de cribado en agar no se veía afectado cuando las placas se inocularon con suspensiones de conidias sin filtrar ni ajustar, y sólo se observaron algunas discrepancias en las placas con agares que contenían posaconazol. Omitir el ajuste del inóculo no condujo a errores en la detección de las cepas que albergaban la sustitución TR_{34}-L98H.

Debido al elevado número de errores muy graves encontrados en los agares con posaconazol, especialmente en cepas que presentaban la sustitución TR_{34}-L98H con niveles bajos de resistencia a este fármaco y que no lograron crecer en presencia de posaconazol, se consideró que esto podría atribuirse a la alta concentración de fármaco en el pocillo (0,5 mg/L). Cabe destacar que el método E.Def 10.1 fue diseñado con concentraciones ajustadas a los puntos de corte vigentes en ese momento. Por lo tanto, se adaptó la concentración final de posaconazol en el agar a los puntos de corte actualizados de EUCAST (v10), estableciéndola en 0,25 mg/L. Esta concentración reducida de posaconazol en las placas de agar podría aumentar los errores graves; sin embargo, también podría reducir los errores muy graves en la detección de cepas TR_{34}-L98H con resistencia de bajo nivel al posaconazol. En un estudio reciente, Lucio y colaboradores encontraron una buena concordancia al reducir la concentración de posaconazol a la mitad (de 0,5 mg/L a 0,25 mg/L) en cepas con sustituciones TR_{34}-L98H, TR_{53} y TR_{46}-Y121F-T289A (Lucio et al., 2022). En el caso de esta tesis, la reducción de la concentración de posaconazol a 0,25 mg/L en los pocillos de agar condujo a una disminución en el número de errores muy graves, especialmente en cepas con sustitución TR_{34}-L98H con resistencia de bajo nivel al posaconazol, sin un incremento en el número de errores graves. En cualquier caso, estas discrepancias resultaron poco preocupantes, ya que las cepas resistentes a este antifúngico lograron crecer en los

pocillos que contenían itraconazol y, por consiguiente, el resultado en el pocillo de agar con posaconazol podría omitirse.

El método de las tiras de difusión en gradiente es el método más utilizado en los laboratorios de Microbiología Clínica, pero tiene la limitación de que no se cuenta con puntos de corte para interpretar los valores de CMIs, como se ha comentado previamente. En esta tesis se estudió el método de las tiras de difusión en gradiente con objeto de proponer unos puntos de corte orientativos para interpretar los valores de CMIs obtenidos frente a cepas de *A. fumigatus sensu lato*.

Estudios anteriores, compararon el método de microdilución de E.Def 9.4 con las tiras de difusión en gradiente, y encontraron acuerdos categóricos elevados, pero aunque incluían cepas de *A. fumigatus* de tipo salvaje y cepas con mutaciones en el gen *cyp51A* (TR_{34}-L98H, TR_{46}-Y121F-T289A, M220 y G54), estaban limitados por el bajo número de estas últimas, los agentes antifúngicos seleccionados y la ausencia de especies crípticas (Idelevich et al., 2018; Arendrup et al., 2017b). Espinel-Ingroff y colaboradores, utilizando el método de microdilución CLSI, propuso puntos de corte epidemiológicos frente a *A. fumigatus sensu stricto* para itraconazol (2 mg/L), voriconazol (0,5 mg/L) y posaconazol (0,25 mg/L) (Espinel-Ingroff et al., 2019; Espinel-Ingroff et al., 2017).

Los puntos de corte propuestos por Espinel-Ingroff y colaboradores fueron similares a los propuestos en esta tesis, itraconazol (1 mg/L), voriconazol (0,5 mg/L) y posaconazol (0,25 mg/L) y detectaron las cepas de *A. fumigatus sensu stricto* resistentes. Los valores de sensibilidad y especificidad fueron superiores al 97%. Los errores detectados fueron escasos, encontrándose sólo un error grave en una cepa con la sustitución TR_{46}-Y121F-T289A.

Se estudiaron también especies crípticas por primera vez, pero en este caso al igual que se observó en las placas de agar que contienen azoles, el rendimiento fue bajo y sólo se pudieron proponer valores de corte de CMIs para voriconazol (>1 mg/L) e isavuconazol (>0,75 mg/L) con un elevado número de cepas que resultaron falsamente sensibles a esos fármacos. El bajo rendimiento con especies crípticas puede deberse, al igual que en el método de las placas que contienen azoles, a la escasa esporulación de

las cepas, que podría dar lugar a inóculos de conidias irregulares y, por consiguiente, a un crecimiento también irregular en las placas de RPMI.

De la misma manera que se hizo con el método de las placas de agar que contienen azoles, se intentó simplificar el método de las tiras de difusión en gradiente omitiendo el filtrado y el ajuste del inóculo. Debido al bajo rendimiento obtenido en las especies crípticas, estas no fueron aquí estudiadas. Los requisitos de preparación de placas con tiras de difusión en gradiente con inóculos filtrados y ajustados son idénticos a los utilizados para inocular placas de microdilución siguiendo el método E.Def 9.4. Aquí se demuestra que el rendimiento del método de tiras de difusión en gradiente apenas se veía afectado por la forma en que se preparaba el inóculo.

El acuerdo esencial entre ambas formas de preparación del inóculo fue del 95,5% para itraconazol y del 98% para voriconazol y posaconazol. Sólo se observaron dos discrepancias cuando se utilizaron suspensiones de conidias sin filtrar ni ajustar; sin embargo, éstas no condujeron a errores categóricos. Se encontró una discrepancia categórica en una cepa que albergaba la sustitución G448S, que se clasificó falsamente como sensible a posaconazol (CMI EUCAST de 0,25 mg/L, ATU) con una CMI en tira de difusión en gradiente de 0,25 mg/L al usar el inóculo sin filtrar ni ajustar (muy cercana al valor de corte de CMI >0,25 mg/L propuesto) y con una CMI al usar el inóculo filtrado y ajustado de 0,38 mg/L. Futuros estudios con un mayor número de cepas deberán clarificar si puede ser necesario establecer un ATU en las CMI de posaconazol obtenidas por tiras de difusión en gradiente, ya que podría existir confluencia de cepas de tipo salvaje y cepas mutantes, al igual que ocurre con las CMIs obtenidas por microdilución en caldo.

Estos puntos de corte deberían ser validados en estudios multicéntricos, pero hasta que se estandaricen, podrían utilizarse en el laboratorio para guiar la interpretación de los resultados de CMIs y poder así alertar sobre la necesidad de ajuste del tratamiento en aquellos casos en los que las CMIs se encuentren por encima, enviando solo en estos casos las cepas a un centro de referencia para confirmar los hallazgos mediante métodos de microdilución.

En estudios futuros será fundamental incorporar los nuevos antifúngicos con actividad frente a *Aspergillus* que se vayan integrando en la práctica clínica (olorofima,

ibrexafungerp, opelconazol, rezafungina y fosmanogepix), con el fin de evaluar la aplicabilidad de los tres métodos analizados. Además, como se ha mencionado previamente, los estudios futuros que utilicen los métodos de placas de agar con azoles y tiras de difusión en gradiente deberían enfocarse en la optimización de estos para las especies crípticas. Esto permitirá determinar si la eficacia del método mejora al aumentar el tiempo de incubación de las placas y al emplear inóculos de conidias estandarizados mediante el uso de una cámara de Neubauer.

La presente tesis tiene ciertas limitaciones. En primer lugar, es necesaria la realización de estudios adicionales en los que se incluyan cepas resistentes con una gama más amplia de mutaciones y con una mayor representación de cepas con la sustitución TR_{46}-Y121F-T289A. En segundo lugar, en el caso del método de placas de agares con azoles y de las tiras de difusión en gradiente, para poder validar las conclusiones extraídas en nuestros estudios, son necesarios estudios externos y multicéntricos.

6. CONCLUSIONES

1. La lectura espectrofotométrica es una alternativa rápida, objetiva y automatizada, a la lectura visual de las CMIs de azoles y anfotericina B frente a *A. fumigatus sensu lato*.

2. La lectura espectrofotométrica de las CMIs de azoles y anfotericina B frente a *A. fumigatus sensu stricto*, utilizando tanto el *endpoint* de inhibición del crecimiento fúngico del 90% como del 95% y comparadas con las lecturas visuales, tienen una elevada concordancia.

3. El método de las placas de agar con azoles (EUCAST E.Def 10.2), es un método de cribado útil, independientemente del tipo de plástico utilizado, para diferenciar entre cepas de *A. fumigatus sensu stricto* sensibles y resistentes a los azoles.

4. El método de las tiras de difusión en gradiente es un método sencillo y útil para el cribado de la resistencia a azoles en cepas de *A. fumigatus sensu stricto* si las CMIs obtenidas se interpretan de la siguiente manera: itraconazol >1 mg/L, voriconazol >0,5 mg/L y posaconazol >0,25 mg/L.

5. Cuando se realiza el cribado de la resistencia a azoles en *A. fumigatus sensu stricto*, ni la omisión de la filtración ni la del ajuste a 0,5 McFarland de las suspensiones de conidias influyó negativamente en el rendimiento del método de las placas de agar con azoles (EUCAST E.Def 10.2), o en el método de las tiras de difusión en gradiente.

6. Los métodos de cribado de resistencia, usando tanto placas con azoles o tiras de difusión en gradiente de concentración, no mostraron un buen rendimiento para la detección de resistencia a azoles frente a cepas pertenecientes a especies crípticas del complejo *Fumigati*.

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8. ANEXO

Comunicaciones como primera autora enviadas a congresos internacionales o nacionales derivadas de esta tesis

2020: 9th Advances Against Aspergillosis & Mucormycosis Conference

- Clinical *Aspergillus* isolates causing aspergillosis in the last 20 years: an overview of aetiology and antifungal resistance to azoles and amphotericin B.

2021: 31st European Society of Clinical Microbiology and Infectious Diseases

- Azole resistance screening in *Aspergillus fumigatus* clinical isolates using azole-containing agar plates (EUCAST E.Def 10.1): low impact of plastic trays used and poor performance against cryptic species.

2022: 32nd European Society of Clinical Microbiology and Infectious Diseases

- Tentative cut-off MIC values using gradient diffusion plastic strips: accurate detection of azole resistance in *Aspergillus fumigatus sensu stricto.*

- Azole resistance screening in *Aspergillus fumigatus* using azole-containing agar plates (EUCAST E. Def 10.1). Good news for the routine clinical mycology! Filtration of inoculum suspensions is not needed.

2023: 33rd European Society of Clinical Microbiology and Infectious Diseases

- EUCAST-obtained MICs of azoles and amphotericin B against *Aspergillus fumigatus sensu stricto*: Spectrophotometric readings and visual inspections showed high agreement.

- EUCAST-obtained olorofim MICs against non-*fumigatus Aspergillus* clinical isolates: high agreements between visual inspections and spectrophotometric readings.

XXVI Congreso Nacional de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica

- Detección de resistencia a azoles en *Aspergillus fumigatus* usando placas de agar (EUCAST E. Def 10.2): la preparación del inóculo no requiere filtrado ni ajuste previo.

2024: **34th European Society of Clinical Microbiology and Infectious Diseases**

- Olorofim showed potent *in vitro* activity against *Aspergillus* spp. clinical isolates collected in a national survey on azole resistance conducted in Spain in 2022 (*ASPE*IN II).

- Gradient diffusion plastic strip for detection of azole resistance in *Aspergillus fumigatus sensu stricto*: inoculum preparation might not require prior filtration and adjustment.

9th Advances Against Aspergillosis and Mucormycosis
Palazzo dei Congressi Lugano, Lugano, Switzerland
27 – 29 February 2020

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Clinical *Aspergillus* isolates causing aspergillosis in the last 20 years: an overview of aetiology and antifungal resistance to azoles and amphotericin B

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INTRODUCTION AND PURPOSE

There is a plethora of reports alerting on the raise in the number of azole-resistant *Aspergillus* spp isolates in different European countries. In contrast, data from Southern Europe is scarce.

The Clinical Microbiology and Infectious Diseases Department of Gregorio Marañón Hospital, a large tertiary hospital located in Madrid, Spain, acts as a watchtower of invasive fungal infections in the hospital where all patients with *Aspergillus* isolation are followed up.

We here report the identification and antifungal susceptibility to amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole of a collection of clinically significant *Aspergillus* spp. isolates from patients cared at our hospital in the last two decades.

MATERIAL AND METHODS

Aspergillus spp. (n=356)

Isolates from 264 patients with proven (n=30) or probable aspergillosis (1 isolate per species and patient)

Admitted to Gregorio Marañon Hospital (Jan 1999 – Dec 2018)

Molecular Identification

Amplification and sequencing of *ß-tub* gene

Phylogenetic analysis

Antifungal Susceptibility EUCAST 9.3.1

AmB, ITC, VOR, POS, and ISA Breakpoints table v 9.0 (Resistance, MIC of ITC and/or VOR ≥ 4 mg/L)

cyp51A gene sequencing in azole-resistant *A. fumigatus* sensu stricto isolates

RESULTS

- The aetiology of agents causing invasive aspergillosis is shown in Figure 1:

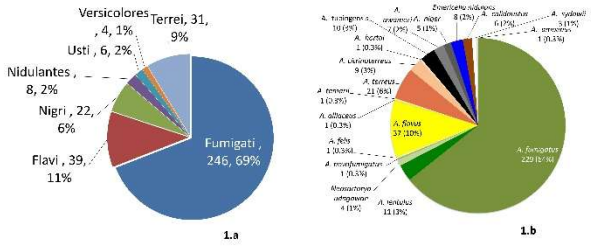

Figure 1. Species distribution by sections (1a) or species identification (1b) of the 356 isolates tested.

- The vast majority of patients were infected by *A. fumigatus* sensu stricto and the number of patients infected by cryptic species was variable and erratic (Figure 3).

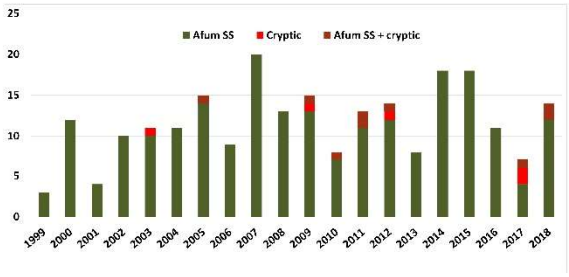

Figure 2. Number of patients infected by *A. fumigatus* complex species per year of the study period.

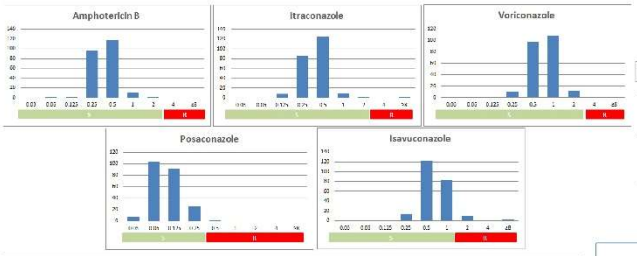

Figure 3. MIC distributions of azoles and amphotericin B against *A. fumigatus* sensu stricto isolates.

- Overall resistance to amphotericin B and azoles was 4.5% and 8.15%, respectively.
- In non-Fumigati sections, azole resistance was found in Terrei (2/31; *A. terreus* sensu stricto, n=1; *A. citrinoterreus*, n=1) and Nidulantes (1/8; *Emericella nidulans*).
- Isolates of Fumigati section showed the highest rate of antifungal resistance with the cryptic species as the ones mostly affected (Table 1).

Table 1. Percentage of resistance (No. of isolates) of the 356 isolates against azoles and amphotericin B.

		Percentage of resistance (No. of isolates)								% of resistance/ non wild type
		0.03	0.06	0.125	0.25	0.5	1	2	4	≥8	
A. fumigatus SS (n=229)	Amphotericin B	0	1	2	96	117	11	2	0	0	0 / 0.9
	Itraconazole	0	0	8	85	125	9	1	0	1	0.45 / 0.9
	Voriconazole	0	0	0	10	97	108	12	1	1	0.9 / 6
	Posaconazole	7	104	91	25	2	0	0	0	0	0.9 / 0.9
	Isavuconazole	0	0	0	14	122	82	9	0	2	5 / 0.9
Cryptic species (n=17)	Amphotericin B	0	0	0	0	1	1	7	5	3	47.5 / 88.2
	Itraconazole	0	0	0	1	5	4	5	0	2	12 / 41
	Voriconazole	0	0	0	0	0	1	11	3	2	29 / 94
	Posaconazole	0	0	5	9	2	1	0	0	0	18 / 17.6
	Isavuconazole	0	0	0	0	0	9	6	2	0	47 / 11.7

Numbers in bold indicate resistance; underlined numbers indicate non wild type isolates based on ECOFFs.

- The percentage of *A. fumigatus* sensu stricto isolates resistant to one or more azoles ranged from 0.4% (itraconazole) to 5% (isavuconazole) (Table 1).
- Most of isavuconazole-resistant isolates had an MIC = 2 mg/L but were susceptible to the remaining azoles and had a *cyp51A* wild type sequence.
- *Cyp51A* gene mutations were found in isolates from 2 patients (Table 2) and showed cross resistance to azoles.

Table 2. Isolates from two patients infected by azole-resistant *A. fumigatus* sensu stricto isolates.

Year	Diagnosis	MIC (mg/L) AmB	ITC	VOR	POS	ISA	*cyp51A* gene	Risk factors (clinical form)
2011	Proven	0.5	2 (I)	≥ 16 (R)	0.5 (R)	8 (R)	G448S	Tympanoctomy (Brain aspergillosis)
2012	Probable	0.25	≥ 16 (R)	4 (R)	0.5 (R)	8 (R)	TR_{34}L98H	Liver disease + HIV (Pulmonary aspergillosis)

CONCLUSION

- *A. fumigatus* complex represented around 70% of the isolates causing invasive aspergillosis at our center.
- The rate of resistance to azoles and amphotericin B in *A. fumigatus* sensu stricto was lower than 1%, highly impacted by the presence of cryptic species, and has not shown any sign of increase over the years.

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ESCMID EUROPEAN SOCIETY OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES

Azole resistance screening in *Aspergillus fumigatus* clinical isolates using azole-containing agar plates (EUCAST E.Def 10.1): low impact of plastic trays used and poor performance against cryptic species

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INTRODUCTION

Broth microdilution methods are the gold standard for antifungal susceptibility testing. Unfortunately, microdilution procedures are time-consuming and technically demanding, which may hamper antifungal susceptibility testing from being widely available in clinical microbiology laboratories. Azole-containing agar allows conducting antifungal susceptibility testing against *Aspergillus fumigatus* routinely.

AIM

The objective of this work is to evaluate the performance of the E.Def 10.1 agar screening method to detect azole resistance in clinical *A. fumigatus* sensu stricto and cryptic species isolates. Moreover, we aim to assess the impact of using different types of plastic trays to prepare the azole-containing agar plates.

METHOD

***Aspergillus* sensu lato (n=143)**

- *A. fumigatus* sensu stricto (n=91) → 36 azole-resistant isolates (Table 1)
- Cryptic species (n=52) → 47 azole-resistant isolates

↓

Antifungal susceptibility to itraconazole, voriconazole and posaconazole

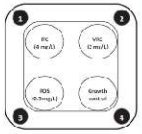
- EUCAST S.3.2 (EUCAST breakpoints v7.0)
- EUCAST E.Def 10.1:
  - Nunc® tissue-treated plates
  - Nunc® non-tissue-treated plates
  - Labclinics® tissue-treated plates

↓

Calculation of sensitivity, specificity and essential and categorical agreement

Table 1. *Cyp51A* mutations of the azole-resistant isolates

Cyp51A mutation	No. of isolates
TR_{34}-L98H	24
G54	5
TR_{46}-Y121F-T289A	1
F46Y-M172V-N248T-D255E-E427K	1
F165L	1
None (*cyp51A* gene wild-type)	4

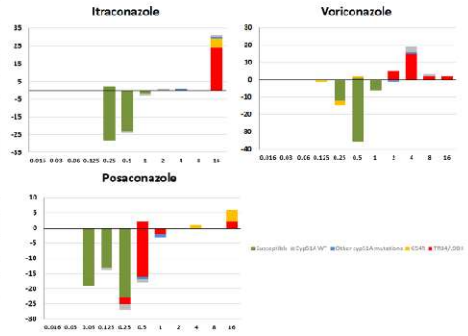


RESULTS

Table 2. Categorical agreements between the agar screening and microdilution methods and sensitivities and specificities of the agar screening method

	A. fumigatus sensu stricto % (No.)					Cryptic species % (No.)				
	Categorical agreement	Very major errors	Major errors	S	Sp	Categorical agreement	Very major errors	Major errors	S	Sp
Itraconazole										
Nunc® tissue-treated plates	97.8	0	2.2 (n=2)	100	96.6	69.2	11.5 (n=6)	19.2 (n=10)	73.9	65.5
Nunc® non-tissue-treated plates	98.9	0	1.1 (n=1)	100	98.3	69.2	9.6 (n=5)	21.1 (n=11)	78.3	62.1
Labclinics® tissue-treated plates	95.6	0	4.4 (n=4)	100	93.1	67.3	11.5 (n=6)	21.1 (n=11)	73.9	62.1
Voriconazole										
Nunc® tissue-treated plates	96.7	1.1 (n=1)	2.2 (n=2)	96.8	96.7	78.8	19.2 (n=10)	1.9 (n=1)	78.7	80
Nunc® non-tissue-treated plates	95.6	1.1 (n=1)	3.3 (n=3)	96.8	95	71.1	25 (n=13)	3.8 (n=2)	72.3	60
Labclinics® tissue-treated plates	95.6	1.1 (n=1)	3.3 (n=3)	96.8	95	78.8	17.3 (n=9)	3.8 (n=2)	80.9	60
Posaconazole										
Nunc® tissue treated plates	73.6	26.4 (n=24)	0	27.3	100	55.8	36.5 (n=19)	7.7 (n=4)	26.9	84.6
Nunc® non tissue treated	74.7	25.3 (n=23)	0	30.3	100	61.5	32.7 (n=17)	5.8 (n=3)	34.6	88.5
Labclinics® tissue treated plates	75.8	23.1 (n=21)	1.1 (n=1)	36.4	98.3	59.6	30.8 (n=16)	9.6 (n=5)	38.5	80.8

Figure 1. Number of *A. fumigatus* sensu stricto isolates falling in each MIC value according to E.Def 9.3.2 method with (bars above the X-axis) or without (bars below the X-axis) fungal growth on Nunc® tissue-treated plates



- Overall, sensitivity and specificity values of the agar screening method were 100% and 93.3%, respectively.
- The type of tray used did not affect sensitivity and specificity values.
- All isolates harbouring TR_{34}-L98H substitutions were classified as resistant to itraconazole and voriconazole by the agar method; however, false susceptibility (very major error) to posaconazole was not uncommon and happened in isolates with posaconazole MICs of 0.25 mg/L.
- Isolates harbouring G54R and TR_{46}-Y121F-T289A substitutions were correctly classified by the agar method as itraconazole/posaconazole resistant and voriconazole-resistant, respectively.
- False resistance (major error) occurred in isolates showing tiny fungal growth.
- Agreements between both procedures against cryptic species were much lower.

Figure 2. Growth pattern of azole-resistant *A. fumigatus* sensu stricto isolates harbouring *cyp51A* gene substitutions

CONCLUSIONS

Azole-containing agar plates proved to be an easy-to-use and reliable method to screen for azole resistance in *A. fumigatus sensu stricto*; the method was minimally impacted by the type of plastic tray used.

The performance against cryptic species was rather poor.

32nd ECCMID EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES Lisbon, Portugal 23–26 April 2022 | ESCMID EUROPEAN SOCIETY OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES | G-M | FEDER | Hospital General Universitario Gregorio Marañón

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Tentative cut-off MIC values using gradient diffusion plastic strips: accurate detection of azole resistance in *Aspergillus fumigatus* sensu stricto

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INTRODUCTION

Although gradient diffusion plastic strips are widely utilized in clinical microbiology laboratories, interpretive criteria are not available for azole susceptibility testing against *Aspergillus fumigatus* sensu stricto.

AIM

The objective of this work is to evaluate the performance of gradient diffusion plastic strips to detect azole resistance in clinical *A. fumigatus* sensu stricto isolates.

***A. fumigatus* sensu stricto (n=89)** → **Antifungal susceptibility to itraconazole, voriconazole, posaconazole and isavuconazole** → **Gradient diffusion plastic strips-obtained MIC distributions** → **Cut-off setting to separate resistant isolates from susceptible ones**

54 azole-susceptible isolates
35 azole-resistant isolates (Table 1)

No. of isolates resistant to itraconazole (n=33), voriconazole (n=30), posaconazole (n=33) and isavuconazole (n=30)

EUCAST 9.4
(EUCAST breakpoints v10)
Gold standard

Gradient diffusion plastic strips
(ETEST®, Biomerieux, Marcy-l'Etoile, France)

MICs were obtained by following the manufacturer´s guidelines after 48h of incubation (Figure 1)

All discrepancies between EUCAST 9.4 and gradient diffusion plastic strips method were retested

Table 1. *Cyp51A* gene substitutions of the azole-resistant isolates

Cyp51A gene substitutions	No. of isolates
TR_{34}-L98H	24
G54	5
TR_{46}-Y121F-T289A	1
F46Y-M172V-N248T-D255E-E427K	1
F165L	1
None (*cyp51A* gene wild-type)	3

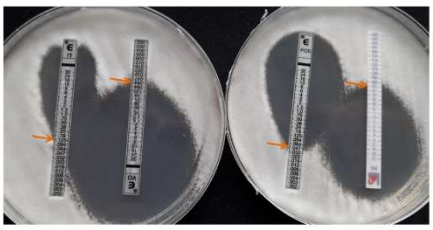
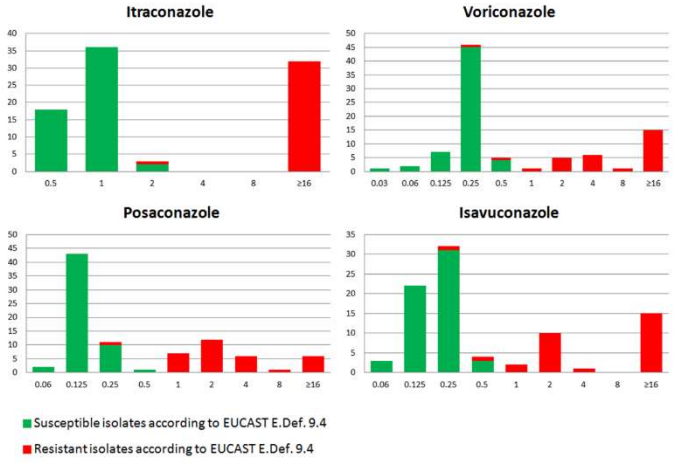


Figure 1. Interpretation of the MICs values using gradient diffusion plastic strips (orange arrows)

RESULTS

Figure 2. Gradient diffusion plastic strips-obtained MIC distributions of azoles against *A. fumigatus*

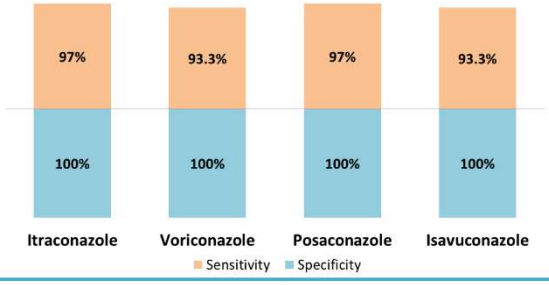


- All isolates with Itraconazole MICs of >2 mg/L, or voriconazole, posaconazole, and isavuconazole MICs of >0.5 mg/L were azole-resistant isolates.
- Using such cut-off values, two resistant *cyp51A*-wild-type gene isolates were incorrectly classified as susceptible to voriconazole and isavuconazole (one of the isolates was also misclassified as susceptible to itraconazole and posaconazole as well) (Table 2 and Figure 2).
- The profile of azole resistance in isolates harbouring G54 or TR_{34}-L98H *cyp51A* gene substitutions was correctly detected by using such cut-off values.
- Sensitivity and Specificity of the cut-off values to detect azole resistance was very high (Figure 3).

Table 2. Isolates false classified as susceptible by the Etest according to the cut-off values proposed

Isolate code	*Cyp51A* gene sequence	E.Def 9.4 method (MIC, in mg/L) / E-test (MIC, in mg/L)			
		Itraconazole	Voriconazole	Posaconazole	Isavuconazole
6545	Wild-type	S(0.25)/0.5	R(4)/0.25	S(0.125)/0.125	R(8)/0.25
6567	Wild-type	R(16)/2	R(2)/0.5	R(0.5)/0.25	R(4)/0.5

Figure 3. Sensitivity and specificity values of gradient diffusion plastic strips to detect azole resistance



CONCLUSIONS

We have been able to set tentative cut-off MIC values to correctly spot azole-resistant *Aspergillus fumigatus* isolates using gradient diffusion plastic strips.

Only few errors (false susceptibility) were found and never occurred in isolates harbouring either the G54 or TR_{34}-L98H *cyp51A* gene substitutions.

The study was supported by grants PI21/00450 from *Fondo de Investigación Sanitaria* (FIS. Instituto de Salud Carlos III; Plan Nacional de I+D+I 2021-2023). The study was co-funded by the European Regional Development Fund (FEDER) 'A way of making Europe'.

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P1598

Azole resistance screening in *Aspergillus fumigatus* using azole-containing agar plates (EUCAST E.Def 10.1). Good news for the routine clinical mycology! Filtration of inoculum suspensions is not needed

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INTRODUCTION

Azole resistance screening in *A. fumigatus* isolates can be routinely carried out by using azole-containing plates (E.Def 10.1 method), a straightforward and easy-to-perform EUCAST method. E.Def 10.1 requires filtering conidial suspensions prior inoculum adjustment, which takes time, increases laboratory workload and is cumbersome.

AIM

The objective of this work is to evaluate whether skipping the filtration step of conidial suspensions prior inoculum adjustment negatively influences the performance of the azole-containing agar method (EUCAST E.Def 10.1) to screen for azole resistance in *A. fumigatus*.

METHODS

A. fumigatus sensu stricto (n=92)

- 54 azole-susceptible isolates
- 38 azole-resistant isolates (Table 1)

Antifungal susceptibility to itraconazole, voriconazole and posaconazole

- EUCAST E.Def 9.4 (EUCAST breakpoints v10)
- EUCAST E.Def 10.1:
  - Conidial suspensions prepared in distilled water (Tween 20 0.1%)
    - Conidial suspensions filtered (Merck Millipore®, Nylon Net Filter 11.0 µm) → Inoculum adjustment 0.5 McFarland → Inoculation of plates
    - Inoculum adjustment 0.5 McFarland → Inoculation of plates

Calculation of sensitivity, specificity and categorical agreement

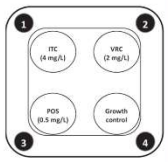

Table 1. *Cyp51A* gene substitutions in azole-resistant isolates

Cyp51A gene substitutions	No. of isolates
TR_{34}-L98H	26
G54	5
TR_{46}-Y121F-T289A	1
F46Y-M172V-N248T-D255E-E427K	1
F165L	1
G448S	1
None (*cyp51A* gene wild-type)	3

RESULTS

- Overall, sensitivity and specificity was 100% and 98.2% regardless the filtration of the conidial suspension.
- Agreements for the agar screening method with either unfiltered or filtered conidial suspensions were high for itraconazole (100%), voriconazole (100%), and posaconazole (97.8%) (Table 2).
- All isolates harbouring TR_{34}-L98H, G54R and TR_{46}-Y121F-T289A substitutions were correctly classified by the agar screening method for unfiltered and filtered suspensions.
- Discrepancies between unfiltered and filtered conidial suspensions were only noted in two isolates harbouring the TR_{34}-L98H substitution, in which filtered conidial suspension led to false susceptibility to posaconazole (isolates 6432 and 6444; Table 3).

Table 2. Categorical agreement values between the studied screening method on azole-containing agar plates using filtered and unfiltered conidia suspensions and the E.Def 9.4 method

Drug	Conidia suspension preparation	Categorical agreement	Errors	
			Very major (false susceptibility)	Major (false resistance)
Itraconazole	Unfiltered	98.9%	1.1% (n=1)	0
	Filtered	98.9%	1.1% (n=1)	0
Voriconazole	Unfiltered	97.8%	1.1% (n=1)	1.1% (n=1)
	Filtered	97.8%	1.1% (n=1)	1.1% (n=1)
Posaconazole	Unfiltered	77.2%	22.8% (n=21)	0
	Filtered	75%	25% (n=23)	0

Table 3. Very major errors (VME, false susceptibility) and major errors (ME, false resistance) found in the isolates tested and using both kinds of inoculums preparation

Isolate code	*cyp51A* gene substitutions	Errors	E.Def 9.4 visual reading (MIC, mg/L)/unfiltered inoculum agar plates/filtered inoculum agar plates		
			Itraconazole	Voriconazole	Posaconazole
3917	G448S	VME	R(2)/S/S	R/R/R	R(0.25)/S/S
6433	F165L	VME	R/R/R	R(2)/S/S	R(0.5)/S/S
4728	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6118	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6293	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6308	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6317	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6319	TR_{34}-L98H	VME	R/R/R	R/R/R	R(1)/S/S
6371	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6374	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6390	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6393	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6402	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6407	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6414	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6422	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.25)/S/S
6432	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/R/S
6444	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/R/S
6503	TR_{34}-L98H	VME	R/R/R	R/R/R	R(1)/S/S
6639	TR_{34}-L98H	VME	R/R/R	R/R/R	R(1)/S/S
6878	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6884	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
6964	TR_{34}-L98H	VME	R/R/R	R/R/R	R(0.5)/S/S
7057	Polymorphism*	ME	S/S/S	S(0.5)/R/R	S/S/S

R, resistant. S, susceptible. * F46Y/M172V/N248T/D255E/E427K

CONCLUSIONS

Unfiltered conidial suspensions for inoculum adjustment does not negatively influence the performance of the E.Def 10.1 method when screening for azole resistance in *A. fumigatus* sensu stricto clinical isolates.

The study was supported by grants PI18/01155 and PI19/00074 from *Fondo de Investigación Sanitaria* (FIS. Instituto de Salud Carlos III; Plan Nacional de I+D+I 2017-2020). The study was co-funded by the European Regional Development Fund (FEDER) 'A way of making Europe'.

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P2012

EUCAST-obtained MICs of azoles and amphotericin B against *Aspergillus fumigatus sensu stricto*: Spectrophotometric readings and visual inspections showed high agreement

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INTRODUCTION

Previous studies show high essential and categorical agreements between MICs of azoles and amphotericin B obtained by spectrophotometric readings and visual inspection against *Aspergillus fumigatus*, according to EUCAST methodology.

Visual MIC readings requires experiences and may lead to some degree of subjectivity. In contrast, spectrophotometric reading is more objective and automated and thus requires less skilled readers.

AIM

The objective of this work is to assess such comparisons, paying special attention to the MIC distributions (modal MIC and wild type upper limits [wt-UL]) obtained by using each endpoint.

METHODS

***A. fumigatus sensu stricto* (n=624) collected at 55 Spanish hospitals**

15 azole-resistant isolates (Table 1)

Antifungal susceptibility to amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole EUCAST 9.4

- **Visual reading**
- **Spectrophotometric readings** (≥95% or ≥90% fungal growth inhibition, read at 540 nm)

- **Essential** (±1 twofold dilution) **and categorical agreement assessment**
- **Setting of wild-type upper limits** (wt-ULs) according to the eye-ball method

Table 1. *Cyp51A* gene mutations in azole-resistant isolates

Cyp51A gene mutation	No. of isolates
TR_{34}-L98H	10
TR_{34}-L98H/S297T/F495I	2
F46Y/M172V/N248T/D255E/E427K	3

RESULTS

Table 2. MIC distributions, essential and categorical agreement between visual and spectrophotometric readings and wt-ULs obtained

Drug	Endpoints used to obtain the MIC	MIC distributions (no. of isolates at each MIC, mg/L)											EA (%)	CA (%)	VME (%)	ME (%)	wt-UL*	EUCAST ECOFF
		0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16						
Amphotericin B	Visual	0	0	2	12	262	315	33	0	0	0	0	-	-	-	-	2	1
	95%	0	0	5	13	283	297	25	0	0	0	1	99.8	99.8	0	0.2	2	
	90%	0	0	7	13	**312**	275	16	0	0	0	1	99.8	99.8	0	0.2	1	
Itraconazole	Visual	1	4	1	36	366	197	4	0	0	0	15	-	-	-	-	1	1
	95%	1	4	2	68	365	162	7	0	0	0	15	98.7	99.4	0.3	0.3	1	
	90%	4	3	8	129	350	110	6	0	0	14	0	96.8	99.5	0.3	0.2	1	
Voriconazole	Visual	0	0	0	3	94	391	123	1	9	3	0	-	-	-	-	2	1
	95%	0	0	0	3	185	360	57	8	9	1	1	97.9	99	0	1	2	
	90%	0	0	7	11	**312**	255	24	9	6	0	0	92.3	99.7	0	0.3	1	
Posaconazole	Visual	6	146	371	82	5	13	1	0	0	0	0	-	-	-	-	0.25	0.25
	95%	8	233	301	56	10	12	1	3	0	0	0	98.4	99	0.2	0.8	0.25	
	90%	21	322	229	33	7	9	0	3	0	0	0	97.3	99	0.3	0.6	0.25	
Isavuconazole	Visual	0	0	0	1	10	388	204	6	2	11	2	-	-	-	-	2	2
	95%	0	0	1	3	22	417	157	8	4	9	3	99.2	99.5	0.2	0.3	2	
	90%	0	0	2	7	81	459	56	7	7	4	1	96.3	99.8	0.2	0	2	

*Two two-fold dilutions higher than the modal MIC
Numbers in **bold** indicate modal MIC; EA = essential agreement; CA = categorical agreement; VME = very major error; ME = major error
Figures in green indicate identical wt-UL, modal MIC or wt-UL and ECOFF (epidemiological cut-off values) among MICs obtained by visual and spectrophotometric reading.
Underlined values indicate resistant isolates (EUCAST breakpoint table v. 10.0 , 2020).

- Overall, essential and categorical agreement was 98.8% and 99.4%, respectively, when spectrophotometric endpoint was ≥95%; and 96.5% and 99.6%, respectively, when spectrophotometric endpoint was ≥90%. No very major errors were found in amphotericin B and voriconazole.
- In itraconazole, two very major errors (0.3%) were found with both spectrophotometric endpoints.
- In posaconazole, one very major error (0.2%) was found when spectrophotometric endpoint was ≥95%, and two very major (0.3%) were found when spectrophotometric endpoint was ≥90%.
- In isavuconazole, one very major error (0.2%) was found with both spectrophotometric endpoints.
- Major errors occurred in all the antifungal drugs when spectrophotometric endpoint was ≥95% (n=16) and in all antifungal drugs except isavuconazole when spectrophotometric endpoint was ≥90% (n=8).
- The fungal growth inhibition endpoints that led to modal MIC and wt-UL values identical to the ones obtained by visual inspections were: itraconazole, posaconazole and isavuconazole (≥95% or ≥90%), amphotericin B and voriconazole(≥95%).

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EUCAST-obtained olorofim MICs against non-*fumigatus Aspergillus* clinical isolates: high agreements between visual inspections and spectrophotometric readings

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INTRODUCTION

We previously showed a high agreement between olorofim MIC values obtained by visual inspection and spectrophotometric readings against *Aspergillus fumigatus sensu lato* following EUCAST methodology (DOI: 10.1128/aac.00849-22).

AIM

The objective of this work is to expand those comparisons against non-*fumigatus Aspergillus* spp. clinical isolates.

METHODS

Non-*fumigatus Aspergillus* isolates (n= 439) collected at 55 hospitals in Spain

Sections
Flavi (n=89)
Nidulantes (n=55)
Nigri (n=192)
Terrei (n=103)

Antifungal susceptibility EUCAST 9.4

- **Visual reading**
- **Spectrophotometric readings** (≥95% or ≥90% fungal growth inhibition, read at 540 nm)

- **Essential agreement assessment** (±1 twofold dilution)
- **Setting of wild-type upper limits** (wt-ULs) according to the eye-ball method (2 two-fold dilutions higher than the modal MIC)

RESULTS

Figure 1. MIC distributions and essential agreement between visual and spectrophotometric readings

Sections	Endpoints used to obtain the MIC	MIC distributions (no. of isolates at each MIC, mg/L)										EA (%)	wt-UL	% non-wild type isolates
		0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	≥0.5			
Flavi (n=89)	Visual	0	0	0	10	**64**	14	0	0	0	1	-	0.06	1.1
	95%	0	1	1	35	**44**	7	0	0	0	1	98.9	0.06	1.1
	90%	0	3	13	**46**	25	1	0	0	0	1	80.9	0.03	1.1
Nidulantes (n=55)	Visual	0	0	3	7	**30**	12	2	0	1	0	-	0.06	1.8
	95%	0	3	1	4	**34**	11	0	0	1	1	96.4	0.06	3.6
	90%	0	3	2	10	**34**	4	0	0	1	1	90.9	0.06	3.6
Nigri (n=192)	Visual	0	0	1	4	**121**	52	12	2	0	0	-	0.06	1.0
	95%	0	0	1	1	61	**92**	31	4	2	0	91.1	0.125	3.1
	90%	0	0	1	9	**119**	51	11	0	1	0	98.4	0.06	0.5
Terrei (n=103)	Visual	0	0	27	**65**	9	1	0	0	0	1	-	0.03	1
	95%	0	3	**49**	46	3	1	0	0	0	1	98.1	0.016	1
	90%	0	14	**67**	19	2	0	0	0	0	1	91.3	0.016	1

Numbers in **bold** indicate modal MIC; EA = essential agreement
Figures in green indicate identical wt-UL and modal MIC obtained by visual and spectrophotometric reading

- With only a few exceptions, olorofim MIC values were up to 0.03 mg/L against Flavi and Terrei, 0.06 mg/L against Nidulantes, and 0.125 mg/L against Nigri.
- Essential agreement between visually-obtained MIC values and spectrophotometric-obtained MIC values were higher when the ≥95% fungal growth inhibition endpoint was used (Flavi and Terrei [98.1%]; Nidulantes [96.4%]), except for section Nigri.
- The fungal growth inhibition endpoints that led to modal MIC and wt-UL values identical to the ones obtained by visual inspections were: Flavi section (≥95%), Nidulantes section (≥95% or ≥90%), Nigri section (≥90%).
- In section Terrei, both endpoints led to lower modal MICs and wt-UL values.

CONCLUSIONS

- MICs of olorofim against non-*fumigatus Aspergillus* were low, showing the activity of the drug against the isolates tested.
- Although current E.Def 9.4 procedure version indicates that MIC readings for azoles and amphotericin B values against *A. fumigatus* should be performed using a 90% fungal growth inhibition endpoint, MIC values obtained by spectrophotometer and by visual inspection showed very high agreement values using the 95% fungal growth inhibition endpoint against non-Fumigati section except Nigri, that showed higher agreement using 90% fungal growth inhibition endpoint.

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Detección de resistencia a azoles en *Aspergillus fumigatus* usando placas de agar (EUCAST E.Def 10.2): la preparación del inóculo no requiere filtrado ni ajuste previo

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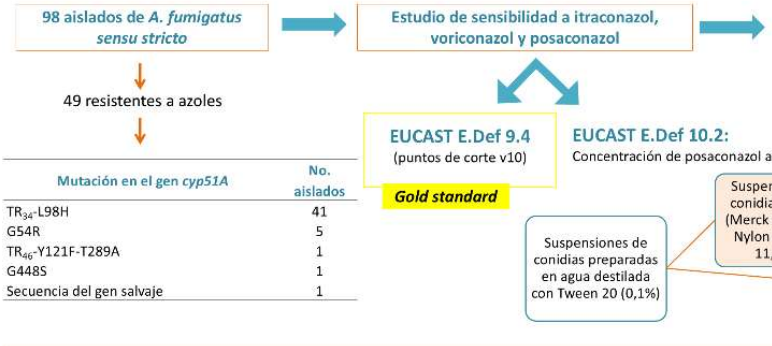
INTRODUCCIÓN Y OBJETIVO

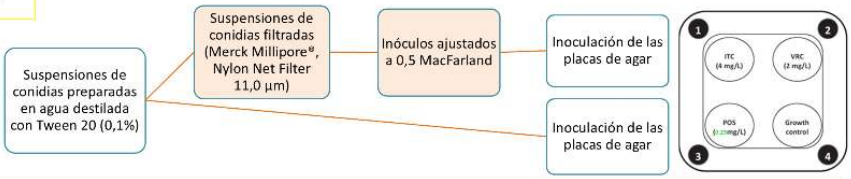
El método de EUCAST de placas de agar suplementado con azoles (E.Def 10.2) permite detectar resistencia a azoles en *A. fumigatus* y es sencillo de implementar en el laboratorio de microbiología clínica. El método recomienda el filtrado de las suspensiones de conidias y su posterior ajuste (0,5 McFarland) lo que conlleva tiempo y aumenta la carga de trabajo.

Se estudió si eliminar el filtrado de las suspensiones de conidias y su posterior ajuste antes de inocular las placas afectaba al rendimiento del método.

MATERIAL Y MÉTODOS



Mutación en el gen *cyp51A*	No. aislados
TR_{34}-L98H	41
G54R	5
TR_{46}-Y121F-T289A	1
G448S	1
Secuencia del gen salvaje	1



Cálculo de sensibilidad, especificidad y acuerdo categórico

- Sensibilidad: probabilidad de que un aislado resistente crezca en uno o más pocillos que contienen azoles.
- Especificidad: probabilidad de que un aislado sensible no crezca en ningún pocillo que contiene azoles.
- Acuerdo categórico: coincidencia en la clasificación de los aislados como S o R tanto por el método de referencia como el procedimiento alternativo.
- Errores mayores (*falsa sensibilidad*): cuando el procedimiento de EUCAST E.Def 9.4 clasifica a un aislado como resistente y el E.Def 10.2 como sensible.
- Errores menores (*falsa resistencia*): cuando el procedimiento de EUCAST E.Def 9.4 clasifica a un aislado como sensible y el E.Def 10.2 como resistente.

RESULTADOS

Tabla 1. Sensibilidad, especificidad y valores de acuerdos categóricos del cribado de la resistencia a azoles utilizando placas de agar y preparando inóculos con y sin filtración y ajuste del McFarland previo.

Antifúngico	Inoculo filtrado y ajustado a 0,5 McF	Sensibilidad	Especificidad	Errores		Acuerdo categórico
				Mayores	Menores	
Itraconazol	No	100%	100%	0	0	99%
	Si	97,9%	100%	1% (n=1)	0	
Voriconazol	No	100%	100%	0	0	100%
	Si	100%	100%	0	0	
Posaconazol	No	93,8%	100%	3,1% (n=3)	0	94,9%
	Si	83,3%	100%	8,2% (n=8)	0	

Tabla 2. Errores mayores (*falsa sensibilidad*) encontrados en los aislados usando las dos formas de preparación del inóculo. Se muestran los valores de CMI en las discrepancias.

Código aislado	Mutaciones en el gen *cyp51A*	E.Def 9.4 (CMI, en mg/L)/agar con inóculo sin filtrar ni ajustar McF/agar con inóculo filtrado y ajustado McF		
		Itraconazol	Voriconazol	Posaconazol
3917	G448S	R(2)/R/S	R/R/R	R(0,25)/R/S
6407	TR_{34}-L98H	R/R/R	R/R/R	R(0,5)/R/S
6422	TR_{34}-L98H	R/R/R	R/R/R	R(0,25)/S/S*
8636	TR_{34}-L98H	R/R/R	R/R/R	R(0,5)/R/S
8682	TR_{34}-L98H	R/R/R	R/R/R	R(0,5)/R/S
9098	TR_{34}-L98H	R/R/R	R/R/R	R(0,5)/S/S*
9244	TR_{34}-L98H	R/R/R	R/R/R	R(0,5)/R/S
10007	TR_{34}-L98H	R/R/R	R/R/R	R(0,5)/S/S*

* Aislados con *falsa sensibilidad* preparando los inóculos sin filtrar ni ajustar.

- La sensibilidad y la especificidad global del método de placas de agar suplementado con azoles fue del 100%, independientemente de la filtración y el ajuste de McFarland.
- El acuerdo categórico de la comparación de ambas formas de preparación del inóculo para itraconazol, voriconazol y posaconazol fue, respectivamente, del 99%, 100% y 94,9%.
- Todos los aislados con mutaciones TR_{34}-L98H, G54R y TR_{46}-Y121F-T289A fueron clasificados correctamente como resistentes a itraconazol y voriconazol.
- En itraconazol, se detectó *falsa sensibilidad* (errores mayores) en un aislado con una mutación G448S con una CMI de 2 mg/L, al utilizar el inóculo filtrado y ajustado a 0,5 McFarland.
- En posaconazol, al utilizar el inóculo sin filtrar y sin ajuste de McFarland, se detectó *falsa sensibilidad* en tres aislados con CMIs de 0,25 mg/L y 0,5 mg/L, portadores de la sustitución TR_{34}-L98H. Al utilizar el inóculo filtrado y ajustado a 0,5 McFarland, se detectó *falsa sensibilidad* en estos mismos tres aislados y en otros cuatro aislados más, portadores de la sustitución TR_{34}-L98H con CMIs de 0,5 mg/L y en un aislado con una mutación G448S con una CMI de 0,25 mg/L.
- No se detectó *falsa resistencia* (errores menores).

CONCLUSIONES

- Las suspensiones de conidias de aislados de *A. fumigatus sensu stricto* preparadas sin filtración ni ajuste de inóculo previo a la inoculación aumentó el rendimiento del procedimiento E.Def 10.2.
- Esta modificación metodológica puede ayudar a implantar el cribado de resistencia a azoles en *A. fumigatus* en el laboratorio de micología clínica.

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Olorofim showed potent *in vitro* activity against *Aspergillus* spp. clinical isolates collected in a national survey on azole resistance conducted in Spain in 2022 (*ASP*EIN II)

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INTRODUCTION

Olorofim is a new antifungal drug belonging to a novel antifungal class (orotomides) and shows *in vitro* antifungal activity against *Aspergillus* isolates, including azole-resistant ones.

We previously showed a high agreement between olorofim MIC values obtained by visual inspection and spectrophotometric readings against *Aspergillus fumigatus sensu lato* following EUCAST E.Def 9.4 methodology (DOI: 10.1128/aac.00849-22).

AIM

The objective of this work is to expand those comparisons against a large collection of *Aspergillus fumigatus sensu lato* as well as other *Aspergillus* sections (Nigri, Terrei, Flavi, and Nidulantes) collected as part of a national survey on azole resistance conducted in Spain in 2022 (*ASP*EIN II study).

METHODS

Aspergillus spp. isolates (n=2,108) collected at 87 Spanish hospitals

Sections
Fumigati (n=1,244)
Nigri (n=351)
Terrei (n=205)
Flavi (n=167)
Nidulantes (n=141)

Antifungal susceptibility to olorofim obtained following EUCAST E.Def 9.4 methodology

- Visual readings
- Spectrophotometric readings (two endpoints [≥95% or ≥90% fungal growth inhibition], read at 540 nm wavelength)

- Essential (±1 two-fold dilutions) agreement assessment
- Setting of wild-type upper limits (wt-ULs) using ECOFFinder software and the eye-ball method (two two-fold dilutions higher than the modal MIC)

RESULTS

Table 1. MIC distributions, essential agreement among visual and spectrophotometric readings (≥95% or ≥90% fungal growth inhibition) and wt-ULs obtained

Section	Endpoints used to obtain the MIC	MIC distributions (no. of isolates at each MIC, mg/L)										EA (%)	wt-UL				
		0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	≥0.5		Statistical wt-UL at each percentage of modelled population				Eye-ball method*
													95%	97.5%	99%	99.9%	
Fumigati (n=1244)	Visual	0	1	7	101	**895**	239	1	0	0	0	-	0.03	0.03	0.03	0.06	0.06
	95%	0	3	14	138	**821**	251	17	0	0	0	98.6	0.03	0.03	0.03	0.06	0.06
	90%	2	6	65	367	**741**	60	3	0	0	0	94.6	0.03	0.03	0.03	0.03	0.06
Flavi (n=167)	Visual	0	0	0	17	**118**	32	0	0	0	0	-	0.03	0.03	0.03	0.03	0.06
	95%	0	1	1	57	**90**	18	0	0	0	0	99.4	0.03	0.03	0.03	0.03	0.06
	90%	0	3	19	**91**	50	4	0	0	0	0	86.8	0.016	0.03	0.03	0.03	0.03
Nidulantes (n=141)	Visual	1	6	20	24	**56**	26	7	0	1	0	-	0.06	0.06	0.125	0.25	0.06
	95%	1	18	15	19	**68**	17	1	0	1	1	94.3	0.06	0.06	0.06	0.125	0.06
	90%	4	19	12	34	**60**	10	0	1	0	1	87.2	0.03	0.06	0.06	0.125	0.06
Nigri (n=351)	Visual	0	0	1	17	**197**	103	29	2	1	1	-	0.03	0.03	0.03	0.06	0.06
	95%	0	0	1	10	102	**165**	63	5	0	5	91.7	0.06	0.06	0.125	0.125	0.125
	90%	0	1	2	27	**195**	104	19	0	2	1	98.9	0.03	0.03	0.06	0.06	0.06
Terrei (n=205)	Visual	0	1	39	**121**	42	2	0	0	0	0	-	0.016	0.016	0.016	0.03	0.03
	95%	0	8	69	**107**	19	2	0	0	0	0	98	0.016	0.016	0.016	0.03	0.03
	90%	0	24	**111**	64	6	0	0	0	0	0	91.2	0.008	0.008	0.016	0.016	0.016

Numbers in **bold** indicate modal MIC; modal MICs in green indicate identical values obtained by visual and spectrophotometric readings; EA = essential agreement; wt-UL = wild-type upper limits; *Two two-fold dilutions higher than the modal MIC

Table 2. MICs of olorofim against azole-resistant isolates

Section	MICs (using visual endpoints) in mg/L	
	Ranges	Modal values
Fumigati (n=69)	0.02-0.06	0.016
Flavi (n=3)	0.08-0.03	0.016
Nidulantes (n=47)	0.001-0.25	0.016
Nigri (n=6)	0.004-0.5	0.016
Terrei (n=12)	0.002-0.03	0.008

- With only a few exceptions, olorofim MIC values were up to 0.03 mg/L against Flavi and Terrei, 0.06 mg/L against Fumigati and Nidulantes, and 0.125 mg/L against Nigri (Table 1).
- MIC distributions obtained using any of the three endpoints led to modal MICs within ±1 two-fold dilutions (Table 1).
- Essential agreements between visually-obtained MIC values and spectrophotometric-obtained MIC values were higher when the ≥95% fungal growth inhibition endpoint was used (Fumigati [98.6%]; Flavi [99.4%]; Nidulantes [94.3%]; Terrei [98%]) except for section Nigri. However, essential agreements when the ≥90% fungal growth inhibition endpoint was used were also high and ≥ 87% for all sections (Table 1).
- The fungal growth inhibition endpoints that led to wt-UL values identical to the ones obtained by visual inspections were: Fumigati, Flavi and Nidulantes section (≥95% or ≥90%), Terrei section (≥95%), Nigri section (≥90%) (Table 1).
- Activity of the drug was unaffected by the presence of azole resistance (Table 2).

CONCLUSIONS

- Olorofim MIC values against most clinically relevant *Aspergillus* spp. were low, including azole-resistant isolates, thus proving the high drug activity.

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Gradient diffusion plastic strip for detection of azole resistance in *Aspergillus fumigatus sensu stricto*: inoculum preparation might not require prior filtration and adjustment

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INTRODUCTION

Gradient diffusion plastic strip are widely utilized for azole susceptibility testing in clinical microbiology laboratories, but they lack proper validation. We previously proposed cut-off values for plastic-strip-obtained MICs of azoles against *A. fumigatus sensu stricto* to separate susceptible and resistant isolates: itraconazole >1.5 mg/L, voriconazole >0.38 mg/L, and posaconazole >0.75 mg/L (DOI: 10.1111/myc.13541).

AIM

The aim of this work is to assess the performance of the method to detect azole resistance in *A. fumigatus sensu stricto* by including a larger number of isolates and to assess whether skipping filtration and inoculum adjustment of conidial suspensions negatively influences its performance.

METHODS

***Aspergillus fumigatus sensu stricto* isolates (n= 98)**

Antifungal susceptibility to itraconazole, voriconazole, and posaconazole

- **Gradient diffusion plastic strips obtained MIC distributions**
- **Cut-off setting to separate resistant isolates from susceptible ones**

- **Essential agreement:** among unfiltered-unadjusted conidial suspensions and filtered-adjusted conidial suspensions
- **Sensitivity:** probability that a resistant isolate had an MIC higher than the cut-off value
- **Specificity:** probability that a susceptible isolate had an MIC lower than the cut-off value

Table 1. *cyp51A* gene substitutions found in the azole-resistant isolates

cyp51A gene substitutions	No. of isolates
TR_{34}-L98H±S297T-F495I	41
G54	5
TR_{46}-Y121F-T289A	1
G448S	1
None (*cyp51A* gene wild-type)	1

EUCAST 9.4 (EUCAST breakpoints v.10) Gold standard

Gradient diffusion plastic strips (ETEST®, Biomerieux, Marcy-l'Etoile, France): MICs were obtained after 48h of incubation

All discrepancies between EUCAST 9.4 and gradient diffusion plastic strips method were retested

Conidial suspensions prepared in distilled water (Tween 20 0.1%)

Inocula filtered (Merck Millipore®, Nylon Net Filter 11.0 µm)

Swab soaked into the suspension and agar RPMI plate streaked

Inocula adjusted to 0.5 McFarland (2×10^6 - 5×10^6 CFU/mL)

Swab soaked into the inoculum and agar RPMI plate streaked

Figure 1. Setting MIC values by using gradient diffusion plastic strips (orange arrow)

RESULTS

- According to the gradient diffusion-plastic-strip-obtained MIC distributions against the isolates, regardless the kind of inocula used, the following cut-off MIC values classified isolates as resistant: >1 mg/L for itraconazole, >0.5 mg/L for voriconazole, and >0.25 mg/L for posaconazole (Figure 1, vertical red line).
- High essential agreement values among MIC values obtained using filtered-adjusted inocula and unfiltered-unadjusted inocula were observed: itraconazole (95.9%), and voriconazole and posaconazole (98%) (Table 1).
- Overall, sensitivity/specificity values of the proposed cut-off values for itraconazole (100%/98%), voriconazole (100%/100%), and posaconazole (≥98%/98%) to screen for azole resistance were high and not impacted by skipping inoculum adjustment and filtering (Table 1). Only two isolates were misclassified and harboured the following *cyp51A* gene substitutions: G448S and TR_{46}-Y121F-T289A (Table 2).

Figure 1. Gradient-diffusion-plastic-strip-obtained MIC distributions against the isolates and using filtered-adjusted conidial inocula (bars above the X-axis) and with unfiltered-unadjusted conidial inoculum (bars below the X-axis). The red lines indicate the proposed cut-offs

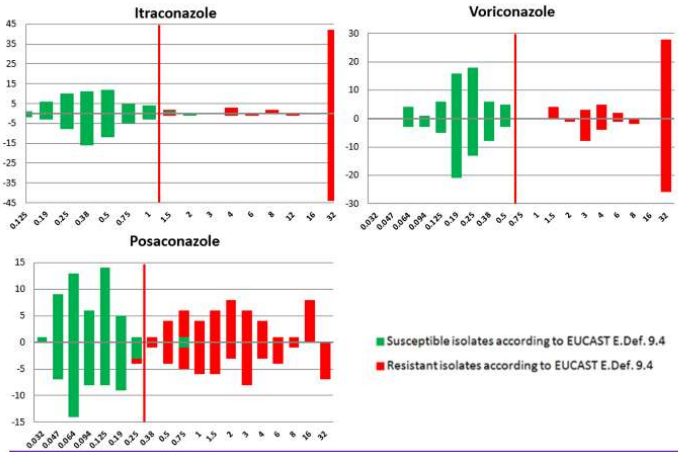

Table 1. Essential agreements between gradient diffusion plastic strips using filtered-adjusted and unfiltered-unadjusted conidia suspensions and their sensitivity and specificity

Drug	Conidia suspension preparation	Essential agreement	Sensitivity	Specificity
Itraconazole	Filtered-adjusted	95.9%	100%	98%
	Unfiltered-unadjusted		100%	98%
Voriconazole	Filtered-adjusted	98%	100%	100%
	Unfiltered-unadjusted		100%	100%
Posaconazole	Filtered-adjusted	98%	100%	98%
	Unfiltered-unadjusted		98%	98%

Table 2. *A. fumigatus sensu stricto* isolates misclassified by gradient diffusion plastic strips according to the proposed cut-off values

Isolate code	*cyp51A* gene sequence	E.Def 9.4 method/gradient diffusion plastic strip with filtered and adjusted inoculum/ gradient diffusion plastic strip with unfiltered and unadjusted inoculum (MIC, in mg/L)		
		Itraconazole	Voriconazole	Posaconazole
3917	G448S	R(2)/1.5/1.5	R(16)/32/32	**R(0.25)**/0.38/0.25
6544	TR_{46}/Y121F/T289A	**S(0.5)**/1.5/2	R(16)/32/32	**S(0.25)**/0.75/0.75

Bold "R" letters in red and "S" letters in green indicate, respectively, resistance or susceptibility according to the microdilution E.Def 9.4 method. MIC, minimum inhibitory concentration.

CONCLUSIONS

- Gradient diffusion plastic strip is a helpful method to screen for azole resistance in *A. fumigatus sensu stricto* isolates when MICs obtained are interpreted as follows: itraconazole >1 mg/L, voriconazole >0.5 mg/L, and posaconazole >0.25 mg/L.
- Preparation of inoculum avoiding filtration and further inoculum adjustment did not negatively influence the performance of the method.

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