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Aspergillus spp.: SUSCETIBILIDADE AOS ANTIFÚNGICOS ISOLADOS E EM ASSOCIAÇÃO POR DIFERENTES METODOLOGIAS E DETECÇÃO DE MECANISMOS DE RESISTÊNCIA A AZÓLICOS

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Farmacêuticas, da Universidade Federal de Santa Maria (UFSM, RS, Brasil), como requisito para obtenção do grau de **Doutor em Ciências Farmacêuticas**

Orientador: Dr. Sydney Hartz Alves
Coorientador: Dr. Régis Adriel Zanette

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Aprovado em 19 de janeiro de 2018:

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EPÍGRAFE

"We can only see a short distance ahead, but we can see plenty there that needs to be done."
(Alan Turing, 1950).

RESUMO

Aspergillus spp.: SUSCETIBILIDADE AOS ANTIFÚNGICOS ISOLADOS E EM ASSOCIAÇÃO POR DIFERENTES METODOLOGIAS E DETECÇÃO DE MECANISMOS DE RESISTÊNCIA A AZÓLICOS

AUTORA: LAURA BEDIN DENARDI
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As aspergiloses aparecem entre as principais causas de morbidade e mortalidade em pacientes em estado de imunossupressão. *Aspergillus fumigatus* é a espécie mais comum causadora de aspergilose invasiva e não-invasiva em humanos, seguida de *Aspergillus flavus*, *Aspergillus niger* e *Aspergillus terreus*. O tratamento destas infecções é efetuado principalmente através da administração de antifúngicos azólicos, sendo voriconazol o fármaco de escolha na aspergilose invasiva. Equinocandinas e anfotericina B também são ativas frente a *Aspergillus* e são atualmente utilizadas em situações específicas ou em associação a azólicos. Resistência aos azólicos tem sido documentada de forma crescente entre os centros médicos pelo mundo, principalmente associada a *A. fumigatus*, e vem se tornando um problema terapêutico de grande relevância. O desenvolvimento de resistência tem sido associado a mutações no gene *cyp*51A de *A. fumigatus*. Duas vias de seleção de resistência têm sido estudadas, a via no paciente, e mais recentemente, uma rota ambiental. Com o objetivo de superar a resistência, vem se estudando alternativas, como o uso de fármacos combinados no tratamento de pacientes com aspergilose refratária ao tratamento padrão. Neste estudo buscou-se determinar o perfil de suscetibilidade de *A. fumigatus* e *A. flavus*, isolados de pacientes com aspergilose ou do ambiente, frente aos antifúngicos azólicos, equinocandinas e anfotericina B, bem como frente a associações destes por diferentes metodologias. Além disso, foram investigados os mecanismos de resistência de *A. fumigatus* de origem clínica ou ambiental que apresentaram resistência a um ou mais antifúngicos azólicos. Os principais resultados observados mostraram que tanto nos isolados ambientais quanto nos clínicos alguns perfis resistentes a azólicos foram detectados, enquanto que as equinocandinas mostraram-se bastante ativas frente as duas espécies de *Aspergillus* e a anfotericina B mostrou atividade mais pronunciada frente a *A. fumigatus* do que a *A. flavus*. Além disso, verificou-se que as metodologias de Etest e crescimento em ágar contendo azólicos são capazes de determinar a concentração inibitória mínima (CIM) e detectar resistência de *A. fumigatus* ao isavuconazol, respectivamente, em concordância com a metodologia padrão de microdiluição em caldo. As interações entre antifúngicos azólicos e equinocandinas frente a *A. fumigatus* variaram dependendo da metodologia empregada, tornando-se muito importante a padronização destes métodos para obtenção de resultados concordantes. Contudo, as interações sinérgicas encontradas *in vitro* e *in vivo* mostram um efeito favorável ao uso destas associações no tratamento das aspergiloses. Nas combinações de azólicos e equinocandinas frente a *A. flavus*, uma maior porcentagem de interações sinérgicas foi observada utilizando como base a leitura da CIM, enquanto que pela leitura da concentração efetiva mínima (CEM) indiferença foi o principal resultado. Ainda, verificou-se a presença de mutações ligadas ao gene *cyp*51A em uma cepa clínica e em uma ambiental de *A. fumigatus* resistentes ao itraconazol, descritas pela primeira vez no Brasil. Conclui-se que as combinações entre azólicos e equinocandinas são promissores para uso no tratamento de pacientes com aspergilose refratária ao tratamento padrão; diferentes perfis de suscetilidade de *Aspergillus* a antifúngicos testados isoladamente e em associação podem ser encontrados dependendo da metodologia utilizada, fazendo-se necessária a padronização dos diferentes testes na busca de metodologias mais fáceis e rápidas que facilitem seu uso em laboratórios de rotina. Finalmente, o primeiro relato da presença de mecanismos de resistência a azólicos ligadas ao gene *cyp*51A em isolados brasileiros de *Aspergillus* gera preocupação com o gerenciamento das aspergiloses nos centros clínicos do Brasil a fim de evitar que as taxas de resistência elevem-se pelo uso indiscriminado de azólicos na clínica e na agricultura.

Palavras-chave: *Aspergillus* spp. Resistência a azólicos. Interações entre antifúngicos. Aspergilosis

ABSTRACT

Aspergillus spp.: SUSCEPTIBILITY TO ANTIFUNGAL DRUGS ALONE AND IN COMBINATION USING DIFFERENT METHODOLOGIES AND DETECTION OF AZOLE RESISTANCE MECHANISMS

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Aspergillosis is a group of diseases among the main causes of morbidity and mortality in immunosuppressed patients. *Aspergillus fumigatus* is the most common species that causes invasive and non-invasive aspergillosis in humans, followed by *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus terreus*. Azole antifungals are the main treatment of these infections, with voriconazole being the drug of choice in invasive aspergillosis. Echinocandins and amphotericin B are also active against *Aspergillus* and are currently used in specific situations or in association with azoles. Azole resistance has been increasingly documented among medical centers around the world, mainly associated with *A. fumigatus*, and has become a major therapeutic problem. The development of resistance has been associated with mutations in the *cyp*51A gene of *A. fumigatus*. Two pathways of resistance selection have been studied, the pathway in the patient, and more recently, an environmental route. In order to overcome resistance, alternatives such as the use of combination drugs have been studied in the treatment of patients with aspergillosis refractory to standard treatment. This study aimed to determine the susceptibility profile of *A. fumigatus* and *A. flavus*, isolated from patients with aspergillosis or from the environment against azole antifungals, echinocandins and amphotericin B, alone and in combination, by using different methodologies. In addition, resistance mechanisms of clinical or environmental strains of *A. fumigatus* that showed resistance to one or more azole antifungals were investigated. The main results showed that in either environmental or clinical strains some azole resistant profiles were detected, while the echinocandins were very active against both species of *Aspergillus.* Amphotericin B showed more pronounced activity against *A. fumigatus* in comparison to *A. flavus*. In addition, Etest and agar plates with azole methodologies have been found to be capable of determining the minimum inhibitory concentration (MIC) and detecting resistance of *A. fumigatus* to isavuconazole, respectively, in accordance with the standard broth microdilution method. The interactions between azole and echinocandin antifungal agents against *A. fumigatus* varied depending on the methodology used, making it very important to standardize these methods to obtain concordant results. However, the synergistic interactions found *in vitro* and *in vivo* showed a favorable effect to the use of these associations in the treatment of aspergillosis. In the azole-echinocandin combinations against *A. flavus*, a higher percentage of synergistic interactions were observed when the MIC was read while by reading the minimum effective concentration (MEC) indifference was the main outcome. Furthermore, mutations linked to the *cyp*51A gene were found in one clinical and one environmental itraconazole-resistant *A. fumigatus* strains. In conclusion, combinations between azoles and echinocandins are promising for use in the treatment of patients with aspergillosis refractory to standard treatment; different *Aspergillus* susceptibility profiles to antifungals tested alone and in association can be found depending on the methodology used, making it necessary to standardize the different tests in order to have easier and faster methodologies that facilitate their use in routine laboratories. Finally, the first report of the presence of mechanisms of resistance to azoles linked to the *cyp*51A gene in Brazilian *Aspergillus* isolates generates concern with the management of aspergillosis in clinical centers of Brazil in order to avoid that the resistance rates increase by the indiscriminate use of azoles in the clinic and in agriculture.

Key words: *Aspergillus* spp. Azole Resistance. Antifungal Interactions. Aspergillosis

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1 INTRODUÇÃO

As infecções fúngicas oportunistas são as principais causas de doenças em pacientes com algum tipo de imunossupressão. *Aspergillus* spp. é o principal gênero de fungos filamentosos responsáveis por causar infecções em diferentes sítios nestes pacientes (HERBRECHT et al, 2012).

O gênero *Aspergillus* é responsável por causar diversas manifestações clínicas patológicas no homem, todas elas reconhecidas como aspergiloses. O espectro das aspergiloses inclui: i) infecção invasiva com risco de vida em pacientes imunocomprometidos; ii) infecção crônica ou subaguda em pacientes com doença pulmonar ou nos seios da face preexistente e com provável defeito na imunidade inata; iii) doença eosinofílica ou alérgica que se manifesta em muitas formas incluindo aspergilose broncopulmonar alérgica, rinossinusite eosinofílica e alveolite alérgica extrínseca; iiii) infecção invasiva localizada como resultado de um trauma ou cirurgia, especialmente ceratites e infecção pós-operatória (SHERIF et al, 2010; DENNING, 2009).

Aspergillus fumigatus é, atualmente, a espécie responsável pela maioria dos casos de aspergilose, seguida de *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus* e *Aspergillus nidulans*, no entanto, nos últimos anos vêm aumentando a taxa de infecções causadas por *Aspergillus* não-*fumigatus* (STEINBACH et al, 2004; MARR et al, 2002) ocorrendo variação entre os centros com relatos de maior incidência de *A. flavus* e *A. terreus* (PASQUALOTTO et al, 2009).

Até a década de 1990, quando o itraconazol foi introduzido, os tratamentos disponíveis para aspergilose eram a flucitosina, e/ou anfotericina B, acompanhado, ou não, de cirurgia. Com a introdução do itraconazol, o primeiro antifúngico azólico oral efetivo para aspergilose, iniciaram-se os estudos sobre profilaxia, seleção da terapia primária, monitorização terapêutica e definições da doença. Coincidentemente, nos anos 90 também ocorreram os primeiros relatos de resistência aos azólicos, que é um problema crescente atualmente. Ao longo dos anos foram se estabelecendo novas alternativas de tratamento com o uso de anfotericina B complexo-lipídico, novos antifúngicos azólicos, como voriconazol, posaconazol e isavuconazol, e ainda uma nova classe com ação específica na parede celular fúngica, as equinocandinas; porém há relatos de resistência mesmo aos antifúngicos mais recentes (DENNING, 2009; MAERTENS et al, 2011).

A resistência de *Aspergillus* spp. aos antifúngicos azólicos tem agravado os problemas terapêuticos, impossibilitando o uso de terapia oral no tratamento da aspergilose o que torna a profilaxia com azólicos, ainda, objeto de muita controvérsia. Atualmente há duas supostas vias de seleção de resistência. Azol-resistência pode ser selecionada nos pacientes durante a terapia com azólicos, e também estudos mais recentes têm mostrado que é possível uma via de seleção de resistência no meio ambiente (CHEN et al, 2005; HOWARD et al, 2009; VAN DEN LINDEN et al, 2011). Em resposta as falhas terapêuticas na clínica, o uso de estratégias que permitam melhor compreensão das vias de resistência, visando o controle mais eficaz no uso de antifúngicos, diagnósticos mais precisos e precoces da aspergilose, assim como novas e mais eficazes alternativas de tratamento se fazem necessárias. A terapêutica antifúngica baseada na combinação de fármacos está entre estas estratégias.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 O gênero *Aspergillus*

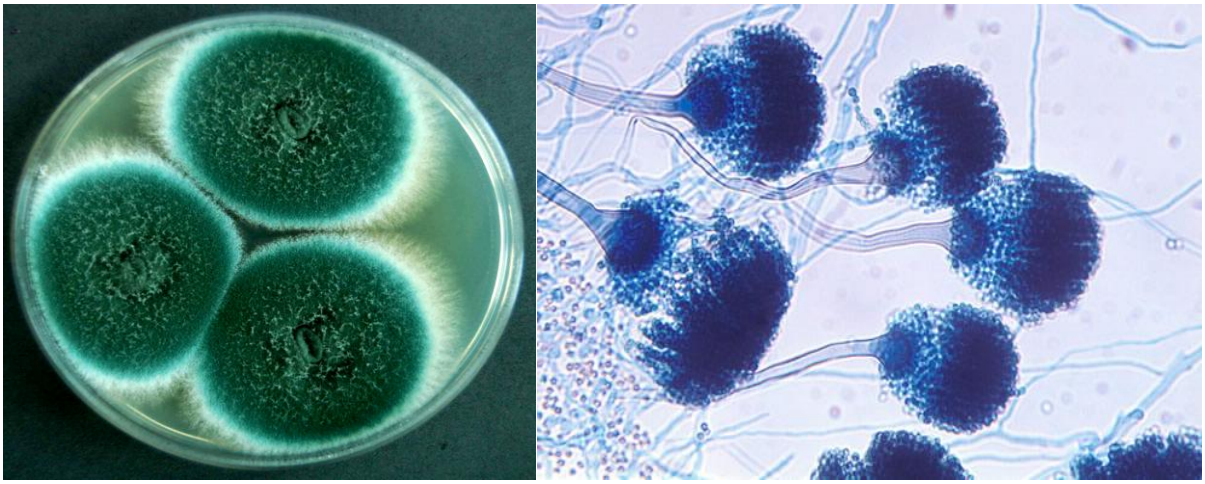
Os fungos filamentosos do gênero *Aspergillus* pertencem à família Aspergillaceae, a classe dos Ascomicetos e à subclasse Euascomycetae (SHARMA & CHWOGULE, 1998; LARONE, 2002). Este gênero compreende mais de 200 espécies, das quais em torno de 34 têm sido descritas como patógenos em humanos (BARNES & MARR, 2006).

As hifas do micélio vegetativo de *Aspergillus* são hialinas e septadas, estes fungos produzem dois tipos de propágulos: os conídios, formados por mitoses, e os ascósporos, formados por meioses. A reprodução assexuada forma estruturas especializadas chamadas de conidióforos, essas estruturas emergem do micélio vegetativo como hifas eretas, septadas, de parede espessa que se expandem formando uma vesícula na região apical. A partir desta vesícula numerosas células conidiogênicas surgem de forma sincronizada, chamadas de fiálides, em cujo interior são produzidos os conídios. Os conídios são formados em cadeias interligadas. As fiálides são formadas por um único elemento ou por curtos ramos (métulas), quando as métulas são formadas as células conidiogênicas são ditas bisseriadas, e na ausência das mesmas são consideradas unisseriadas. O conjunto de vesículas, fiálides e conídios é denominado cabeça aspergilar (Figura 1) (LARONE, 2002).

Os conídios reprodutivos são produzidos em grande número, e por apresentar um tamanho pequeno e uma camada externa hidrofóbica ficam suspensos no ar por várias horas, em repouso, encontram-se quiescentes mantendo-se viáveis por meses. A concentração média dos propágulos de *Aspergillus* spp no ar é de 0,2 a 15 conídios/ m³ (PARK & MEHRAD, 2009).

Em seu aspecto macroscópico as colônias apresentam-se com superfície de coloração branca no início da sua maturação, todavia dependendo da espécie a sua cor pode se tornar verde, amarela, laranja, castanha ou preta. No seu verso a colônia apresenta-se geralmente branca, acastanhada ou dourada. A textura da colônia é algodonosa tornando-se pulverulenta com a produção dos conídios (Figura 1) (LARONE, 2002).

Figura 1: Aspecto macroscópico e microscópico da colônia de *Aspergillus fumigatus* em ágar *Czapek* a 25 °C por cinco dias.



Fonte: http://fungi.myspecies.info/sites/fungi.myspecies.info/files/DSC_0033.jpg
http://www.aspergillus.org.uk/sites/default/files/pictures/combined_images/041a.jpg

As espécies de *Aspergillus* têm uma vasta distribuição, são típicos do solo, comum em climas quentes, mas também podem ser encontrados em climas extremos. A grande maioria das espécies possui capacidade de se desenvolver em ambientes extremos, a exemplo de *A. fumigatus* que é termotolerante, capaz de crescer em temperatura de até 55 °C (HEDAYATI et al, 2007).

A. fumigatus e *A. flavus* são fungos ubíquos. *A. fumigatus* é abundante no ar durante o tratamento de resíduos biológicos. Infecções nosocomiais por estas duas espécies em pacientes imunocomprometidos pode ser devido à falta de higiene ou obras de construção nas proximidades. *A. fumigatus* é responsável por aproximadamente 90% das aspergiloses invasivas diagnosticadas, o que se justifica devido à alta presença desta espécie no ar, e ao

pequeno tamanho de seus conídios que podem ser facilmente inalados e penetram facilmente nas áreas mais profundas dos pulmões. Apesar de *A. flavus* ser capaz de causar infecção em humanos, principalmente no trato respiratório superior, a maior preocupação relacionada à presença desta espécie contaminando vegetais como milho, amendoim, algodão e nozes é sua capacidade de produção de aflatoxinas, metabólito secundário com grande potencial carcinogênico (HEDAYATI et al, 2007).

A. niger e *A. terreus* são menos comuns causando infecções em relação às duas espécies descritas acima. Enquanto que *A. niger* é osmotolerante e principal agente etiológico fúngico de otomicoses, *A. terreus* é um saprófita do solo e produtor de muitos metabólitos secundários, sendo causa comum de aspergilose broncopulmonar invasiva ou alérgica, cutânea, oftálmica e onicomicoses (LASS-FLORL et al, 2012; PERSON et al, 2010).

2.1.1 Taxonomia

Recentemente a taxonomia de *A. fumigatus* tem sido revisada pela incorporação de informações baseadas na sequência de seu DNA. Até agora, as sequências ß-tubulina parcial e calmodulina são os mais promissores lócus para identificação de *Aspergillus* em nível de espécie. *Aspergillus* seção Fumigati (também denominada complexo *Aspergillus fumigatus*) previamente identificada como *A. fumigatus,* baseada nas características macroscópicas e microscópicas, agora contém 33 diferentes espécies, 10 anamorfas e 23 teleomorfas (SAMSOM, et al, 2007).

A secção Flavi (também denominada complexo *Aspergillus flavus*) previamente identificada como *Aspergillus flavus*, atualmente contém 20 espécies anamorfas e cinco teleomorfas (HEDAYATI et al, 2007; PILDAIN et al, 2008). Apesar de vários estudos sobre esta seção terem sido realizados (PILDAIN et al, 2008; GEISER et al, 2000) nos últimos anos, a identificação em nível de espécie tem sido difícil devido à sobreposição de algumas características morfológicas consideradas importantes e a variação de características dentro de uma mesma espécie (SAMSOM et al, 2007).

2.2 Patologia das aspergiloses

A principal via de entrada de *Aspergillus* spp. no organismo é através da inalação dos conídios. As espécies de *Aspergillus* produzem um grande número de conídios secos e hidrofóbicos que são facilmente inalados (GUARRO et al, 2010).

A aspergilose compreende uma variedade de manifestações clínicas que dependem fundamentalmente do estado imune e/ou anatômico do hospedeiro, do grau de alergenicidade e da frequência de exposição aos propágulos fúngicos. Deste modo, a grande maioria dos indivíduos saudáveis entra em contato com o fungo e o elimina sem que ele cause qualquer dano, porém um desiquilíbrio fungo/hospedeiro pode levar ao desenvolvimento de diversas formas de aspergilose (SHIBUYA et al, 2004).

Consideram-se três categorias de aspergilose: i) infecções invasivas, caracterizadas pelo crescimento de hifas nos tecidos (aspergilose pulmonar invasiva, rinossinusite invasiva, aspergilose traqueobrônquica, aspergilose pulmonar necrotizante crônica), ii) colonização de superfícies mucosas sem invasão tecidual (bola fúngica pulmonar, doença pulmonar obstrutiva crônica, bronquiectasias) e iii) hipersensibilidade (aspergilose broncopulmonar alérgica, asma, sinusite alérgica, pneumonite) (PARK & MEHRAD, 2009).

Os pacientes de risco para aspergilose são aqueles com prolongada neutropenia, em tratamento com esteroides, transplantados halogênicos ou de pulmão com doença granulomatosa crônica, internados em unidades de terapia intensiva e portadores de imunodeficiências herdadas ou adquiridas (GUARRO et al, 2010; PATTERSON et al, 2016). Nestes pacientes a taxa de morbidade e mortalidade pode variar de 20 a 60% quando há o desenvolvimento de aspergilose invasiva, dependendo do estado imune e da ocorrência de disseminação ou não (PAGANO et al, 2007). Cavidades não pulmonares podem também ser colonizadas, a exemplo das cavidades sinusais (GUARRO et al, 2010).

É em geral aceito que muitos dos pacientes imunocomprometidos se contaminem fora do hospital, e a doença torna-se clinicamente evidente durante a hospitalização, quando tratamentos imunossupressivos são administrados (EINSELE et al, 1998). Nos pacientes imunocompetentes, além de manifestações alérgicas, bola fúngica pulmonar também pode ocorrer (LATGÉ, 1999).

Relata-se que a frequência de isolamento de *Aspergillus* spp. a partir de amostras do trato respiratório é de 16,3 casos em cada 1000 pacientes hospitalizados com doença pulmonar obstrutiva crônica (DPOC) (GUINEA et al, 2010). Transplantados de órgãos sólidos apresentam a maior frequência de aspergilose invasiva como complicação. Em transplantados hepáticos a incidência é de 1-9%, e em transplante pulmonar é de 5-20% (DE

PAUW et al, 2008). Em pacientes submetidos a transplante de células hematopoiéticas observa-se que a aquisição da aspergilose tem um pico de incidência precocemente (<20 dias) após o transplante, e outro pico em torno de 100 dias após o procedimento (MARR et al, 2002). Um dos fatores que tem contribuído para esta incidência é o uso de altas doses de corticoides utilizadas no tratamento da doença do enxerto contra o hospedeiro (WALD et al, 1997).

Complexo *A. fumigatus* é o mais prevalente em infecções pulmonares invasivas, já que possui um pequeno conídio (23 µm) que favorece sua deposição nos alvéolos pulmonares (GUARRO et al, 2010). Infecções invasivas muitas vezes se originam de uma infecção no trato respiratório superior, como sinusites nasais ou a partir de infecções superficiais. Complexo *A. fumigatus* também são os principais agentes de aspergilose em pacientes com imonudefciência primária, causando doença oportunista, caracterizada como colonização ou invasão, geralmente acompanhada por reações alérgicas (GUARRO et al, 2010).

Este complexo também é responsável pela maioria dos casos de alergia, apesar de outras espécies como *A. flavus*, *A. niger* e *A. oryzae* também causarem reações alérgicas. Mais de 20 alérgenos produzidos por *A. fumigatus* já foram caracterizados (CHAKRABARTI et al, 2002). Outras infecções oportunistas causadas por complexo *A. fumigatus* incluem otomicoses, infecções oculares, onicomicoses e endocardites (PASQUALOTTO et al, 2009). Esta espécie se destaca pelos seus fatores de virulência, tem uma excepcional versatilidade fisiológica, sendo capaz de crescer em altas temperaturas e apresenta reação tecidual bastante intensa (SEVERO, 1997; TARRAND et al, 2005).

Complexo *A. flavus* é a segunda causa principal de aspergilose invasiva e não-invasiva, depois do complexo *A. fumigatus* (DENNING et al 1998; MORGAN et al, 2005). Sinusites fúngicas e infecções cutâneas são mais frequentemente associadas a esta espécie. As sinusites podem ser alérgicas levando à bola fúngica ou tornando-se invasivas, essa última normalmente ligada à imunossupressão inata. Este complexo possui um conídio maior que complexo *A. fumigatus*, o que facilita sua deposição no trato respiratório superior, podendo ser esta a razão de serem os agentes mais comuns neste sítio (MORGAN et al, 2005). Complexo *A. flavus* também é responsável por cerca de 80% das ceratites fúngicas, que ocorrem principalmente em clima seco e quente, e mais de 40% dos casos de aspergilose em feridas, frequentemente de origem nosocomial (PASQUALOTTO & DENNING, 2006).

Outras síndromes clínicas que podem ser causadas por espécies de *Aspergillus* são pericardites (PASQUALOTTO & DENNING, 2006), infecção do sistema nervoso central

(HUSSAIN et al, 1995), osteomielites (FISCHER et al, 1992) e infecção no trato urinário (KUETER et al, 2002), porém são de ocorrência bastante rara e normalmente causadas por *Aspergillus* não-*flavus* e *fumigatus* (PAGANO et al, 1996).

2.3 Diagnóstico

O diagnóstico da aspergilose invasiva é baseado largamente nos achados clínicos e histopatológicos. Porém, as limitações na definição dos sinais clínicos e na obtenção dos dados histológicos fazem com que sua utilização como únicos métodos na obtenção do diagnóstico não seja possível (DEL PALACIO et al, 2003).

O diagnóstico micológico, baseado na observação direta e no isolamento do agente a partir dos espécimes clínicos, é de grande importância para identificação precisa do agente etiológico e para avaliação da suscetibilidade aos agentes antifúngicos (DEL PALACIO et al, 2003). Há um consenso geral de que as culturas são fundamentais para o diagnóstico definitivo e a determinação das espécies em pacientes com infecção fúngica invasiva (DE PAUW et al, 2008). As diferentes espécies de *Aspergillus* crescem bem em meios comuns de isolamento. O meio padrão para identificação das espécies é o ágar Czapek a 25 °C (PAZOS et al, 2005).

Técnicas de diagnóstico rápidas, recentemente introduzidas, têm se mostrado vantajosas na obtenção de um diagnóstico precoce. Dentre essas técnicas fazem parte a tomografia computadorizada de alta resolução e a detecção do fungo por métodos não-culturais, baseados na detecção de antígenos fúngicos circulantes, principalmente galactomanana e (1-3)-ß-D-glucana, polissacarídeos componentes da parede celular de *Aspergillus* spp., os quais são liberados durante o crescimento das hifas. Com a detecção destes dois componentes, no soro do paciente, é possível determinar fatores como carga fúngica, taxa de crescimento, grau de invasão e a presença de anticorpos anti-*Aspergillus* (PAZOS et al, 2005; HOENIGL et al, 2014). No entanto, análises de galactomanana e (1-3)-ß-D-glucana como marcadores para diagnóstico de aspergilose invasiva, não são consideradas específicas uma vez que a galactomanana também é encontrada na parede celular de algumas espécies de *Penicillium* e a (1-3)-ß-D-glucana é encontrada em outros fungos além de *Aspergillus*, como *Candida* spp., *Fusarium* spp. e *Acremonium* spp. (KUPFAHL et al, 2007; HOPE et al, 2005).

O diagnóstico utilizando metabólitos primários ou secundários tem sido bastante estudado, a exemplo da gliotoxina, uma toxina produzida especificamente por *Aspergillus*

spp. durante o processo de infecção. Esta toxina já foi detectada em pulmão e soro de pacientes infectados com *A. fumigatus* e tem sido considerado um marcador de aspergilose bastante promissor. A análise quantitativa no soro é realizada por cromatografia líquida acoplada à espectrometria de massas (LEWIS et al, 2005; VIDAL-GARCÍA et al, 2016).

Técnicas moleculares surgem como ferramentas rápidas e precisas na detecção de aspergilose e identificação dos agentes. Estas técnicas são especialmente recomendadas quando culturas atípicas e/ou resistência antifúngica são observadas (YOO et al, 2008; PATTERSON et al, 2016). A amplificação do DNA de *Aspergillus* diretamente do sangue e dos tecidos através da técnica de reação em cadeia da polimerase (PCR) vem se tornando cada vez mais popular à medida que métodos de extração tornam-se comercialmente disponíveis e as plataformas se tornam mais automatizadas (WHITE et al, 2010; CRUCIANI et al, 2015). No entanto, a técnica ainda não foi incluída nas definições da EORTC/MSG - "*The European Organisation for Research and Treatment of Cancer/Mycoses Study Group*" por não haver padronização da metodologia (DE PAW et al, 2008).

Em adição, sequenciamento do gene *cyp51A* de *A. fumigatus* tem sido utilizado como principal método para detecção de mecanismos de resistência a antifúngicos azólicos e, mais recentemente, foi disponibilizado comercialmente um kit para PCR multiplex em tempo real denominado AsperGenius®. Ele rapidamente diagnostica infecções por *Aspergillus* e identifica simultaneamente azol-resistência. Dentro de 2,5 horas a detecção e caracterização é realizada em amostras do trato respiratório inferior de pacientes infectados com *Aspergillus* (WHITE et al, 2017).

2.4 Tratamento – Problema Terapêutico – Resistência

Três classes de agentes antifúngicos estão disponíveis para o tratamento das aspergilose: poliênicos, triazólicos e equinocandinas (BASSETTI et al, 2014). Os triazólicos são os agentes preferidos para tratamento e prevenção da aspergilose invasiva na maioria dos pacientes, enquanto que a anfotericina B desoxicolato e seus derivados lipídicos são opções apropriadas para a terapia inicial e de resgate quando o voriconazol não pode ser administrado, e finalmente, as equinocandinas são eficazes na terapia de resgate (sozinhas ou em combinação) mas seu uso rotineiro como monoterapia para o tratamento primário de aspergilose invasiva não é recomendado (PATTERSON et al, 2016).

2.4.1 Antifúngicos azólicos

Os azólicos representam a maior classe de antifúngicos utilizadas na clínica por mais de duas décadas. Exercem os seus efeitos na membrana da célula fúngica. Inibem a enzima 14-α-esterol desmetilase impedindo a conversão de lanosterol para ergosterol. Este mecanismo resulta na acumulação de metilesteróis tóxicos na membrana e inibição do crescimento da célula fúngica. Os azólicos diferem na sua afinidade pela enzima 14-α-esterol desmetilase e essa diferença é, em grande parte, responsável pela sua potência antifúngica variável e espectro de atividade. A inibição cruzada de enzimas do citocromo P450 humano é responsável pela maior parte dos efeitos secundários clínicos que têm sido descritos com estes agentes. Os azólicos itraconazol, voriconazol, posaconazol e isavuconazol demonstram atividade fungicida contra *Aspergillus* spp. (THOMPSON III & PATTERSON, 2010; DONNELLEY et al, 2016).

Voriconazol é o tratamento de escolha para aspergilose invasiva. É um triazólico de segunda geração, com estrutura molecular semelhante ao fluconazol, disponível sob a forma de comprimido, suspensão oral e intravenosa. É metabolizado pelo fígado com apenas 5% do fármaco inalterada aparecendo na urina. Exibe farmacocinética não linear em adultos, com a concentração máxima no plasma e a área sob a curva aumentando desproporcionalmente com o aumento da dose. Dentre os principais efeitos adversos relacionados ao tratamento com voriconazol estão os distúrbios visuais transitórios, hepatotoxicidade, que pode ser limitante de dose, erupção cutânea, náuseas, vômitos, diarreia e alucinações visuais ou auditivas (PATTERSON et al, 2016; WALSH et al, 2008).

Itraconazol é um triazólico de primeira geração, utilizado no tratamento das aspergiloses alérgicas e não-invasivas, podendo ser utilizado na aspergilose invasiva quando o paciente não responde à terapia padrão, no entanto é raramente recomendado nesta última em vista que muitos estudos relatam isolados de *A. fumigatus* resistentes ao itraconazol (VERWEIJ et al, 2007; SNELDERS et al, 2008; DENNING et al, 1997). Está disponível em cápsulas, solução oral e parenteral. As reações mais observadas ao itraconazol são transitórias e incluem náuseas e vômitos, hipertrigliceridemia, hipocalemia e níveis elevados de enzimas hepáticas (PATTERSON et al, 2016).

Posaconazol é um triazólico de segunda geração, com estrutura molecular semelhante ao itraconazol. Está disponível como suspensão oral, comprimidos de liberação lenta e formulação intravenosa. Foi aprovado pelo FDA (*Food and Drug Administration*) para profilaxia de aspergilose invasiva em pacientes com neutropenia devido à quimioterapia de

indução da remissão para leucemia mieloide aguda e síndrome mielodisplásica utilizando dose de 200 mg, três vezes ao dia. Doses maiores de posaconazol (800 mg de duas a quatro vezes ao dia) também podem ser utilizadas como terapia de salvamento, quando voriconazol não for eficiente. Os efeitos tóxicos são geralmente leves, incluindo diarreia e náuseas, e não parecem estar relacionadas às concentrações de fármaco. A comprovada eficácia de profilaxia com posaconazol em pacientes de risco e o uso de voriconazol no tratamento de aspergilose invasiva tem substituído anfotericina B e itraconazol para essas indicações (ULLMANN et al, 2007; THOMPSON III & PATTERSON, 2010).

O isavuconazol foi aprovado em 2015 pelas agências de controle de medicamentos europeia (*The European Medicines Agency)* e americana (*US Food and Drug Administration*) para o tratamento da aspergilose invasiva. Isavuconazol é o princípio ativo do pro-fármaco isavuconazonium. Possui amplo espectro de ação e meia-vida de cinco dias. Está comercialmente disponível tanto em formulação oral como intravenosa e geralmente é bem tolerado. O perfil de toxicidade é semelhante ao dos outros triazóis, com uma taxa similar de transtornos gastrointestinais, mas com base em experiência limitada, menor taxa de fotossensibilidade, distúrbios da pele e distúrbios hepatobiliares e visuais em comparação com voriconazol (DONNELLEY et al, 2016; PATTERSON et al, 2016).

2.4.1.1 Resistência aos azólicos: Vias de seleção e mecanismos

Falhas clínicas envolvendo isolados de *A. fumigatus* resistentes aos antifúngicos azólicos têm sido reportadas, especialmente nas últimas décadas em diversos centros médicos pelo mundo, como Espanha (MELLADO et al, 2007), Bélgica (LAGROU et al, 2008), Dinamarca (ARENDRUP et al, 2008), Suécia (CHRYSSANTHOU, 1997), França (DANNAOUI et al, 2001), Reino Unido (DENNING et al, 1997, HOWARD et al, 2006) e Países Baixos (VERWEIJ et al, 2007).

A resistência aos azólicos está comumente associada a mutações no gene *cyp51*, o qual codifica a enzima alvo destes antifúngicos. O genoma de *A. fumigatus* possui duas cópias deste gene, o *cyp51A* e o *cyp51B*, que codificam de maneiras diferentes a enzima 14-α-esterol desmetilase. Numerosos mecanismos têm sido descritos e consistem de um ponto de mutação ou polimorfismo de nucleotídeo único (SNP) em *cyp51A* bem como combinações de mudanças genéticas (HOWARD et al, 2009). Até agora, somente SNPs localizados no *cyp51A* têm sido encontrados correspondendo a um fenótipo azol-resistente, enquanto que raramente

são descritas mutações no *cyp51B*, e estas não estão associadas à azol-resistência (SNELDERS et al, 2012).

A estrutura da proteína 14-α-esterol desmetilase, codificada pelo *cyp51A*, em cepas selvagens de *A. fumigatus*, contém dois canais de entrada ligados, o canal 1 e o canal 2, imersos nas membranas do retículo endoplasmático (GOLLAPUDY et al, 2004; XIAO et al, 2004). Esses canais permitem a entrada de substratos de esteróis altamente lipofílicos e compostos azólicos, para ligar-se ao seu sítio de atividade, ou restringir a entrada de outros metabólitos. Os códons 54 e 220 estão localizados em alças que estão em estreita proximidade com o canal de entrada 2. Qualquer mutação nestes códons resulta na substituição de resíduos hidrofóbicos menores por maiores, o que resulta no fechamento do canal 2, perturbando assim o encaixe das moléculas grandes dos antifúngicos. Embora dois canais de acesso estejam presentes, mudanças em aminoácidos, em qualquer canal, afetam o encaixe de compostos azólicos em toda a proteína, podendo gerar os fenótipos resistentes (VERWEIJ et al, 2007; MELLADO et al, 2007).

Além de mutações em códons únicos e específicos, também têm sido descritas mutações em *tandem repeats* (TRs). As TRs são sequências de DNA de 2 a 200 nucleotídeos repetitivas que não ocorrem apenas sequencialmente, mas também diretamente adjacente. Elas podem ser completamente idênticas ou parcialmente degeneradas. O número dessas cópias repetitivas de codificação geralmente varia entre diferentes isolados, levando a expansão ou contração de blocos de aminoácidos. As mutações em TRs mais descritas em *A. fumigatus* resistentes a azólicos são: *TR_{34}/L98H*, *TR53* e *TR46/Y121F/T289A* (SNELDERS et al, 2012; VERWEIJ et al, 2007; MELLADO et al, 2007).

Azol-resistência pode ser selecionada no paciente durante a terapia com azólicos, especialmente naqueles com lesões cavitárias, aspergiloma ou outra, onde o fungo se reproduz por esporulação assexuada, fato que facilita a transferência de genes de resistência a esporos (VERWEIJ et al, 2007).

Em muitos casos de resistência aos azólicos, descritos até agora, os pacientes apresentavam histórico de terapia prévia com azólicos e a maioria havia sido tratada para aspergilose crônica. Este modo de seleção de resistência foi caracterizado por alta diversidade de mecanismos de resistência, principalmente ligados ao gene *cyp51A* (HOWARD et al, 2009). Casos em que vários isolados de *A. fumigatus* de um mesmo paciente apresentaram diferentes mecanismos de resistência prova que os fungos são capazes de adquirir diferentes tipos de mecanismos na seleção de resistência no paciente (CAMPS et al, 2012).

Outra rota de desenvolvimento de resistência, que tem sido foco de pesquisas, é a ambiental (VERWEIJ et al, 2007; SNELDERS et al, 2012). Em um estudo prospectivo multicêntrico de vigilância nacional, realizada nos Países Baixos entre 2007 e 2009, para determinar os efeitos da resistência sobre estratégias de gestão de pacientes e saúde pública, foi notado que 64% dos pacientes de quem foram isoladas cepas de *A. fumigatus* resistentes, não apresentavam histórico de tratamento prévio com azólicos. Como aspergilose não é uma doença contagiosa e a transmissão de um paciente a outro é altamente improvável, este fato sugere que os esporos inalados pelos pacientes já eram azol resistentes. Além disso, mais de 90% dos isolados destes pacientes apresentaram o mesmo mecanismo de resistência, o *TR_{34}/L98H*, que consiste na duplicação de 34 pares de bases na região promotora do gene *cyp51A* ligada a uma substituição de leucina na posição 98 por resíduo de histidina. Sendo este um alelo de resistência também encontrado em cepas ambientais, uma diferente rota de seleção de azol-resistência se confirmou (van der LINDEN et al, 2011).

É possível que azol-resistência se desenvolva no nicho ecológico de *A. fumigatus*, que é principalmente solo e material orgânico. Essa possibilidade é suportada, uma vez que, em outro estudo realizado nos Países Baixos, cepas de origem ambiental apresentaram resistência cruzada a triazóis de uso clínico e todas apresentaram o mesmo mecanismo de resistência, *TR_{34}/L98H* (SNELDERS et al, 2009).

A. fumigatus não é um fitopatógeno, porém como habita o solo e compostos são expostos a uma variedade de agentes fungicidas, utilizados para combater outros fungos causadores de doenças em plantas. Os inibidores da 14-α-desmetilase (DMIs) são fungicidas bastante utilizados na agricultura e também como revestimento de couro e madeira pra evitar o crescimento de fungos. Eles possuem estrutura semelhante aos azólicos de uso clínico, porém há um número muito maior de DMIs (VERWEIJ et al, 2009).

Em outro estudo realizado também nos Países Baixos foram testados 31 agentes fungicidas, herbicidas e reguladores do crescimento em plantas frente a cepas de *A. fumigatus* provenientes de pacientes com aspergilose contendo ou não a mutação *TR_{34}/L98H*. A maioria dos compostos não demonstrou atividade *in vitro*, porém bromuconazol, tebuconazol, difenoconazol e propiconazol, fungicidas com estrutura bastante similar aos triazóis médicos, apresentaram boa atividade frente às cepas sensíveis mas não frente aquelas com a presença da mutação *TR_{34}/L98H* (SNELDERS et al, 2012). Possivelmente o uso de fungicidas triazólicos na agricultura resulte na seleção de resistência cruzada aos triazólicos de uso médico.

2.4.2 Antifúngicos Poliênicos

Antifúngicos poliênicos têm sido usados no tratamento da aspergilose invasiva há mais de 40 anos. Historicamente é considerado que a atividade da anfotericina B envolve a ligação irreversível do fármaco com o ergosterol na membrana plasmática da célula fúngica, resultando na formação de poros transmembrana que permitem a saída de íons, despolarizando e desorganizando a membrana, levando à acidificação intracelular, e à precipitação dos componentes citoplasmáticos que conduz à morte da célula (BRAJTBURG & BOLARD, 1996). No entanto, evidências sugerem que a anfotericina B forma grandes agregados extramembranosos que extraem ergosterol das bicamadas lipídicas gerando a morte da célula fúngica (ANDERSON et al, 2014).

Anfotericina B é solubilizada com desoxicolato para administração intravenosa, uma vez que possui baixíssima absorção oral, sendo algumas vias alternativas de administração intraperitoneal, intravítrea, intratecal, irrigação da bexiga e aerossolização. Embora novas opções terapêuticas tenham mudado um pouco o seu papel, anfotericina B complexada com lipídios permanece como umas das mais importantes opções terapêuticas para aspergilose, devido ao seu amplo espectro de atividade e limitada resistência cruzada com antifúngicos triazólicos. Estudos recentes têm demonstrado a importância da determinação precisa de espécies de *Aspergillus* durante a terapia com anfotericina B, pois algumas espécies não-*fumigatus*, particularmente *A. terreus* e *A. flavus*, são relativamente resistentes à anfotericina B (LEWIS, 2010).

Resistência de *A. fumigatus* à anfotericina B é um fenômeno raro, porém estudos sugerem que mutações na via de biossíntese do ergosterol (primariamente gene *ERG3* codificando α-8,7 isomerase ou α-5,6 desaturase) leva ao acúmulo de esteróis alternativos na membrana celular fúngica e reduz a ligação à anfotericina B (CHAMILOS & KONTOYIANNIS, 2005; KONTOYIANNIS & LEWIS, 2002). Outro mecanismo proposto é a ação da anfotericina B como agente oxidante. Acredita-se que a resistência se dá devido ao aumento de enzimas neutralizantes (catalase e superóxido dismutase) que conferem resistência ao estresse oxidativo na célula (BLUM et al, 2008).

2.4.3 Equinocandinas

As equinocandinas são a primeira classe de antifúngicos que tem ação na parede celular fúngica, sendo potentes inibidores da síntese de glucanas. A parede celular fúngica talvez

represente o alvo ideal para o tratamento terapêutico de fungos patogênicos de seres humanos, uma vez que a grande maioria da massa da parede celular dos fungos é composta de polímeros de carboidratos, os quais estão completamente ausentes do corpo humano (PFALLER et al, 2009).

Esta classe possui três representantes: caspofungina, micafungina e anidulafungina. Tem ação frente a *Aspergillus* spp. e *Candida* spp., possuindo um favorável perfil farmacocinético e farmacodinâmico, além de excelente perfil de segurança (PFALLER et al, 2009). Os regimes de tratamento com equinocandinas para aspergilose invasiva ainda estão evoluindo. Elas são consideradas fungistáticas frente a *Aspergillus* spp., mas têm efeito fungicida ao crescimento celular nas pontas das hifas, onde a parede celular é menos rígida devido à ausência de ligações cruzadas entre glucana e quitina, podendo levar a lise das mesmas. Além disso, a sua considerável eficácia, *in vivo*, parece ser derivada de um aumento da resposta imunitária local fármaco-induzida (PERLIN & HOPE, 2010).

Caspofungina é a única equinocandina aprovada pelo FDA para aspergilose invasiva como terapia de resgate ou em pacientes que são intolerantes ao tratamento de primeira linha (WALSH et al, 2008). Micafungina e anidulafungina ainda não foram formalmente aprovadas para tratamento da aspergilose invasiva nos EUA e Europa em razão do não estabelecimento de doses eficazes (BASSETTI et al, 2014). No entanto um estudo realizado por pesquisadores japoneses mostrou que a micafungina tem eficácia e segurança comparável a caspofungina em uma dose 50 a 150 mg/kg/dia (HANADATE et al, 2011); ainda, em outro estudo comparando micafungina e caspofungina, no tratamento de primeira linha de aspergilose, os autores relataram não haver diferença aparente entre os grupos de pacientes tratados, assim como a ocorrência de efeitos adversos foi muito semelhante entre os dois grupos (KOHNO et al, 2013).

Resistência a equinocandinas é incomum entre as espécies sensíveis como *Candida*, porém os relatos de resistência vêm aumentando, ao longo do uso dessa classe na clínica. Em *Candida albicans* e *Candida glabrata* essa resistência é atribuída à mutação em FKs1p, a maior subunidade da enzima glucana sintase (CLEARY et al, 2008; PERLIN et al, 2007). Em infecções por *Aspergillus* resistência as equinocandinas são muito raras. Têm sido observadas espécies com sensibilidade reduzida a equinocandinas, como *A. lentulus* (BALAJEE et al, 2005) e *A. flavus* (ESCHERTZHUBER et al, 2007; HEDAYATI et al, 2007). Mutações em gene Fks1 também podem conferir resistência em *A. fumigatus* (ROCHA et al, 2007). Essas mutações diminuem a afinidade da glucana sintase pelas equinocandinas em mais de mil

vezes, resultando em cepas com elevadas CEMs e conferindo resistência cruzada entre a classe (PERLIN et al, 2007).

Perlin & Hope (2010) sugerem que equinocandinas teriam destaque como uma classe de antifúngicos usados em combinação com outras classes, uma vez que são fungistáticas e a exposição prolongada pode levar ao surgimento de cepas com sensibilidade reduzida.

2.4.4 Terapias de Combinação

As infecções fúngicas refratárias ao tratamento com antifúngicos convencionais de primeira escolha podem responder mudando-se a classe terapêutica ou combinando-se os agentes. As vantagens de se utilizar agentes antifúngicos em combinação, assim como, agentes antifúngicos combinados com não-antifúngicos, têm sido demonstradas por muitos pesquisadores frente a diversos gêneros de difícil tratamento como *Fusarium* spp., *Candida* spp., *Exophiala* spp., *Aspergillus* spp. e *Pythium insidiosum* (VITALE et al, 2003; TE DORSTHORST et al, 2004; CUENCA-ESTRELLA et al, 2005; STERGIOPOULOU et al, 2008; STERGIOPOULOU et al, 2009; VENTURINI et al, 2011; DENARDI et al, 2013; JESUS et al, 2014).

Em especial, frente ao gênero *Aspergillus* já se tem documentado alguns efeitos favoráveis no uso de combinações de fármacos de diferentes classes. Cuenca-Estrella et al., (2005) demonstrou sinergismo na associação entre caspofungina e voriconazol frente a isolados de *A. fumigatus* resistentes ao itraconazol; em outro estudo a associação caspofungina e voriconazol, como tratamento de primeira linha de 40 pacientes transplantados de órgãos sólidos foi associada à redução da mortalidade, em comparação com a formulação lipídica de anfotericina B (SINGH et al, 2006); Seyedmousavi et al. (2013) demonstraram efeitos sinérgicos *in vitro* e *in vivo* entre anidulafungina e voriconazol em isolados de *A. fumigatus* sensíveis e resistentes ao voriconazol; Lewis & Kontoyiannis (2005) detectaram uma diminuição de quatro vezes na concentração efetiva de voriconazol quando testado em combinação com micafungina frente a isolados de *A. fumigatus* e *A. terreus*; Elefanti et al (2013) observaram efeitos sinérgicos entre voriconazol e anfotericina B associados a equinocandinas frente a *Aspergillus* spp.

Potente sinergismo também foi encontrado por Afeltra et al (2003) frente a isolados de *A. fumigatus* resistentes ao itraconazol quando associou-se itraconazol com fármacos membrana-ativos, como amiodarona, lanzoprazol e nifedipino. Acredita-se que o sinergismo é

devido ao menor efluxo de itraconazol da célula, um dos mecanismos de resistência dos fungos, já que esses compostos são potentes inibidores das bombas de efluxo, melhorando assim, a ação do itraconazol na célula fúngica. Ainda, Yekutiel et al (2004) relataram sinergismo em 29 de 31 isolados de *Aspergillus* spp. quando associou-se caspofungina a sulfametoxazol (leitura da CIM) e sinergismo ou aditividade em 12 dos 31 isolados com leitura da (CEM).

A combinação de diferentes classes de antifúngicos entre si, bem como com fármacos usados para o tratamento de outras patologias pode melhorar a eficácia do tratamento de infecções fúngicas refratárias ao tratamento convencional, fazendo com que ocorra uma diminuição na resistência aos antifúngicos, melhore a eficácia e o tempo de tratamento do paciente, bem como reduza os efeitos adversos causados por altas doses de antifúngicos. No entanto, as combinações ainda são incertas e exigem mais estudos e controle clínico (WALSH et al, 2008).

3 PROPOSIÇÃO

Nesta tese objetivou-se:

- Determinar o perfil de suscetibilidade de *A. fumigatus* e *A. flavus* isolados de pacientes com diferentes formas clínicas de aspergilose e do ambiente, frente aos antifúngicos azólicos, às equinocandinas, e à anfotericina B.
- Avaliar a suscetibilidade de isolados clínicos de *A. fumigatus* e *A. flavus* frente a associações de antifúngicos azólicos e equinocandinas através das metodologias de microdiluição em caldo.
- Comparar as metodologias de Etest e microdiluição em caldo na avaliação da suscetibilidade de *A. fumigatus* frente as combinações de azólicos e equinocandinas.
- Avaliar a combinação de posaconazol e anidulafungina em modelo murino de aspergilose pulmonar causada por *A. fumigatus*.
- Determinar a concordância entre as metodologias de Etest e crescimento em ágar contendo antifúngico com microdiluição em caldo para medir a atividade de isavuconazol frente a cepas clínicas de *A. fumigatus*.
- Investigar os mecanismos de resistência de *A. fumigatus* de origem clínica e ambiental que apresentaram elevadas CIMs a um ou mais antifúngicos azólicos a fim de pesquisar a presença de mutações relacionadas à azol-resistência em cepas brasileiras.

CAPÍTULO 1

In vitro ANTIFUNGAL SUSCEPTIBILITY OF CLINICAL AND ENVIRONMENTAL ISOLATES OF *Aspergillus fumigatus* and *Aspergillus flavus* IN BRAZIL

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Abstract

The *in vitro* susceptibility of 105 clinical and environmental strains of *Aspergillus fumigatus* and *Aspergillus flavus* to antifungal drugs, such as amphotericin B, azoles, and echinocandins, was evaluated by the broth microdilution method proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Following the EUCAST-proposed breakpoints, 20 % and 25 % of the clinical and environmental isolates of *A. fumigatus*, respectively, were found to be resistant to itraconazole (Minimal Inhibitory Concentration, MIC > 2.0 mg/L). Voriconazole showed good activity against *A. fumigatus* and *A. flavus* strains, except for one *A. fumigatus* clinical strain where the MIC was 4.0 mg/L. Posaconazole (≤ 0.25 mg/L) also showed appreciable activity against both the species of *Aspergillus,* except for the six *A. fumigatus* strains with relatively higher MICs (0.5 mg/L). The MICs for Amphotericin B ranged from 0.06 to 1.0 mg/L for *A. fumigatus*, but were much higher (0.5 to 8.0 mg/L) for *A. flavus*. Among the echinocandins, caspofungin showed a geometric mean of 0.078 and 0.113 against the clinical and environmental strains of *A. flavus*, respectively, but had elevated minimal effective concentrations (MECs) for seven of the *A. fumigatus* strains. Anidulafungin and micafungin exhibited considerable activity against both *A. fumigatus* and *A. flavus* isolates, except for one environmental isolate of *A. fumigatus* that showed an MEC of 1 mg/ L to micafungin. Our study proposes that a detailed investigation of the antifungal susceptibility of the genus *Aspergillus* from different regions of Brazil is necessary for establishing a response profile against the different classes of antifungal agents used in the treatment of aspergillosis.

Key-Words: *Aspergillus flavus*, *Aspergillus fumigatus*, echinocandins, azoles, environmental, susceptibility

Introduction

Aspergillosis includes a wide range of diseases caused by the filamentous fungus *Aspergillus* that affects mainly the respiratory tract of immunocompromised patients. It is characterized by invasive and chronic pulmonary infection, allergic reaction, or fungal growth. *Aspergillus fumigatus*, followed by *Aspergillus flavus*, is the most common species causing aspergillosis.[1,2] The treatment of these diseases is based on the use of azole antifungal drugs, such as voriconazole (VCZ), which is the treatment of choice, itraconazole (ITZ), posaconazole (PCZ), and more recently, isavuconazole (ISZ).[3,4] Nevertheless, many studies have reported resistance of *A. fumigatus* to the azole antifungal drugs that is often due to the cross-resistance to the agricultural triazoles. Resistance rates vary widely across medical centers around the world, with some studies showing high resistance rates[5,6,7] and others with rates even lower than 1 %.[8,9] As an alternative to the use of azoles, lipid formulations of amphotericin B (AMB) and echinocandins are also being used nowadays in the treatment of aspergillosis.[3,4]

Owing to the widespread use of azoles in the treatment of aspergillosis and the potential consequence of acquiring azole resistance, antifungal susceptibility studies of the concerned fungal species have become increasingly important in order to understand their resistance profile in each medical center and improve the treatment settings. In this study, we evaluated the susceptibility profile of *A. fumigatus* and *A. flavus*, isolated from patients with different forms of aspergillosis and from the environment in southern Brazil, against azoles, AMB, and echinocandins by using the broth microdilution methodology.

Materials and methods

Aspergillus strains

Twenty-five clinical strains of *A. fumigatus* and twenty of *A. flavus* were isolated from patients with sinusitis, invasive or cutaneous aspergillosis; twenty strains of *A. fumigatus* and

forty of *A. flavus* were isolated from maize crops belonging to the Laboratory of Mycological Research of the Federal University of Santa Maria in southern Brazil. Analyses of macro and micromorphology, growth of *A. fumigatus* at 48 °C, and sequencing of the internal transcribed spacer (ITS 1 and 2) regions were performed to identify the species. ITS region amplifications were performed via PCR using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTAT TGATATG-3′) primers, as described previously.[10] The strains were stored in 10 % glycerol at –80 °C and were subcultured on Potato Dextrose Agar (PDA) at 27 °C for three days or until sporulation. In all the tests, *A. fumigatus* ATCC 204305 and *A. flavus* ATCC 204304 strains were used as the internal quality controls.

Antifungal Susceptibility Testing

The minimal inhibitory concentrations (MICs) of ITZ, PCZ, VCZ (Sigma-Aldrich, São Paulo, Brazil), and AMB (Leadiant Biosciences, Maryland, USA) and the minimal effective concentrations (MECs) of the echinocandins such as anidulafungin (AFG; Pharmacia & Upjohn Co. Kalamazoo, MI, USA), caspofungin (CAS; Laboratoires Merck Sharp & Dohme-Chibret, Clermont-Ferrand, France), and micafungin (MFG; Astellas Pharma Tech Co., Takaoka, Toyama, Japan) were determined following the recommendations of the EUCAST protocol for filamentous fungi (EUCAST, 2008). All the antifungal drugs were solubilized in dimethyl sulfoxide (DMSO) and the working solutions were prepared in RPMI 1640 medium with L-glutamine (Sigma-Aldrich, São Paulo, SP, Brazil). The final drug concentrations ranged from 0.063 to 32.0 mg/L for ITZ and VCZ, 0.008 to 4.00 mg/L for PCZ, 0.016 to 8.00 for AMB, and 0.0005 to 1.00 mg/L for the echinocandins.

Conidial suspensions were obtained from sporulated *Aspergillus* cultures and adjusted to contain 2–5 × 10^6 conidia/mL by counting in a hemocytometer. To obtain a final concentration of 2–5 × 10^5 conidia/mL, 1:10 dilutions were prepared in sterile distilled water. For the microplate preparation, in each well, 100 µL of the final conidial suspension were

added to 100 µL of each of the antifungal drug concentrations. Growth and negative control are included in all tests. The microplates were incubated at 35 °C. The MEC readings for echinocandins, defined as the lowest concentration leading to the growth of the abnormal, branched, and short hyphae as compared to those forming long and unbranched hyphae in the growth control, were performed 24 h post-incubation. The MIC readings for azoles and AMB, defined as the lowest concentration that completely inhibits the growth compared to that obtained in case of the control, were taken 48 h post-incubation.

Data Analyses

Geometric mean (GM), MIC/MEC_{50} (minimal inhibitory/effective concentration that inhibits the growth of 50 % of the strains) and MIC/MEC_{90} (minimal inhibitory/effective concentration that inhibits the growth of 90 % of the strains) values were calculated by Microsoft Office Excel 2016 (Microsoft Informatica Ltda., São Paulo, Brazil). In order to define the susceptibility of *A. fumigatus* and *A. flavus* to azole antifungal agents and AMB, the EUCAST breakpoints were used.[12,13] The results with AMB were also analyzed according to the breakpoints suggested by Elefanti et al.[15] and CLSI (M38-A2, 2008) method[14]. For echinocandins, the CAS ECVs (epidemiological cut-off values) proposed by Espinel-Ingroff et al.[16] were used in the interpretation of the results.

Results

The *in vitro* activities (GM, MIC/MEC_{50}, MIC/MEC_{90} and MIC/MEC range) of each antifungal drug against the *A. fumigatus* and *A. flavus* clinical and environmental strains are presented in the Table 1 and Figure 1, while the Figure 2 shows the percentages of susceptibility, intermediate profile and resistance against azoles and AMB considering the breakpoints as described above.

Against to ITZ the clinical and environmental isolates of *A. fumigatus* showed susceptible profile for 52 and 50% of the strains, respectively. Resistance (MIC ≥ 4.0 mg/ L)

was observed in 20% of clinical strains and 25% of environmental strains and intermediate profile (MIC = 2.0 mg/ L) was observed in 28% of clinical strains and 25% of environmental strains. *A. flavus* isolates showed a good susceptibility profile to ITZ, only 7.5% (3 strains) from environmental were resistant (MIC ≥ 4.0 mg/ L).

VCZ and PCZ showed to be effective against the clinical and environmental isolates of *A. fumigatus* and *A. flavus*. Except for 1 clinical isolate that showed MIC = 4.0 mg/L for VCZ, resistance was not observed in any other *Aspergillus* isolate against this antifungal. PCZ showed resistant profile (MIC = 0.5 mg/L) for 5 clinical isolates and 1 environmental isolate of *A. fumigatus* and 100% susceptible profile for both isolates of *A. flavus*.

AMB MICs for *A. fumigatus* ranged from 0.5 to 1.0 mg/L (clinical isolates) and 0.06 to 1.0 mg/L (environmental isolates), whereas for *A. flavus* the MICs ranged from 0.5 to 2.0 mg / L to clinical isolates and 0.5 to 8.0 mg / L to environmental isolates.

Echinocandins showed good activity against both *Aspergillus* species, with GMs of 0.024 for MFG, 0.009 for AFG and 0.078 for CAS in clinical isolates and 0.085 for MFG, 0.019 for ANF and 0.113 for CAS in environmental isolates of *A. flavus*. For *A. fumigatus* was observed MEC = 1.0 mg/L of CAS against 3 clinical isolates (GM = 0.188) and 1 environmental isolate (GM = 0.070). In addition 1 environmental isolate also had MIC = 1.0 mg / L for MFG. The ANF MECs ranged from 0.001 to 0.125 mg/L in clinical isolates and 0.001 to 0.06 mg / L in environmental isolates.

Discussion

The *in vitro* antifungal susceptibility of *A. fumigatus* and *A. flavus*, recovered from patients and environmental sources in Brazil, to azoles, AMB, and echinocandins have been described in this study. In general, all the antifungal drugs showed appreciable activity against both the species. However, *A. fumigatus* strains were found to be less susceptible to ITZ, while *A. flavus* strains were less susceptible to AMB.

Several studies on these fungal strains have reported resistance to azole antifungals, especially ITZ, with high rates reported in European countries, such as the Netherlands and United Kingdom, where azole resistance rates reach 38 %.[17,18] This resistance has been attributed to the long-term azole therapy in patients with chronic aspergillosis, in addition to cross-resistance to the agricultural triazoles. [5,19]

In Brazil, epidemiological data on the susceptibility of *Aspergillus* isolates recovered from both patients as well as the environment are quite scarce. Although resistance is observed in patients treated with azoles, susceptibility testing is not common in the routine laboratory tests. In the current study, we demonstrated 20 to 25 % rate of ITZ resistance in clinical and environmental isolates of *A. fumigatus*, which is alarming considering the facts that triazoles are the first line of treatment for aspergillosis and that azole fungicides are heavily used in Brazilian agriculture. [20]

Resistance to triazoles have already been described in many other Latin American countries. In Colombia, 19 environmental strains of *A. fumigatus*, collected from flower fields and other sites in the city of Bogota, were reported to be azole-resistant and contained alterations in the *Cyp*51A gene, mainly 46 bp tandem repeat combined with Y121F and T289A point mutations (TR46/Y121F/T289A).[21] In Argentina, an *A. fumigatus* strain, recovered from a patient with keratitis, has also been shown resistant to ITZ, and harbored the G54E mutation in *Cyp*51A.[22] In contrast, a multicenter international surveillance network performed by van der Linden et al.[23] tested 3788 *Aspergillus* strains from different countries for azole resistance, among which 64 clinical strains recovered from Brazilian patients showed no resistance to ITZ. In addition, Negri et al.[24] recently reported non-resistance to ITZ and PSC, and only intermediate MICs (2.0 mg / L) for few strains to VCZ in a study with 221 Brazilian clinical strains of *A. fumigatus*.

VCZ and PCZ were also found to have good activity against *A. fumigatus* strains. While VCZ has been used in the clinic recently, PCZ is yet to be allowed for the treatment of patients in Brazil. Although five clinical strains of *A. fumigatus* required MIC = 0.5 mg/L of PCZ, which is just above the breakpoint concentration, we believe that a significant opinion with respect to its azole antifungal susceptibility can be given only after PCZ is clinically accepted in the Brazilian medical centers. Only one clinical strain of *A. fumigatus* showed an MIC of 4.0 mg/L for VCZ, which shows that this azole is considered a good treatment choice; however, adequate use should always be taken into account for this profile to be maintained. In contrast to our results, a study by van Ingen et al.[18] in the Netherlands showed that when resistant to ITZ, approximately 92 to 97 % of *A. fumigatus* strains were also resistant to VCZ and PCZ. The higher use of VCZ and PCZ in medical centers around the world may explain this contrast.

On the other hand, all azole antifungals showed considerably good activity against *A. flavus*, except three strains of environmental origin that were resistant to ITZ (MIC = 4.0 (n = 2) and 8.0 mg/ L (n = 1)). Many supportive studies have shown *A. flavus* to be quite susceptible to azole antifungals. Shivaprakash et al.[25] tested 188 clinical and environmental isolates of *A. flavus* from India and the Netherlands, all of which were susceptible to the triazoles tested, except three strains that required MICs of 4.0 mg/L. In Brazil, Gonçalves et al.[26] also showed good response of *A. flavus* to azole antifungals, with an MIC range from 0.25 to 2.0 mg/L.

Following the EUCAST[11] breakpoints, both the isolates of *A. fumigatus* and *A. flavus* were found to be 100 % susceptible to AMB. However, considering the CLSI breakpoints [14], 76 % of clinical *A. fumigatus* showed an intermediate profile while 10 % of the clinical and environmental isolates of *A. flavus* showed a resistant profile (MIC of AMB ≥ 2.0 mg/L). If we base our analyses on the breakpoints proposed by Elefanti et al. in 2014,[15] the same

susceptibility profile is obtained for *A. fumigatus* as that obtained from CLSI breakpoints; however, 85 % of the clinical and 72.5 % of the environmental *A. flavus* isolates showed a resistant profile with MIC of AMB ≥ 1.0 mg/L.

Although the interpretations from different proposed breakpoints may differ, AMB was seen to have lesser activity against *A. flavus* than against *A. fumigatus*. This could be due to mutations in the ergosterol biosynthesis pathway that lead to a decrease in ergosterol concentration in the fungal cellular membrane, thereby decreasing the targets of AMB action in *A. flavus*.[27] Our results are in agreement with those of other studies that evaluated the susceptibility profile to AMB of *A. flavus*. Gonçalves et al.[26] reported 49 % resistance to AMB (MIC≥ 2.0 mg/ L) in 77 clinical isolates belonging to the section *Flavi*. Sabatelli et al.[28] also demonstrated higher activity of AMB against *A. fumigatus* (MIC_{90} = 1.0 mg/L; n = 1.119) than that against *A. flavus* (MIC_{90} = 2.0 mg/L; n = 89) isolates. More recently, Taghizadeh-Armaki et al.[29] also showed reduced susceptibility of environmental and clinical strains of *A. flavus* to AMB.

Of the three echinocandins tested here, only CAS and MFG showed increased MECs (≥ 0.5 mg/L) against six and one clinical isolates of *A. fumigatus*, respectively. The MECs of CAS against *A. fumigatus* were lower for environmental strains than for the clinical ones, whereas for *A. flavus*, the mean MECs showed the opposite trend in the two strains.

ANF and MFG showed lower mean MECs compared to CAS for both *A. fumigatus* and *A. flavus* strains. AFG was found capable of causing alterations in hyphae at concentrations lower than those of MFG for all the isolates, except for *A. fumigatus* clinical strains in which the same mean MEC was observed for both MFG and AFG. Other authors have reported better activity of ANF against *Aspergillus* spp.[30,31] compared to the other echinocandins.

CAS is successfully used in salvage therapy for invasive aspergillosis, while the two other echinocandins mentioned here have not yet been recommended clinically owing to the

lack of reliable and effective dose definitions. Echinocandin resistance is not common in *Aspergillus*; however, a few reports of CAS resistance have been emerging.[31] Although it has not been possible to establish a reliable relationship between the MEC and clinical response, based on the ECVs reported by Elefanti et al.[16], we observed a lower action of CAS in some of the clinical *A. fumigatus* strains studied here, hinting at the possibility of resistance development during treatment.

Information regarding the antifungal susceptibility of *Aspergillus* in Brazil and other Latin American countries is still scarce because of the lack of routine susceptibility tests in most of the clinical laboratories. Although the current study could not evaluate a large number of strains, it could be clearly concluded that a significant percentage of the clinical and environmental strains of *A. fumigatus* were azole-resistant, whereas *A. flavus* especially showed less susceptibility to AMB, and echinocandins presented elevated MECs against some strains. Larger studies with strains from different regions of Brazil are urgently required, not only to increase the awareness about the antifungal susceptibility of this important fungal pathogen in our country, but also to implement a better management of aspergillosis in our medical centres, thereby reducing the resistance rates.

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Declaration of Interest

The authors declare no conflicts of interest.

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Table 1 *In vitro* susceptibility of *A. fumigatus* and *A. flavus* clinical and environmental isolates against azoles, echinocandins and amphotericin B.

Drugs	Minimal Inhibitory Concentration (MIC) / Minimal Effective Concentration (MEC) (mg/L)															
	A. fumigatus (clinical isolates; n = 25)				*A. fumigatus* (environmental isolates; n = 20)				*A. flavus* (clinical isolates; n = 20)				*A. flavus* (environmental isolates; n = 40)			
	Range	**GM**	**50%**	**90%**	**Range**	**GM**	**50%**	**90%**	**Range**	**GM**	**50%**	**90%**	**Range**	**GM**	**50%**	**90%**
ITZ	1.00−16.00	2.00	1.00	16.00	1.00−16.00	1.803	1.00	4.00	1.00−2.00	1.189	1.00	2.00	0.50−8.00	1.414	1.00	2.00
VCZ	0.50−4.00	1.357	1.00	2.00	0.25−2.00	0.707	0.50	2.00	0.50−1.00	0.871	1.00	1.00	0.50−2.00	1.017	1.00	2.00
PCZ	0.06−0.50	0.169	0.125	0.50	0.03−0.50	0.065	0.06	0.125	0.125−0.25	0.171	0.125	0.25	0.03−0.25	0.188	0.125	0.125
AMB	0.50−1.00	0.847	1.00	1.00	0.06−1.00	0.329	0.25	0.50	0.50−2.00	0.966	1.00	1.00	0.50−8.00	0.917	1.00	1.00
MFG	0.001−0.125	0.007	0.008	0.031	0.002−1.00	0.031	0.03	0.125	0.008−0.006	0.024	0.03	0.06	0.004−0.25	0.085	0.125	0.25
AFG	0.001−0.125	0.007	0.004	0.063	0.001−-0.006	0.007	0.008	0.015	0.002−0.03	0.009	0.008	0.016	0.002−0.125	0.019	0.016	0.06
CAS	0.06−1.00	0.188	0.125	1.00	0.03−1.00	0.070	0.06	0.125	0.008−0.250	0.078	0.06	0.125	0.03−0.25	0.113	0.125	0.25

ITZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole; AMB, amphotericin B; MFG, micafungin; AFG, anidulafungin; CAS, caspofungin; GM, geometric mean; 50%, minimal inhibitory concentration / minimal effective concentration that inhibited the growth of 50% of the isolates; 90%, minimal inhibitory concentration / minimal effective concentration that inhibited the growth of 90% of the isolates.

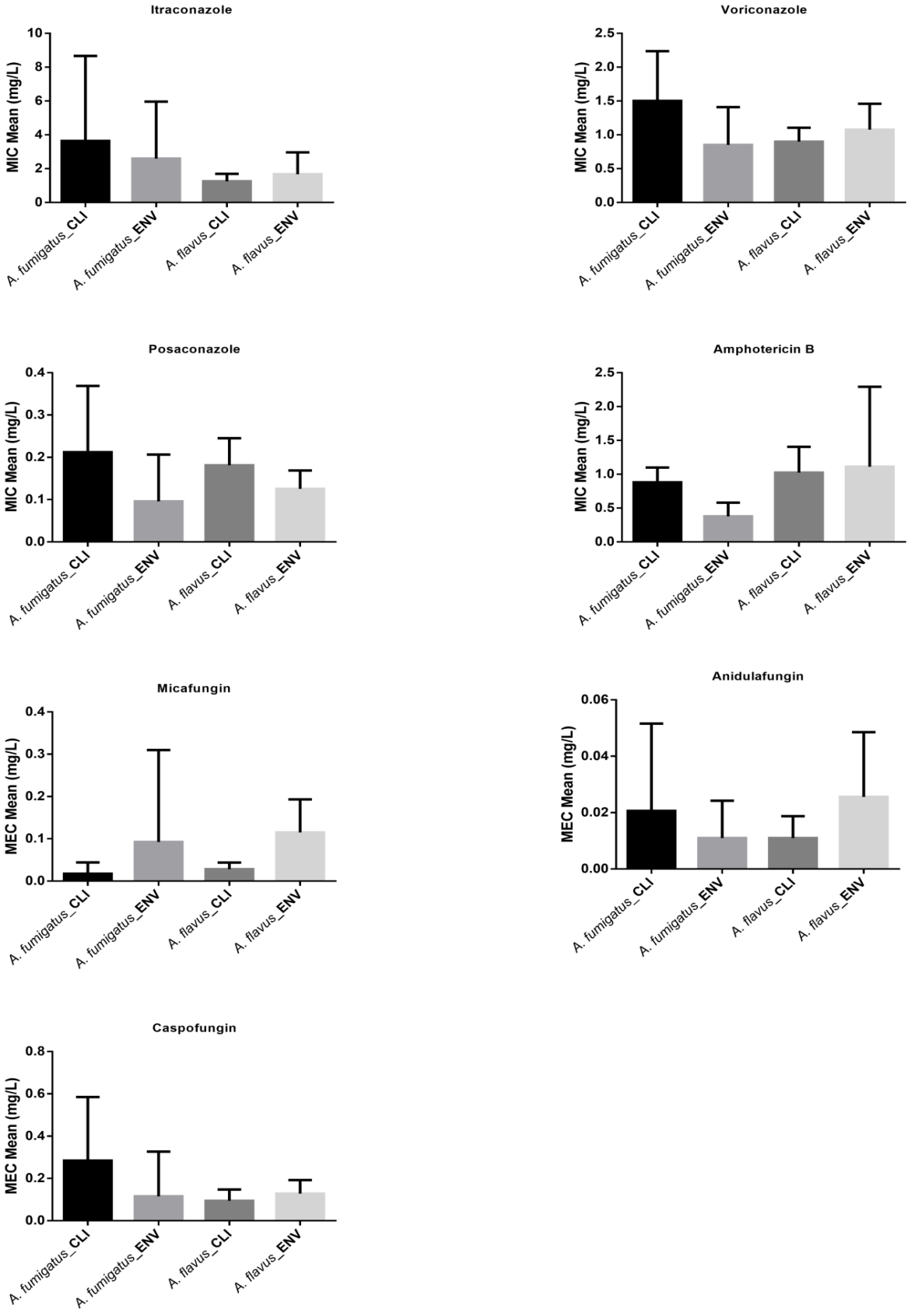

Figure 1 Minimal Inhibitory Concentration (MIC) and Minimal Effective Concentration (MEC) mean of itraconazole, voriconazole, posaconazole, amphotericin B, micafungin, anidulafungin and caspofungin for *A. fumigatus* and *A. flavus* clinical (CLI) and environmental (ENV) isolates.

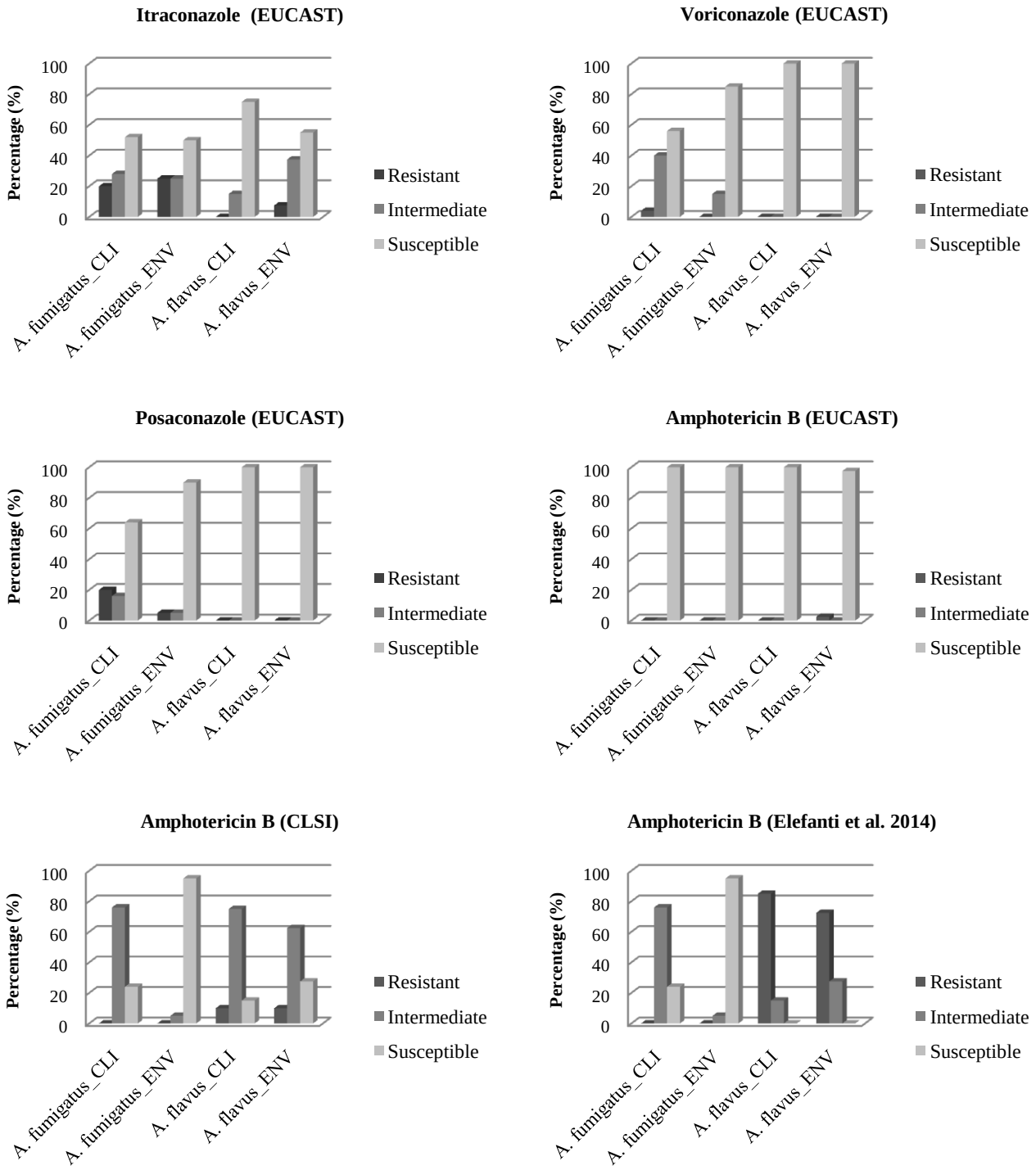

Figure 2 Susceptibility profile of *Aspergillus fumigatus* and *Aspergillus flavus* clinical (CLIN) and environmental (ENV) isolates for azoles, following the EUCAST breakpoints, and amphotericin B by EUCAST, CLSI and Elefanti et al. 2014 proposed breakpoints.

CAPÍTULO 2

Clinical and Environmental Azole-Resistant *Aspergillus fumigatus* Harboring TR34/L98H and M220R Mutations Isolated in Brazil

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ABSTRACT

Aspergillus fumigatus is an airborne fungal pathogen that has been shown to develop resistance since it is exposed to azole antifungals during infection or in the environment. This study reported the occurrence of TR34/L98H and M220R mutation in two triazole-resistant *A. fumigatus* strains obtained from a patient with aspergillosis and from the environment in Brazil. Susceptibility testing to azoles, echinocandins and amphotericin B was carried out by EUCAST broth microdilution method. Mutations were detected by real time PCR and full sequence of *cyp*51A. Although there is no epidemiological data showing high percentages of azole-resistant *A. fumigatus* strains in Brazil, it is the first time that triazole-resistant *A. fumigatus* with TR34/L98H and M220R mutations in *cyp*51A are described in this country. These results suggest that mutations related to azole resistance are present in Brazilian strains and adequate management of aspergillosis is necessary to avoid increasing resistance rates.

Key-Words: *Aspergillus fumigatus*, TR34/L98H, azole resistance, M220R, environment.

INTRODUCTION

Aspergillus fumigatus is an opportunistic fungal pathogen that is widespread in the environment. It is responsible for causing various forms of infection mainly in immunocompromised patients, such as acute or chronic invasive aspergillosis, allergic bronchopulmonary aspergillosis, and aspergilloma (Paulussen et al, 2017; Kosmidis & Denning, 2015).

Therapy for these infections is primarily based on the use of azole antifungal drugs. Voriconazole (VCZ) is the first line of treatment and posaconazole (PCZ) mainly used in the prophylaxis of aspergillosis in patients at risk, while itraconazole (ITZ) and, more recently, isavocunazole (ISZ) are used in specific cases (Frampton et al, 2008; Denning et al, 2002;). Although the use of azoles have revolutionized the treatment of aspergillosis the first isolates of resistant *Aspergillus* were reported in 1997 and this number has been increasing over the years, becoming a major concern in the management of aspergillosis (Howard et al, 2009; Denning et al, 1997).

A. fumigatus isolates with resistant profile and carrying mutations azole resistance-related are expending and being detected not only in Europe, where most of these cases are detected (Snelders et al, 2009; Snelders et al, 2008; Verweij et al, 2007; Mellado et al, 2004), but in other parts of the world, (Liu et al, 2015; Ahmad et al, 2014; Chowdhary et al, 2012; Lockhart et al, 2011). Two major pathways of azole resistance acquisition in *A. fumigatus* have been considered: a) prolonged azoles exposure of patients with chronic aspergillosis; b) development of resistance in the environment due to pressure of DMIs (demethylation inhibitory) fungicides used in agricultural crops (Snelders et al, 2012; Verweij et al, 2009).

In Brazil, demethylation inhibitory fungicides (DMIs) are used mainly in the control of Asian rust in soybean crop (caused by the fungus *Phakopsora pachyrhizi*)

(Embrapa, 2013; Godoy, 2012) and in the control of foliar fungi diseases in maize (mainly caused by the ascomycetes *Cercospora zeae-maydis*) (da Costa & Cota, 2012). The most used DMIs are tebuconazole, epoxiconazole, cyproconazole, propiconazole, prothioconazole, metconazole, tetraconazole and difeconazole (FRAC, 2).

Despite the high use of triazole fungicides in Brazilian agriculture, data correlating cross-resistance of *A. fumigatus* between triazoles for clinical and environmental use have not been documented in Brazil, as well as epidemiological data reporting clinical resistance to this class of antifungal drugs are quite scarce. Therefore, the objective of this study was to identify possible mutations in *A. fumigatus* strains isolated from a patient (n = 1) and environment (n = 1) in Brazil that showed high minimal inhibitory concentrations (MIC) to azoles.

MATERIALS AND METHODS

Clinical *A. fumigatus* isolate - case report

A 62-year-old male patient with a history of treated tuberculosis at 46 years of age, presenting the following symptoms: a productive cough for 2 years that had worsened for 3 months, malaise and low fever was admitted to a hospital in southern Brazil. This patient had received previous treatment with levofloxacin and prednisone due to a diagnosis of pneumonia. Chest computed tomography showed ill-defined infiltrated lung areas and small cavities in one of the pulmonary lobes. The research for mycobacteria and blood culture was negative as well as direct examination and sputum culture was negative for fungi. Histopathological examination revealed septated hyaline hyphae at a 45º angle. The second culture of bronchoalveolar lavage (BAL) was positive for *A. fumigatus*. Based on the clinical evidences the patient was diagnostic with chronic necrotizing pulmonary aspergillosis (CNPA). Therapy with itraconazole

200 mg a day was started, as there was no significant improvement in clinical status, therapy was modified to voriconazole (200 mg every 12 h). After treatment with voriconazole the symptoms decreased, however, the culture of a new BAL sample showed again growth of *A. fumigatus*. The patient then received amphotericin B (1.0 mg per kilogram once daily) and the case had a good evolution.

Environmental isolation

The environmental *A. fumigatus* was isolated from maize harvested from a crop in southern Brazil by the in-house plating method in surface with Dichloran Rose Bengal Chloramphenicol Medium (DRBC, Himedia®). For the plating of samples, 1 g of ground maize seeds was weighed and diluted in 9 mL of sterile peptone water (0,1%) (Himedia®) (1:10 dilution). The suspension was mixed in shaker for 10 min and more two dilutions were made from the first suspension (1:100 and 1:1000). From each of the three dilutions, 0.1 mL aliquot was transferred to *Petri* dishes with the DRBC culture medium. The plates were incubated at 37° C until observation of visible fungal growth. *A. fumigatus* were then isolated and identified.

***Aspergillus fumigatus* strains**

The *AFu*1 (clinical) and *AFu*2 (environmental) strains were identified by macro and micro morphology, growth capacity at 48 ° C, sequencing of ITS1-5.8s-ITS2 region (White et al, 1990) and the ß-tubulin gene as described previously (Samson et al, 2007; Glass et al, 1995).

Susceptibility testing to antifungal drugs

To determine the susceptibility profile of *A. fumigatus* isolates to antifungal drugs broth microdilution assay was performed following to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocol (EUCAST, 2008). ITZ, amphotericin B (AMB), anidulafungin (AFG) and caspofungin (CAS) were assayed in a

concentration range from 0.016 to 16 mg/L; VCZ and PCZ from 0.008 to 8.0 mg/L, ISZ from 0.004 to 4.0 mg/L and micafungin (MFG) from 0.002 to 2.0 mg/L. Minimal effective concentrations (MECs) by microscopy and minimal inhibitory concentrations (MICs) by visual readings were determined at 24 and 48h, respectively. For interpretation of azole resistance the followed breakpoints were used, ITZ and VCZ, MIC ≥ 4.0 mg/L, POS ≥ 0.5 mg/L and ISZ ≥ 2.00 (Arendrup et al, 2016; Hope et al, 2013; Arendrup et al, 2012).

Screening of mutations in *Cyp*51A gene

To detect the presence of mutations the real-time PCR and the full sequence of *Cyp*51A assay were performed for both strains. *A. fumigatus* strains were cultured on Sabouraud dextrose agar at 27º C until sporulation, after that the DNA was extracted with breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0) and phenol-chloroform-isoamylalcohol was used for DNA precipitation.

For full sequence assay PCR was performed using the Phusion Green High-Fidelity DNA polymerase Kit (Thermo Fisher Scientific, The Netherlands) following the manufacturer's instructions. Amplification was performed using the cycling parameters, 98º C for 30 s, 40 cycles of 98º C (10s), 58º C (30s) and 72ºC (4 min), 72º for 10 min, and one final cycle at 20º C. The forward primer 5'-ATGGTGCCGATGCTATGG-3' and reverse primer 5'-CTGTCTCACTTGGATGTG-3' were used for both PCR and subsequently *Cyp*51A gene sequencing. To identify mutations the sequences were aligned with a reference *cyp*51A sequence (GenBank accession no. AF338659).

The real-time PCR was performed following the methodology proposed by Klassen et al, 2010 using the LightCycler 480 (Roche Diagnostics, Almere, The

Netherlands). For detection of *Cyp*51A promoter polymorphism a reaction volume of 10 µL containing~ 1ng of DNA, 0.5 µM of primers (51APrF and 51APrR), 0.5 U of FastStart Taq DNA polymerase (Roche Diagnostic), 0.2 mM of dNTPs, 1x Resolight dye (Roche Diagnostic), and 2.5 mM $MgCl_2$ in 1x reaction buffer was used. For other mutation points detection the primers and probes used were G54F (0.3 µM), G54R (0.2 µM), G54P (0.2 µM), L98HF (0.1 µM), L98HR (0.5 µM), L98HPwt (0.2 µM), L98HPmut (0.2 µM), M220F (0.5 µM), M220R (0.2 µM), M220P (0.2 µM), G138F (0.2 µM), G138R (0.5 µM) and G138P (0.2 µM) in a final volume concentration of 10 µL containing ~ 1ng of DNA, 1.0 U of FastStart Taq DNA polymerase, 0.2 mM of dNTPs, 1x Resolight dye and 2.0 mM $MgCl_2$ in 1x reaction buffer.

RESULTS AND DISCUSSION

Results showed the presence of a tandem repeat of 34 bp in the promoter region of the *cyp*51 gene associated with a point of mutation in the codon L98H (TR34/L98H) in the clinical isolate of *A. fumigatus* (*AFu*1) and a mutation point in the M220 amino acid (M220R) in the environmental isolate (*AFu*2). Both strains showed high MICs to itraconazole (> 16 mg / L); however *AFu*1 was resistant to the other azoles, while *AFu*2 strain was resistant only to isavocunazole and showed intermediate susceptibility to voriconazole, whereas against AmB and echinocandins both strains showed to be sensitive (Table 1).

Table 1. Susceptibility of *A. fumigatus* isolates harboring azole-related mutations.

Strains	Mutations	Antifungal Susceptibility (mg/L)							
		AMB	ITZ	VCZ	PCZ	ISA	ANF	CAS	MFG
*AFu*1	TR34/L98H	0.500	>16.00	4.000	0.500	4.000	0,125	0.500	0.016

*AFu*2	M220R	0.500	>16.00	2.000	0.250	4.000	0.063	0.250	0.031

*AFu*1, *A. fumigatus* clinical isolate; *AFu*2, *A. fumigatus* environmental isolate; AMB, amphotericin B; ITZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole; ISA, isavocunazole; ANF, anidulafungin; CAS, caspofungin; MFG, micafungin.

TR34/L98H mutation have been found in both, clinical and environmental *A. fumigatus* strains, since it is the most resistance mechanism reported in the environmental strains. It is believed that the generation of this mutation occur in the environment, and when the patients are infected with these strains, resistance to the treatment is observed even though they have never been treated with clinical azoles (Snelders et al, 2008; Verweij et al, 2009).

Camps et al, (2012) reported that isolates containing the TR34/L98H mutation have a common ancestor, since high genetic relationships were found between different isolates containing this mutation compared to those wild-type or containing other mutations. However, three hypotheses regarding the origin of this mechanism have been addressed including geographic migration of resistant conidia or independent local development and subsequent selection of the mutation or both is occurring at the same time (Vermeulen et al, 2013). Although the higher genetic correlation among the isolates containing the TR34/L98H mutation than those that do not harbor this mutation, the detection of this mechanism of resistance in regions other than Europe reinforces the hypothesis that the origin can occur by both ways, migration of resistant conidia or independent local development.

Mutations in codon 220 of the *cyp*51A gene have been correlated with itraconazole resistance and different patterns of high MICs to the others azole antifungal drugs. The *cyp*51 protein has two input channels (1 and 2) that allow the azoles to bind to their site of action. The M220 codon is located in loops close to channel 2 and any mutation in this codon leads to the closure of this channel, which affects the fitting of

the azoles throughout the protein (Gollapudy et al, 2004; Mellado et al, 2004). So far, mutation in this codon has only been described in clinical strains and its origin has been attributed to the prolonged treatment of patients with azole antifungals (Mellado et al, 2004; Garcia-Effron et al 2005). In this study we found a M220R mutation in a strain of environmental origin, which showed high MIC for itraconazole but not for the other azoles, showing that the mutations at codon 220 may also occur in the environment.

Although the detection of resistance mechanisms in two strains of *A. fumigatus* does not represent great concern regarding the general context of azole resistance in the Brazilian context, we believe that this number is underestimated due to the lack of adequate diagnosis of fungal diseases, as well as the susceptibility tests in Brazilian hospitals that makes it difficult to study of azole resistance.

CONCLUSION

This study is the first report of azole-resistant *A. fumigatus* isolates harboring TR34/L98H or M220R mutations in Brazil. Although more studies on the epidemiology of clinical and environmental isolates of *A. fumigatus* are needed, the results shown here are a first warning regarding the possible emergence of azole resistance in *A. fumigatus*, generating a concern about the management of infections caused by this fungus as well as on the control in the use of triazole fungicides in agriculture, thus avoiding an increase in resistance rates to azoles treatment.

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DECLARATION OF INTEREST

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CAPÍTULO 3

Comparison between Etest and Broth Microdilution Methods for Testing Itraconazole-Resistant *Aspergillus fumigatus* Susceptibility to Antifungal Combinations

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ABSTRACT

The checkerboard-broth microdilution assay (BMD) is the most frequently used method for the *in vitro* evaluation of drug combinations. However, its use to evaluate the effect of antifungal drugs on filamentous fungi is sometimes associated with endpoint-reading difficulties, and different degrees of interaction are assigned to the same drug combination. We evaluated combinations of the azoles, itraconazole, posaconazole, and voriconazole, with the echinocandins, anidulafungin, caspofungin, and micafungin, against 15 itraconazole-resistant *Aspergillus fumigatus* clinical strains via the checkerboard-BMD and Etest assay. Readings after 24 and 48 h, considering the two reading endpoints, the minimum inhibitory concentration (MIC) and minimum effective concentration (MEC), were performed for both methods. Our results showed that the correlation coefficients between the BMD and Etest methods were quite diverse for to the drug combinations tested. The highest correlation coefficients of the Etest with the BMD assays (MEC and MIC reading) were the Etest-MIC reading at 24 h and the Etest-MEC reading at 48 h. Improvements in experimental conditions may increase the correlation between the two methods and ensure that Etest assay can be safely used in the evaluation of antifungal combinations against *Aspergillus* species.

Keywords: Antifungal combinations; *Aspergillus fumigatus*; Etest; broth microdilution

1 INTRODUCTION

Aspergillosis, caused by the species *Aspergillus fumigatus*, is a fungal infection associated with high morbidity and mortality [1-2]. There has been a notable increase in the incidence of aspergillosis over the past three decades [3-5]. Triazole antifungals are preferred agents to treatment and prevention of pulmonary aspergillosis syndromes, particularly voriconazole (VCZ) and posaconazole (PCZ) are recommended for the primary treatment and prophylaxis, respectively and itraconazole (ITZ) as a second treatment line, especially in chronic and allergic infectious [6-7]. However, several cases of azole resistance have been documented in medical centres around the world [8-11]. Echinocandins are another class of antifungals used as an alternative treatment for aspergillosis. They are usually used in combination with azoles, generating an effective alternative approach for aspergillosis treatment [12-15]. The synergistic effect of the azole-echinocandin combination may be very beneficial to reduce the incidence of resistance and toxicity. Thus, we found it very important to evaluate the interactions between these two classes of antifungals.

The checkerboard method, which is based on the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, is the most frequently used method to evaluate drug interactions *in vitro*. However, when this method is used for drugs against filamentous fungi, especially drugs that have different reading endpoints, as is the case in azoles-echinocandins combinations in which the determination of the reading endpoint is growth inhibition of 100% for azoles and changes in the hyphal morphology for echinocandins, the determination of the fractional inhibitory concentration index (FICi) is difficult to carry out. Therefore, some methods have been developed to overcome this problem, such as the modified checkerboard microdilution method that uses the MTT

dye [3-(4, 5-dimethyl-2-thiazyl) 2, 5-diphenyl-2H-tetrazolium bromide] [16], isothermal microcalorimetry [17], response surface model [18], and Etest. The antifungal drug combinations by Etest, an easy and fast diffusion method, were evaluated only against yeast but not to filamentous fungi [19]. In this study, we investigated the interactions between azoles and echinocandins *in vitro* by using Etest and checkerboard methods to determine the potential therapeutic effect of azole-echinocandin combinations against ITZ-resistant *A. fumigatus* infections.

2 MATERIALS AND METHODS

2.1 Study design

Effects of echinocandins (anidulafungin (ANF), caspofungin (CAS), and micafungin (MFG)) and azoles (ITZ, PCZ and VCZ) against ITZ-resistant *A. fumigatus* clinical strains were assessed via broth microdilution (BMD) according to EUCAST guidelines [20] and Etest methods, using RPMI, Sigma-Aldrich®/ Bacto Agar, Difco® Bacto® supplemented with 2% glucose.

2.2 Chemicals and Etest strips

Standard ITZ, PCZ, and VCZ powders were obtained from Sigma-Aldrich, São Paulo, SP, Brazil. ANF (Pharmacia & Upjohn Co. Kalamazoo, MI, USA), CAS (Laboratories Merck Sharp & Dohme-Chibret, Clermont Ferrand, France), and MFG (Astellas Pharma Tech Co., Takaoka, Toyana, Japan) were obtained from the corresponding manufacturers. All the Etest strips were acquired from the same manufacturer (bioMérieux Brazil, Rio de Janeiro, RJ, Brazil).

2.3 Clinical isolates

A. fumigatus isolates were obtained from the fungus culture collection of the Mycological Research Laboratory (LAPEMI) of the Federal University of Santa Maria, Brazil. Fifteen ITZ-resistant isolates were tested using both assays. To ensure the

reproducibility of all the tests, reference strains of *A. fumigatus* (ATCC 204305) and *A. flavus* (ATCC 204304) were used as internal quality control strains.

The clinical isolates were identified based on macro- and micro-morphology and this identification was confirmed by sequencing the internal transcribed spacer (ITS) regions [21]. All isolates were stored in saline until the time of use. To perform the tests, isolates were subcultured on potato dextrose agar (PDA, Himedia®). The resistance detection was based on susceptibility tests using BMD and Etest methods, and growth in the presence of agar containing different concentrations of ITZ (4.0 to 32.0 mg/L).

2.4 Broth microdilution (BMD)

To evaluate the susceptibility of *A. fumigatus* to drugs alone and in combination, stock solutions of ITZ, VCZ, PCZ, MFG, CAS, and ANF were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich®). All solutions were diluted in RPMI 1640 medium (supplemented with L-glutamine and 2% glucose) buffered to pH 7.0 with 3-(N-morpholino) propanesulfonic acid (MOPS) (RPMI, Sigma-Aldrich®).

2.4.1 Determination of MICs and MECs

The individual MICs and MECs were determined in accordance with the EUCAST E.DEF 9.3 methodology [20] for spore-forming moulds. In brief, 100 μL of each antifungal concentration was dispensed in 96-well microplates, in columns 1 to 10, whereas columns 11 and 12 served as the negative (inoculum-free) and positive (drug-free) controls, respectively. The final concentration of the azoles and echinocandins in the wells ranged from 0.063 to 32.00 mg/L for ITZ and VCZ, 0.008 to 4.00 mg/L for PCZ, and 0.001 to 0.50 mg/L for the three echinocandins.

For inoculum preparation, conidial suspensions were harvested from cultures grown for 5–7 days at 37 °C in PDA, with saline solution supplemented with

0.1% Tween 20. These suspensions were filtered through an 11-µm-pore filter fitted to a syringe to remove the hyphae. Then, the suspensions were adjusted to contain 2–5 × 10^6 conidia/mL by counting in a haemocytometer chamber. To obtain a final working inoculum concentration of 2–5 × 10^5 cfu/mL, a 1:10 dilution was made in sterile distilled water. All adjusted inoculum were quantified by plating on Sabouraud agar plates. A total of 100 µL of the adjusted inoculum was dispensed into the microdilution wells. The microplates were incubated at 35 °C and readings were taken after 24 and 48 h. For all drugs, MIC was defined visually as the lowest concentration that completely inhibits growth compared with the positive control, while MEC was defined, especially for echinocandins, as the lowest concentration resulting in abnormal, short, and branched hyphae, in contrast to the unbranched and long hyphal elements of the positive control well observed under an inverted microscope after 24 h [20-22].

2.4.2 Drug interactions

The interactions between azoles and echinocandins were studied using the checkerboard microdilution technique in 96-well microplates as described below. Drugs were dispensed as follows, 50 µL of each azole concentration was dispensed in columns 1 to 10, and then 50 µL of each echinocandin concentration was added to rows A to G. The final antifungal concentrations in the wells ranged from 0.016 to 8.00 mg/L for ITZ and VCZ, 0.002 to 1.00 mg/L for PCZ, and 0.001 to 0.125 mg/L for the three echinocandins. A volume of 100µL of the standardized inoculum (2–5 × 10^5 cfu/mL) was added and after incubation at 35 °C, readings were taken after 24 and 48 h. Each isolate was tested three times on different days. Since azoles and echinocandins show different endpoints readings, both 100% and 50% (MEC reading for echinocandins) of inhibition were considered MIC endpoints.

In order to assess the nature of drug interactions, the following equation, which determines the fractional inhibitory concentration index (FICi) was applied: $\text{FICi} = FIC_A + FIC_B = \frac{C_A^{comb}}{C_A^{alone}} + \frac{C_B^{comb}}{C_B^{alone}}$, where $C_A{}^{alone}$ and $C_B{}^{alone}$ are the concentrations of drugs *A* and *B* alone, respectively, and $C_A{}^{comb}$ and $C_B{}^{comb}$ are the concentrations of drugs *A* and *B* in combination, respectively. FICis were calculated for all wells that were considered MICs (at 24 h, MEC / 50% inhibition; at 48 h, MIC / 100% inhibition) for each isolate and the three replicates. FICi(s) mean were calculated from the triplicates to define drug-drug interactions. FICi ≤ 0.5 indicated synergism; 0.5 < FICi < 4.0 indicated non-significant interactions; and FICi ≥ 4.0 indicated antagonism [23].

2.5 Etest method

RPMI, Sigma-Aldrich®/ Bacto Agar, Difco® Bacto® supplemented with 2% glucose (Merck®) was used for Etest®. To prepare the inoculum, 5 mL of saline containing 0.1% Tween 20 was added to tubes containing *A. fumigatus* colonies after 48 or 72 h of growth in PDA Himedia®, at 35 °C. Then, the tubes were scraped smoothly to obtain a conidial suspension, which was transferred to another tube and left to sediment for 2–5 min, after which the supernatant was transferred to a new tube. Cells were counted using a haemocytometer, and adjusted to 10^6 cfu/mL [24,25]. Nine-millimetre-diameter plates containing 30 mL of RPMI/Bacto Agar were inoculated with a swab and excess liquid was removed by pressing the swab against the tube wall. Then, the swab was slipped on the plate three times at a 60° angle. The plates were left for 15 min at room temperature (24 °C ± 2) dry and then the strips were applied.

2.5.1 Individual antifungal activity

To determine the individual antifungal activity of each drug, the strips of each antifungal (ANF, CAS, MFG, ITZ, VCZ, and PCZ) were applied onto the plate by using tweezers to ensure that the whole surface area of the strip is in contact with the

agar and that there are no blisters. The plates were incubated at 35 °C and readings were taken after 24 and 48 h for all antifungal drugs.

2.5.2 Combined antifungal activity

The combined antifungal activity was determined using the diffusion method [19]. First, the antifungal strip was applied directly onto the agar as described above. The plates were allowed to stand at room temperature (24 °C ± 2) for 2 h to allow diffusion of the antifungal. Then, this strip was removed and the new strip with the second antifungal was applied onto the same place. The plates were incubated at 35 °C and readings were taken after 24 and 48 h.

2.5.3. Interpretation of results

For azoles, the Etest MIC was recorded as the lowest drug concentration at which the border of the elliptical zone of inhibition intercepted the Etest strip. For echinocandins, the Etest MEC was read at the intersection of the compact growth and slight growth (distinct colonies) zones with the Etest strip (Fig 1). The distinct colonies were collected and short, stubby branches and star-like hyphal morphologies were observed under the microscope. Evaluation of drug interactions via Etest was carried out using the same criterion as for BMD, by calculated the FICi(s).

2.6 Correlation between BMD and Etest results for single and combination tests

In order to compare the MIC/MEC values obtained from BMD and Etest methods, values from the Etest method were elevated to the next twofold dilution to match the drug scheme of the BMD method. Essential agreement between both methods was concluded when the MIC/MEC values fell within ±2 dilutions of the twofold dilution scheme, as analysed previously.

Regarding combination tests, in order to compare the outcomes of both BMD and Etest methods, the Pearson’s correlation analyses [26] was performed via the

software package GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA). A *P* of 0.05 was considered significant.

3 RESULTS

The MIC and MEC characteristics of all *A. fumigatus* isolates assessed using both BMD and Etest methods are presented in Table 1. The agreement percentage between BMD and Etest significantly high among azoles, with concordance rates of 100% for VCZ, 93.33% for ITZ, and 86.66% for PCZ. For all echinocandins, the agreement index was lower, CAS, MFG and ANF showed concordance rates of 46.66%, 73.33% and 53.33%, respectively (Fig 2). With the exception of VCZ, all antifungal agents showed higher MICs/MECs against *A. fumigatus* strains when tested using Etest (24 and 48 h) compared to BMD (Table 1).

BMD and Etest methods revealed different interactions between azoles and echinocandins against *A. fumigatus* strains depending on the drug combination, the endpoint, and the method used. The FICi(s) ranges of combinations determined using BMD and Etest are presented in Table 2 and the percentages of synergism, indifference and antagonism are showed in Fig 4 for both methods. Through BMD method, reading of the MEC or MIC did not seem to significantly influence in the final results, higher percentages of indifference were observed for both endpoint readings; only combinations of ITZ with CAS (by MEC) and ITZ with ANF (by MIC) showed synergism higher than 50% (60% and 53.33%, respectively). The Etest method yielded higher percentages of synergism than those obtained using the BMD method, especially when MIC values were taken into consideration. In the Etest-MEC readings at 24 and 48 h, the results show higher indexes of indifference for most combinations between azoles and echinocandins. Low or zero percentages of antagonism were observed for all reading conditions in the Etest method.

For the Etest MIC readings taken after 24 h, all the combinations of azoles and echinocandins were synergistic with percentage range from 60 to 100% of synergism (Fig 4), except PCZ-CAS combination showed a higher percentage of indifference (73.33%) when tested using this method (Fig 4).

Regarding Etest MIC readings taken after 48 h, the synergistic interactions observed at 24 h persisted, but with a decrease of about 10% in the synergism percentage in some combinations, except combinations of VCZ with CAS that showed 60% indifference. The PCZ-CAS combination remained with indifferent results in 48 h (86.66% indifferent) (Fig 4).

Etest MEC readings demonstrated lower percentages of synergism than Etest-MIC readings. At 24 h, the combinations of ITZ and PCZ with ANF, and VCZ and PCZ with CAS showed percentages of synergism from 53.0 to 86.66%, while the other associations showed indifferent results. At 48h the combinations of VCZ with each of the echinocandin were indifferent whereas the combinations of ITZ and PCZ showed more synergistic results, only ITZ-CAS and PCZ-MFG combinations were mostly indifferent (Fig 4).

The correlation data and experimental conditions of the methods are presented in Fig 3. The Pearson's correlation coefficient (r) between BMD-checkerboard and Etest results ranged from 0.289 to 0.980 among the azole-echinocandin combinations. However, most of the combinations showed correlation indexes of above 0.7 for all Etest and BMD conditions tested, indicating a good correlation between both methods.

The two most consistent conditions of the Etest with the BMD assays (MEC and MIC reading) were the Etest-MIC reading at 24 h and the Etest-MEC reading at 48 h. However, the correlation indices between the different test conditions were dependent on the antifungal drug combinations. In the ITZ-ANF combination, the reading of the

MEC in the BMD was more concordant with the reading of the MEC in the Etest at both reading times (r = 0.953 / Etest-24 h; r = 0.973 / Etest-48 h), as the reading of the MIC in the BMD had better agreement with the reading of the MIC in the Etest (r = 0.826 / Etest-24 h; r = 0.853 / Etest-48 h).

In the VCZ-ANF combination Etest reading taking into account the MEC (at 24 and 48 h), had a better correlation at both endpoints in the BMD. In the combinations involving VCZ with the other echinocandins, the results were quite diverse, depending on the condition and time of reading. The best correlation for the VCZ-CAS combination was between BMD-MIC with Etest-MEC-24 h (r = 0.919). The best correlation in the VCZ-MFG combination was BMD-MIC with Etest-MIC-24 h (r = 0.980).

Unlike the VCZ-ANF combination, the PCZ-ANF combination showed a greater correlation between the methods when the MIC was read in Etest, with both BMD-MEC and BMD-MIC. In the PCZ-MFG combination, the highest correlation indices were observed between Etest-MIC-48 h and BMD-MEC (r = 0.952) and MIC (r = 0.951) While in the PCZ-CAS combination, we observed the lowest correlation indices between the two methods; the most consistent was BMD-MEC with Etest-MEC-24 h (r = 0.857).

4 DISCUSSION

Azole and echinocandin combinations have been used in the treatment of aspergillosis to obtain better outcomes than with monotherapy, especially in refractory fungal infections [27-31]. However, *in vitro* and *in vivo* studies conducted to test azole-echinocandin combinations against *Aspergillus* species have resulted in conflicting outcomes. The ideal method for evaluating the interactions between azoles and echinocandins against *A. fumigatus* has not yet been defined. Although BMD-

checkerboard is considered the gold standard method, it has some limitations, especially the subjective interpretation of MEC that depends on the reader, which may be one of the reasons for the discrepancy between the results obtained through the different methods. In this study, we observed that the mode of action of the echinocandins could also be detected via Etest, as demonstrated by the formation of halos with colonies of different morphologies, which provides a better visualization of the MEC in comparison with the microscopic reading in BMD.

Our results showed a high degree of concordance between EUCAST_BMD and Etest methods when comparing the individual activity of an azole antifungal against ITZ-resistant *A. fumigatus* strains. Other authors have already demonstrated the agreement between CLSI_BMD and Etest in previous studies. For example, Pfaller et al. [32] evaluated 376 *Aspergillus* isolates and demonstrated 97.6% and 95.8% agreement between both methods for VCZ and ITZ, respectively. Espinel-Ingroff et al [33] evaluated the effects of VCZ, PCZ, and ITZ on 87 *Aspergillus* isolates, among which 64 were *A. fumigatus*, showing 93.3 to 100% agreement in case of VCZ, independent of the Etest incubation time. For ITZ and PCZ, the results were more dependent on the incubation time; for *A. fumigatus*, ITZ showed better concordance after 24 h (90.3%) and PCZ after 48 h (95.2%). Lamoth and Alexander [34], assessed the agreement between both methods for VCZ and PCZ against 290 clinical moulds, including 74 *A. fumigatus* isolates, showing 95 and 89% concordance for VCZ and PCZ, respectively. The results of our study confirm that Etest is a reliable and reproducible susceptibility test for *A. fumigatus* with respect to the azoles and that it is possible to detect the resistance to ITZ, concordant with the EUCAST_BMD findings obtained here.

Regarding echinocandins, the correlation between the two methods was lower. This can be attributed to the greater difficulty in interpreting the MEC readings. Fuller et al. [35] have already shown poor agreement when CAS was tested via Etest (MIC, 24 h on agar RPMI) and CLSI_BMD (MEC, 24 h) against 345 *Aspergillus* isolates, with an agreement percentage of only 26%. In contrast to our findings, Martos et al. [24] evaluated the Etest and CLSI_BMD methods for 67 clinical isolates of *Aspergillus* to three echinocandins demonstrating excellent agreement between both methods, 100% for MFG and ANF, and 82.4% for CAS. Lamoth and Alexander [34], also demonstrated high agreement between the two methods for CAS (96%) and MFG (100%) against 74 *A. fumigatus* clinical isolates.

In combination assays, different degrees of interactions between azoles and echinocandins against ITZ-resistant *A. fumigatus* strains were observed based on the method used and the reading conditions. In general, the combinations evaluated via Etest, in all conditions, presented higher percentages of synergism than those evaluated using BMD.

Among the most frequently studied combinations against *A. fumigatus*, susceptible and resistant to ITZ and/or other azoles, are the combinations of VCZ with echinocandins. Many authors have reported a synergistic interaction between VCZ and ANF by different *in vitro* and *in vivo* methods [14,16,17,36,37]; however, in most studies, synergistic results were dependent on the method, antifungal dose, endpoint, and susceptibility of *Aspergillus* strains to azoles. Here, we found different results for this combination, which were completely synergistic by Etest-MIC-24 h, until a lack of synergism in BMD was indicated by MIC and MEC.

The same occurred in the combination of VCZ with MFG (100% synergism by Etest MIC at 24 h, and no synergism by BMD). In a study by Chandrasekar et al.

[38], a pig model of invasive aspergillosis was conducted and its results were concordant with our BMD results, in which the combined therapy between VCZ and MFG was not able to overcome monotherapy. In the same time, another *in vitro* study conducted by Lewis and Kontoyannis [39] tested this combination by the checkerboard method with MTT and showed that the activity of VCZ increased by the addition of MFG, however, this study tested only one isolate of *A. fumigatus*.

In the VCZ-CAS combination, we observed a lower synergism index by Etest and BMD, however the interactions were concordant by both methods. Other studies showed conflicting interactions depending on the method; Zhang et al [40] demonstrated indifference in VCZ with CAS combinations in a murine model of invasive aspergillosis by *A. fumigatus*, while Kirkpatrick et al [12] demonstrated high effectiveness of this combination in a guinea pig model of invasive aspergillosis.

In the combinations of PCZ with echinocandins, Etest demonstrated higher percentages of synergism than BMD, especially with ANF and MFG. With CAS, both methods are in agreement, showing higher percentages of indifference. A few studies have demonstrated the effectiveness of these combinations, especially PCZ combination with CAS, showing *in vitro* [15,41] and *in vivo* [14] synergism depending on the susceptibility of the *Aspergillus* strains to the azoles.

Regarding the combinations of ITZ with echinocandins, we found consistent results between the two methods, except for the combination of ITZ with ANF in which the BMD-MEC method showed contrasting results with other experimental conditions. Published articles have demonstrated different *in vitro* interactions between ITZ and echinocandins depending on the experimental conditions. For example, Cuenca-Estrella et al. [42] showed 64.33% synergism in the combination of ITZ with CAS by checkerboard assay in the MEC reading, but no synergism was observed in the MIC

reading. Shalit et al [43] also found controversial results. They demonstrated 100% synergism by reading the MEC and 100% indifference by reading the MIC by the checkerboard assay. On the contrary, *in vivo* studies have shown excellent interaction between ITZ and CAS or MFG [44-46] .

This study still has several limitations. For example, we were unable to define the ideal reading conditions by Etest for all drug combinations. We mainly concluded that, for most interactions, the MEC reading in the Etest method seem to have a better correlation with the BMD in its final result, which is the definition of the type of interaction between the antifungal drugs.

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6 DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest.

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Table 1.EUCAST broth microdilution (BMD) method and Etest susceptibility results for itraconazole-resistant *Aspergillus fumigatus* strains against azoles an echinocandins.

Method	Drug	Range	GM	MIC/MEC_{50}	MIC/MEC_{90}
Etest - 24 h - MIC	Itraconazole	4.000 - 32.00	12.699	16.00	32.00
	Voriconazole	0.250 - 6.000	0.899	1.000	3.000
	Posaconazole	0.023 - 0.750	0.254	0.380	0.750
	Caspofungin	32.00 - 32.00	32.00	32.00	32.00
	Micafungin	32.00 - 32.00	32.00	32.00	32.00
	Anidulafungin	32.00 - 32.00	32.00	32.00	32.00
Etest - 48 h - MIC	Itraconazole	4.000 - 32.00	12.699	16.00	32.00
	Voriconazole	0.250 - 6.000	0.899	1.000	3.000
	Posaconazole	0.125 - 1.500	0.451	0.500	1.500
	Caspofungin	32.00 - 32.00	32.00	32.00	32.00
	Micafungin	32.00 - 32.00	32.00	32.00	32.00
	Anidulafungin	32.00 - 32.00	32.00	32.00	32.00
Etest - 24 h - MEC	Itraconazole	4.000 - 32.00	12.699	16.00	32.00
	Voriconazole	0.250 - 6.000	0.899	1.000	3.000
	Posaconazole	0.023 - 0.750	0.254	0.380	0.750
	Caspofungin	0.190 - 0.500	0.327	0.380	0.500
	Micafungin	0.006 - 0.125	0.019	0.016	0.094
	Anidulafungin	0.002 - 0.047	0.012	0.016	0.032
Etest - 48 h - MEC	Itraconazole	4.000 - 32.00	12.699	16.00	32.00
	Voriconazole	0.250 - 6.000	0.899	1.000	3.000
	Posaconazole	0.125 - 1.500	0.451	0.500	1.500
	Caspofungin	0.190 - 0.500	0.359	0.380	0.500
	Micafungin	0.008 - 0.500	0.023	0.016	0.032
	Anidulafungin	0.002 - 0.250	0.013	0.016	0.032
BMD - 24 h - MEC	Caspofungin	0.008 - 0.500	0.061	0.030	0.060
	Micafungin	0.002 - 0.060	0.018	0.030	0.060
	Anidulafungin	0.002 - 0.030	0.004	0.002	0.008
BMD - 48 h - MIC	Itraconazole	4.000- 16.00	6.350	4.000	16.00
	Voriconazole	0.250 - 2.000	1.149	1.000	2.000
	Posaconazole	0.006 - 0.500	0.207	0.250	0.500

Etest - 24 h - MIC, Etest method read by MIC at 24 h; Etest - 48 h - MIC, Etest method read by MIC at 48 h; Etest - 24 h - MEC, Etest method read by MEC at 24 h; Etest - 48 h - MEC, Etest method read by MEC at 48 h; BMD - 24 h - MEC, broth microdilution read at 24 h by MEC (Minimal Effective Concentration); BMD - 48 h - MIC, broth microdilution read at 48 h by MIC (Minimal Inhibitory Concentration); GM, geometric mean; MIC/MEC_{50}, minimal inhibitory/effective concentration that inhibits 50% of the strains (mg/L); MIC/MEC_{90}, minimal inhibitory/effective concentration that inhibits 90% of the strains (mg/L).

Table 2. FIC indexes based on 100 % of inhibition growth (MIC, Minimal Inhibitory Concentration) and distinct morphology (MEC, Minimal Effective Concentration; 50 % of inhibition growth) endpoints by broth microdilution (BMD) and Etest methods for itraconazole-resistant *Aspergillus fumigatus* strains.

Drug Combinations	FIC index Average (FIC index Range)					
	Etest-24h-MIC	Etest-48h-MIC	Etest-24h-MEC	Etest-48h-MEC	BMD-24h-MEC	BMD-48h-MEC
ITZ + CAS	0.153 (0.008 – 0.563)	0.226 (0.012 – 0.844)	0.529 (0.021 – 1.063)	0.756 (0.042 – 2.031)	0.661 (0.019 – 2.06)	0.608 (0.258 – 1.125)
ITZ + MFG	0.226 (0.001 – 0844)	0.349 (0.001 – 1.125)	0.616 (0.096 – 1.334)	1.341 (0.065 – 11.89)	3.149 (0.060 – 30.25)	0.727 (0.50 – 1.25)
ITZ + ANF	0.144 (0.001 – 0.844)	0.190 (0.001 – 1.125)	1.296 (0.063 – 16.00)	1.724 (0.032 – 16.00)	4.931 (0.37 – 30.02)	0.595 (0.245 – 1.25)
VCZ + CAS	0.418 (0.02 – 1.02)	0.474 (0.065 – 1.023)	0.578 (0.128 – 1.316)	0.819 (0.412 – 1.566)	2.434 (0.075 – 16.62)	1.148 (0.255 – 4.25)
VCZ + MFG	0.149 (0.002 – 0.392)	0.296 (0.087 – 0.773)	1.534 (0.043 – 5.461)	1.471 (0.054 – 7.938)	2.687 (0.75 – 15.02)	1.670 (0.53 – 4.25)
VCZ + ANF	0.181 (0.05 – 0.392)	0.313 (0.091 – 0.772)	2.709 (0.133 – 20.96)	3.809 (0.096 – 23.56)	5.768 (0.560 – 30.03)	1.913 (0.516 – 8.03)
PCZ + CAS	1.168 (0.191 – 8.267)	0.768 (0.265 – 1.324)	1.386 (0.148 – 8.641)	0.365 (0.037 – 0.683)	2.151 (0.136 – 9.30)	1.572 (0.508 – 4.182)
PCZ + MFG	0.456 (0.096 – 2.785)	0.354 (0.011 – 0.772)	0.875 (0.128 – 2.522)	1.504 (0.251 – 8.063)	1.622 (0.28 – 5.00)	1.499 (0.516 – 4.19)
PCZ + ANF	0.309 (0.005 – 1.532)	0.304 (0.064 – 0.772)	0.575 (0.095 – 2.043)	1.003 (0.130 – 5.899)	1.595 (0.126 – 3.08)	1.276 (0.371 – 2.034)

BMD-24h-MEC, broth microdilution with endpoint reading at 24 h; BMD-48h-MIC, broth microdilution with endpoint reading at 48 h; Etest-24h-MEC, Etest by MEC with endpoint reading at 24 h; Etest-24h-MIC, Etest by MIC with endpoint reading at 24 h; Etest-48h-MEC, Etest by MEC with endpoint reading at 48 h; Etest-48h-MIC, Etest by MIC with endpoint reading at 48 h; ITZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole; CAS, caspofungin; MFG, micafungin; ANF, anidulafungin.

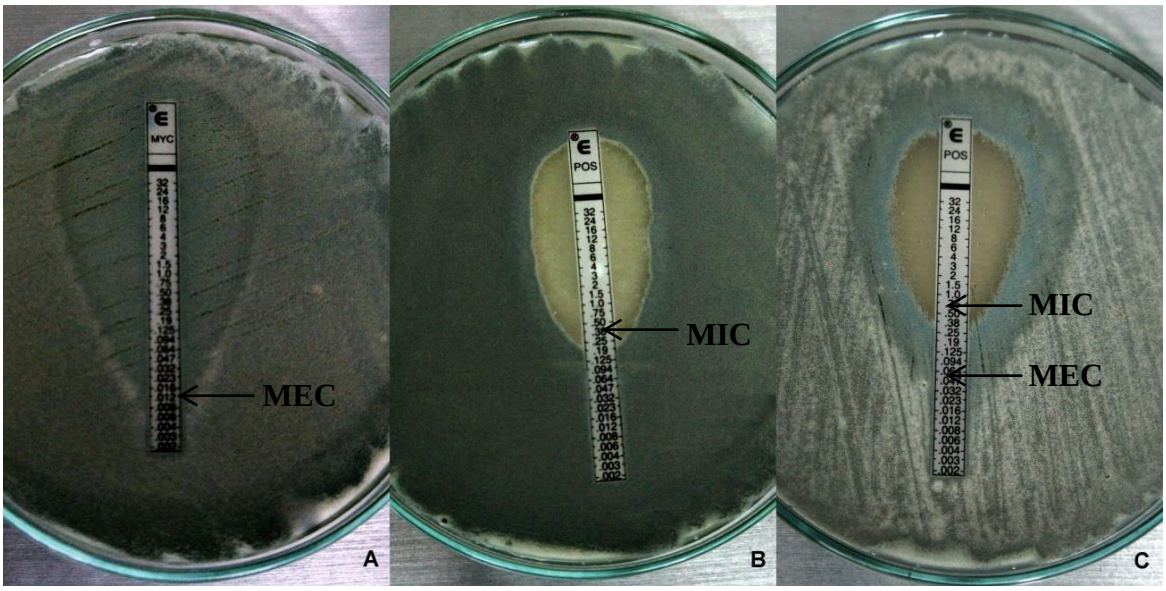


Fig 1. Susceptibility tests performed using Etest® against itraconazole-resistant *A. fumigatus* strains growing at 48 h. A) Micafungin activity alone. B) Posaconazole activity alone. C) Posaconazole associated with micafungin. MEC, minimal effective concentration; MIC, minimal inhibitory concentration.

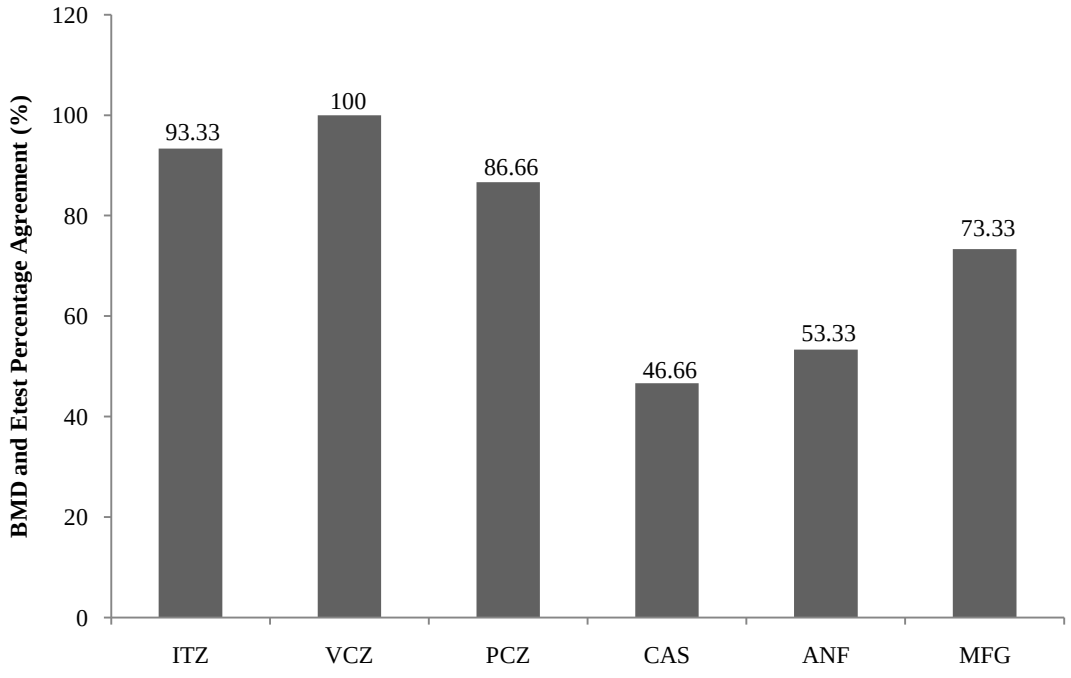
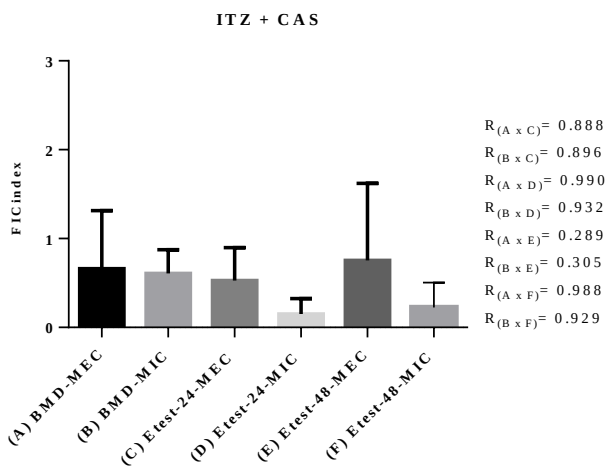
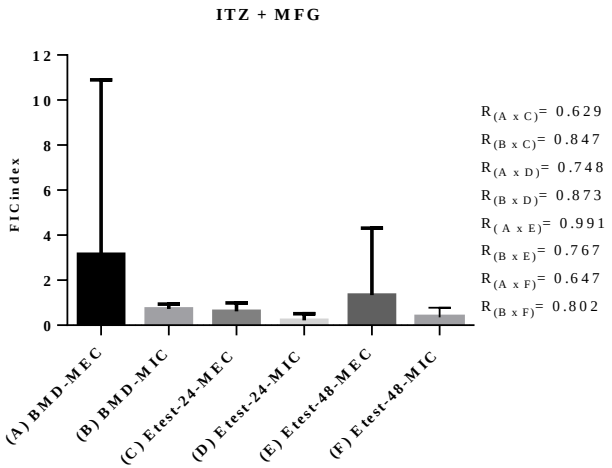
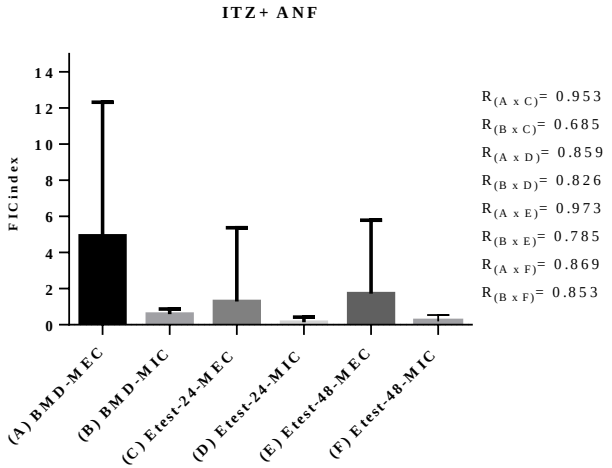
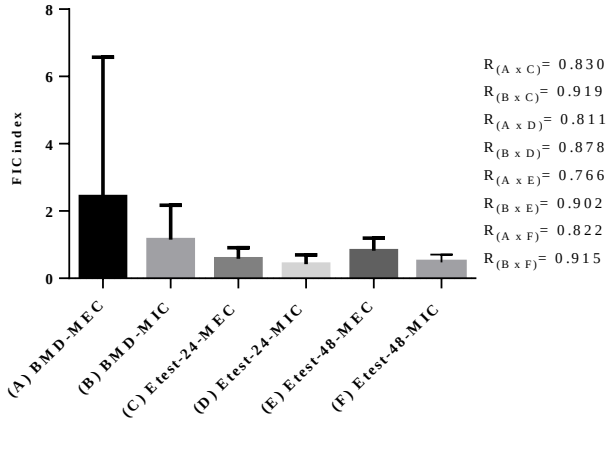


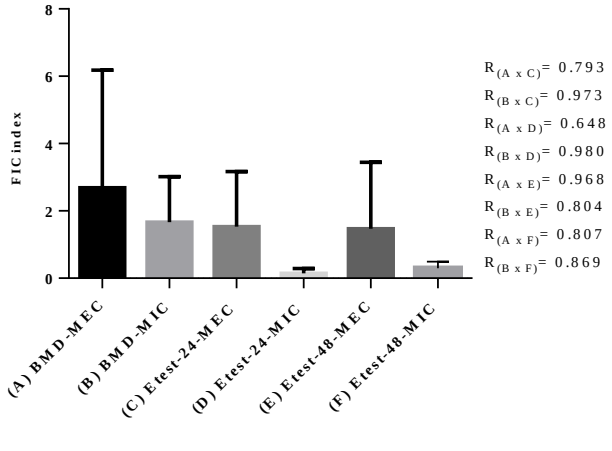
Fig 2. Percent agreement between Etest and Broth Microdilution (BMD) results. ITZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole; CAS, caspofungin; ANF, anidulafungin; MFG, micafungin.

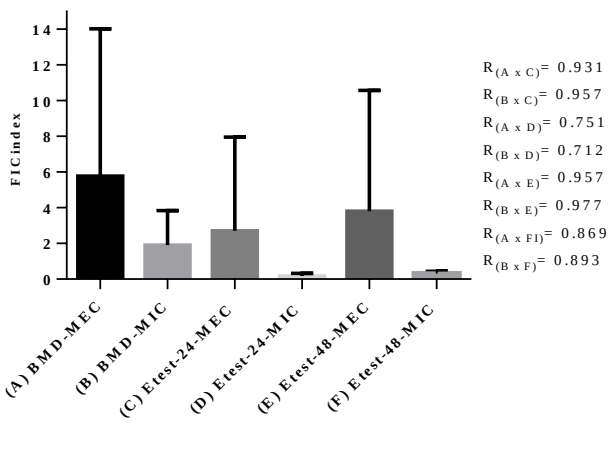

ITZ + CAS
FICindex
3
2
1
0
(A) BMD-MEC
(B) BMD-MIC
(C) Etest-24-MEC
(D) Etest-24-MIC
(E) Etest-48-MEC
(F) Etest-48-MIC
R(A x C)= 0.888
R(B x C)= 0.896
R(A x D)= 0.990
R(B x D)= 0.932
R(A x E)= 0.289
R(B x E)= 0.305
R(A x F)= 0.988
R(B x F)= 0.929


ITZ + MFG
FICindex
12
10
8
6
4
2
0
(A) BMD-MEC
(B) BMD-MIC
(C) Etest-24-MEC
(D) Etest-24-MIC
(E) Etest-48-MEC
(F) Etest-48-MIC
R(A x C)= 0.629
R(B x C)= 0.847
R(A x D)= 0.748
R(B x D)= 0.873
R(A x E)= 0.991
R(B x E)= 0.767
R(A x F)= 0.647
R(B x F)= 0.802


ITZ+ ANF
FICindex
14
12
10
8
6
4
2
0
(A) BMD-MEC
(B) BMD-MIC
(C) Etest-24-MEC
(D) Etest-24-MIC
(E) Etest-48-MEC
(F) Etest-48-MIC
R(A x C)= 0.953
R(B x C)= 0.685
R(A x D)= 0.859
R(B x D)= 0.826
R(A x E)= 0.973
R(B x E)= 0.785
R(A x F)= 0.869
R(B x F)= 0.853


VCZ+ CAS
FICindex
8
6
4
2
0
(A) BMD-MEC
(B) BMD-MIC
(C) Etest-24-MEC
(D) Etest-24-MIC
(E) Etest-48-MEC
(F) Etest-48-MIC
R(A x C)= 0.830
R(B x C)= 0.919
R(A x D)= 0.811
R(B x D)= 0.878
R(A x E)= 0.766
R(B x E)= 0.902
R(A x F)= 0.822
R(B x F)= 0.915


VCZ + MFG
FICindex
8
6
4
2
0
(A) BMD-MEC
(B) BMD-MIC
(C) Etest-24-MEC
(D) Etest-24-MIC
(E) Etest-48-MEC
(F) Etest-48-MIC
R(A x C)= 0.793
R(B x C)= 0.973
R(A x D)= 0.648
R(B x D)= 0.980
R(A x E)= 0.968
R(B x E)= 0.804
R(A x F)= 0.807
R(B x F)= 0.869


VCZ + ANF
FICindex
14
12
10
8
6
4
2
0
(A) BMD-MEC
(B) BMD-MIC
(C) Etest-24-MEC
(D) Etest-24-MIC
(E) Etest-48-MEC
(F) Etest-48-MIC
R(A x C)= 0.931
R(B x C)= 0.957
R(A x D)= 0.751
R(B x D)= 0.712
R(A x E)= 0.957
R(B x E)= 0.977
R(A x FI)= 0.869
R(B x F)= 0.893

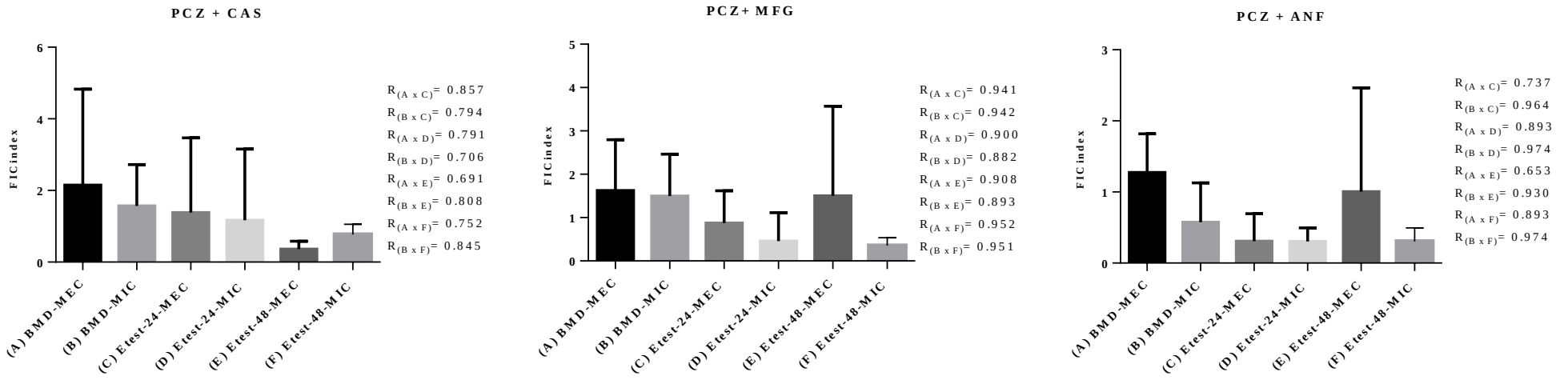


Fig 3. Fractional Inhibitory Concentration Indexes (FIC index) average with standard deviation (SD) of antifungal interactions by broth microdilution (BMD) and Etest methods for *Aspergillus fumigatus* itraconazole-resistant strains and Pearson correlation coefficient (R) between each BMD and Etest condition method. BMD-MEC (A), broth microdilution (Mininal Effective Concentration) with endpoint reading at 24 h; BMD-MIC (B), broth microdilution (Minimal Inhibitory Concentration) with endpoint reading at 48 h; Etest-24-MEC (C), Etest by MEC with endpoint reading at 24 h; Etest-24-MIC (D), Etest by MIC with endpoint reading at 24 h; Etest-48-MEC (E), Etest by MEC with endpoint reading at 48 h; Etest-48-MIC (F), Etest by MIC with endpoint reading at 48 h; ITZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole; CAS, caspofungin; MFG, micafungin; ANF, anidulafungin.

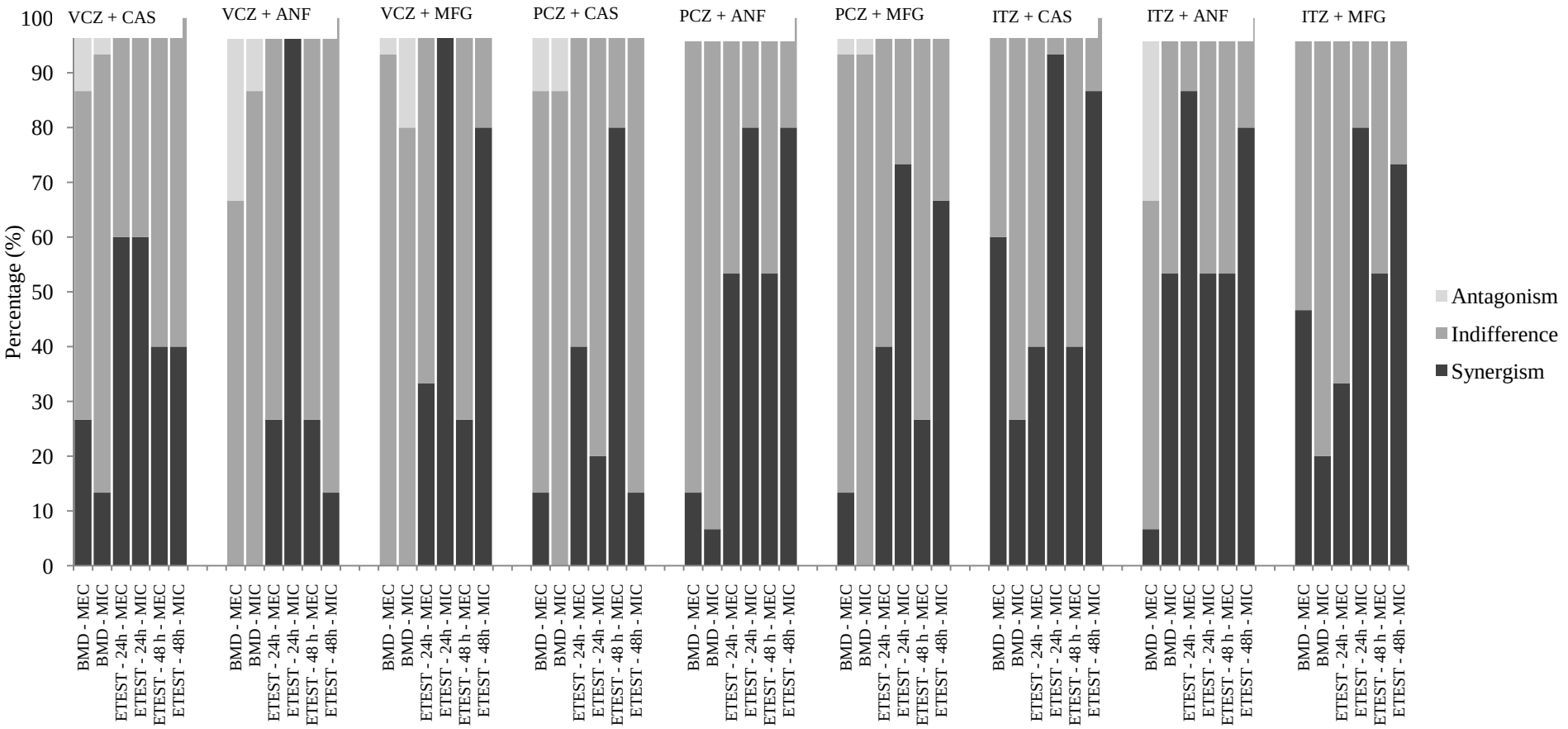


Fig 4. Percentages of antifungal interactions by broth microdilution (BMD) and Etest methods for *Aspergillus fumigatus* itraconazole-resistant strains. BMD-MEC, broth microdilution read in 24 h; BMD-MIC, broth microdilution read in 48 h; Etest -24 h- MIC, Etest method read by MIC at 24 h; Etest -48 h-MIC, Etest method read by MIC at 48 h; Etest -24h-MEC, Etest method read by MEC at 24 h; Etest -48h-MEC, Etest method read by MEC at 48 h; ANF, anidulafungin; CAS, caspofungin; MFG, micafungin; ITZ, itraconazole; PCZ, posaconazole; VCZ, voriconazole; MIC, Minimum Inhibitory Concentration; MEC, Minimum Effective Concentration.

CAPÍTULO 4

Evaluation of the Efficacy of a Posaconazole and Anidulafungin Combination in a Murine Model of Pulmonary Aspergillosis due to Infection with *Aspergillus fumigatus*

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ABSTRACT

Posaconazole (PSC) in combination with anidulafungin (AFG) was evaluated in a murine model of pulmonary aspergillosis. Immunosuppressed animals were infected via the nasal cavity with two different *A. fumigatus* strains. The animals received PSC (oral, 20 mg/kg/day) and/or AFG (i.p., 10 mg/kg/day) for 7 days. On day 8, the mice were euthanized and fungal burdens were determined from the lungs. Survival curves were constructed for mortality analysis. Compared to untreated groups, groups singly treated with PSC or AFG showed a reduced fungal burden in the lungs (P = 0.0001–0.006) and prevention of mortality (66.66% to 83.33% of survival). Combination treatment with PSC and AFG significantly reduced the fungal burden (or sterilized the lungs) compared to the findings in the untreated and monotherapy groups and improved the survival rate to 100%. The PSC and AFG combination therapy was highly effective and should be evaluated in larger-scale experiments.

Keywords: *Aspergillus fumigatus*, Combination therapy, Anidulafungin, Posaconazole

1 INTRODUCTION

Azole and echinocandin antifungal combinations have been recommended by international guidelines as salvage therapy in cases of difficult-to-treat *A. fumigatus* infections (Mikulsa et al., 2011). *In vitro* and *in vivo* studies have demonstrated that the combined use of these classes of antifungal agents, which have different targets in the fungal cell, may be more effective in the treatment of aspergillosis. This is especially true in cases where treatment with voriconazole (VCZ) alone is ineffective due to infection with azole antifungal-resistant *A. fumigatus* (Martin-Vicente et al., 2016; Siopi et al., 2016; Mavridou et al., 2015; Zhang et al., 2013; Tascini et al., 2003).

Since VCZ is the first-line treatment in aspergillosis, most studies have been focused on the combination of this antifungal with echinocandins. Especially good results have been found on using the VCZ-AFG combination, compared to those achieved with monotherapy with either agent, with decreased residual fungal burden and increased survival (Seydemousavi et al., 2013a; Seydemousavi et al., 2013b; van de Sande et al., 2009). However, Seydemousavi and colleagues (Seydemousavi et al., 2013a) demonstrated that this combination may not be advantageous over monotherapy in treatment of infection with VCZ-resistant *A. fumigatus* isolates.

Although PSC is currently indicated for prophylaxis, but not for the treatment of aspergillosis, other authors have already shown that combinations of PSC and echinocandins may have a synergistic effect against *A. fumigatus* strains (Martin-Vicente et al., 2016; Mavridou et al., 2015; Cacciapuoti et al., 2006). Therefore, we evaluated the efficacy of PSC and AFG combination therapy in a murine model of pulmonary aspergillosis.

2 MATERIALS AND METHODS

2.1 Microorganisms

A clinical and an environmental isolate (AFU19 and A07023, respectively) from our collection (Mycological Research Laboratory – LAPEMI, Federal University of Santa Maria – UFSM, Santa Maria, Brazil) and the reference *A. fumigatus* (ATCC 204305) and *A. flavus* (ATCC 204304) strains were used in this study. The isolates were identified by sequencing the internal transcribed spacer (ITS) and ß-tubulin gene. DNA extraction was performed using the MasterPure yeast DNA purification kit (Illumina Latin America, São Paulo, Brazil), following the manufacturer's instructions; they were stored at –80ºC until the time of use. To perform the tests, the isolates were subcultured on potato dextrose agar (PDA) at 35ºC for 72 h.

2.2 *In vitro* susceptibility

The minimal inhibitory concentration (MIC) and minimal effective concentration (MEC) of itraconazole (ITZ), VCZ, PSC and AFG were determined following the EUCAST E.DEF 9.3 methodology (Arendrup et al., 2015). Reference strains of *A. fumigatus* (ATCC 204305) and *A. flavus* (ATCC 204304) were used as internal quality control strains.

2.2.1 Interactions activity

The activity of the AFG and PSC combination was assessed by the checkerboard microdilution method, where the concentrations of PSC and AFG ranged from 1 to 0.002 mg/L and from 0.125 to 0.004 mg/L, respectively. The fractional inhibitory concentration indexes (FICi) were calculated using the equation $\text{FICi} = FIC_A + FIC_B = \frac{C_{PSC}^{comb}}{C_{PSC}^{alone}} + \frac{C_{AFG}^{comb}}{C_{AFG}^{alone}}$ where C_{PSC}^{alone} and C_{AFG}^{alone} are the MIC of PSC and AFG alone, respectively, and C_{PSC}^{comb} and C_{AFG}^{comb} are the MIC of PSC and AFG in combination, respectively. Considering that the MIC reading is performed for PSC (100% of inhibition at 48h) and the MEC reading is performed for AFG (50% of inhibition at 24h), both reading were relayed in the association. FICis were calculated for all wells that were considered MECs at 24 h or MICs at 48h for

each isolate and the three replicates. The fractional inhibitory concentration index (FICI) was calculated and the effect of the combination was classified. Synergy was defined when FICI≤ 5; indifference when 0.5< FICI ≤ 4; and antagonism when FICI > 4.0 (Odds et al., 2003).

2.3 Animals

Six-week-old female Swiss mice (Central Animal Laboratory of the Federal University of Santa Maria - UFSM, Santa Maria, Brazil) weighing 25 to 30 g were used for all studies. All procedures were approved by Ethics Committee on the Use of Animals of UFSM (protocol number CEUA 5317090216). Mice were housed in cages with sterilized shavings in groups of six or ten and allowed access to food and sterile water *ad libitum*.

2.4 Infection model

Mice were immunosuppressed with a combination of cyclophosphamide (intraperitoneal; reconstituted in sterile distilled water) (Genoxal, Prasfarma, Barcelona, Spain) and cortisone acetate (subcutaneous, reconstituted in sterile PBS with 0.02% Tween 80) (Sigma-Aldrich, SP, Brazil). An initial dose of 250 mg/kg of both drugs was given on day 2 prior to infection. To extend the duration of the neutropenia, a new cycle of immunosuppression was administered on day 3 post-infection with a lower dose of cyclophosphamide (200 mg/kg) and the same dose of cortisone acetate (Sheppard et al., 2004).

For infection, *A. fumigatus* strains were subcultured in PDA for 5 days at 37 ° C. On the day of infection, the inoculum was prepared by collecting and suspending the conidia in saline solution with 0.05% Tween 20. The conidial suspension was quantified in hemocytometer and adjusted to a concentration of 1×10^7 to 2×10^7 conidia/mL (Lepak et al., 2013). Viability was confirmed by counting on Sabouraud Dextrose Agar (SDA) plates. Mice were anesthetized for infection induction with an intramuscular injection (50 µL) of ketamine (37.5

mg/mL) and xylazine (5 mg/mL). The conidial suspension (50 µL) was deposited in the nasal cavity of the mice while the diaphragm was lightly pressed; when it was released, the conidia were aspirated into the lungs.

2.5 Treatment design

The therapy started 24 h after the infection. Mice received PSC, (Noxafil, Schering Corporation, Kenilworth, USA) (20 mg/kg, diluted in PBS, orally by gavage, once daily) (Seyedmousavi et al., 2014) or AFG (Ecalta, Pfizer Ltda, São Paulo, Brazil) (10 mg/kg, diluted in sterile water, intraperitoneal, once daily) (Seyedmousavi et al., 2013) or both drugs in combination at the same concentrations as the monotherapies. Six mice were used in each treatment regimen and ten were used in the control groups. Control animals received PBS (i.p.), or sterile water by gavage, or both, as placebo. The therapy was continued for seven days and the animals were inspected twice a day. On day 8, all surviving animals were euthanized by injection of pentobarbital (250 mg/kg) with prior anesthesia by ketamine-xylazine. Those animals that showed exacerbated infection (loss of more than 25% of their body weight, severe dyspnea, or lethargy) were euthanized before the end of the experiment. At the time of euthanasia, lungs were aseptically harvested and processed.

2.6 Lung processing and organism quantitation

Fungal burdens in the lungs of all animals were determined by CFU counting. The lungs were weighed and placed in sterile plastic bags containing 5 mL of saline solution with gentamicin (0.025 g/L) and chloramphenicol (0.4 g/L) (Sigma-Aldrich, São Paulo, Brazil). The bags were closed and the lung tissues were homogenized with manual pressure by rolling a large pen toward the bottom of the bag (25 repetitions). From the homogenate, dilutions (1:10, 1:100, and 1:1000) were performed in saline solution and placed in SDA +

chloramphenicol (0.05 g/L) plates. The plates were incubated at 37ºC for 24 to 48 h. The CFU per mouse lung was then calculated (Graybill et al., 1984; Patterson et al., 1991).

2.7 Statistical Analysis

The colony counts obtained from lung burden studies were converted to $\log_{10}$ per entire lung and compared using the Mann-Whitney U-test. *P* values were considered significant up to the level of 0.05. Residual fungal burden and survival curves were constructed by the Kaplan-Meier method. Mice that did not survive until the end of the study were assigned an arbitrary number of $\log_{10}{}^{5}$ CFU, chosen to overcome the CFU of mice sacrificed and to ensure that death was a poorer result than with any residual fungal burden survival (Shih, 2002). All statistical analyses were performed using the GraphPad Prism software package, version 5.0 (GraphPad Software, San Diego, CA).

3 RESULTS

For the AFU19 and A07023 strains, the MECs of AFG were 0.016 and 0.004 mg/L, respectively. The ITZ MICs were 8.0 mg/L for AFU19 and 4.0 mg/L for A07023, whereas the PSC MICs were 0.250 mg/L for AFU19 and 0.125 mg/L for A07023. The VCZ MIC was 2.0 mg/L for both strains. *In vitro* checkerboard assays showed indifferent or synergistic results in the association of PSC and AFG against the *A. fumigatus* strains, depending on the endpoint. MEC readings produced FICis of 0.683 for the AFU19 and 0.677 for the A07023 strain; MIC readings produced FICis of 0.254 and 0.666 for the AFU19 and A07023 strains, respectively.

Figure 1 compares the residual fungal burden in the lungs of mice infected with *A. fumigatus* strains and left untreated to those treated with PSC or AFG as monotherapies, or with both drugs in combination. Figure 2 shows the survival curves for all groups. Monotherapy with PSC or AFG was able to reduce the residual fungal burden in the lungs of

mice in all treated groups (Figure 1), as well as increase the survival rate in the treated groups compared to the findings achieved in the untreated animals (Figure 2) (P = 0.0001–0.006).

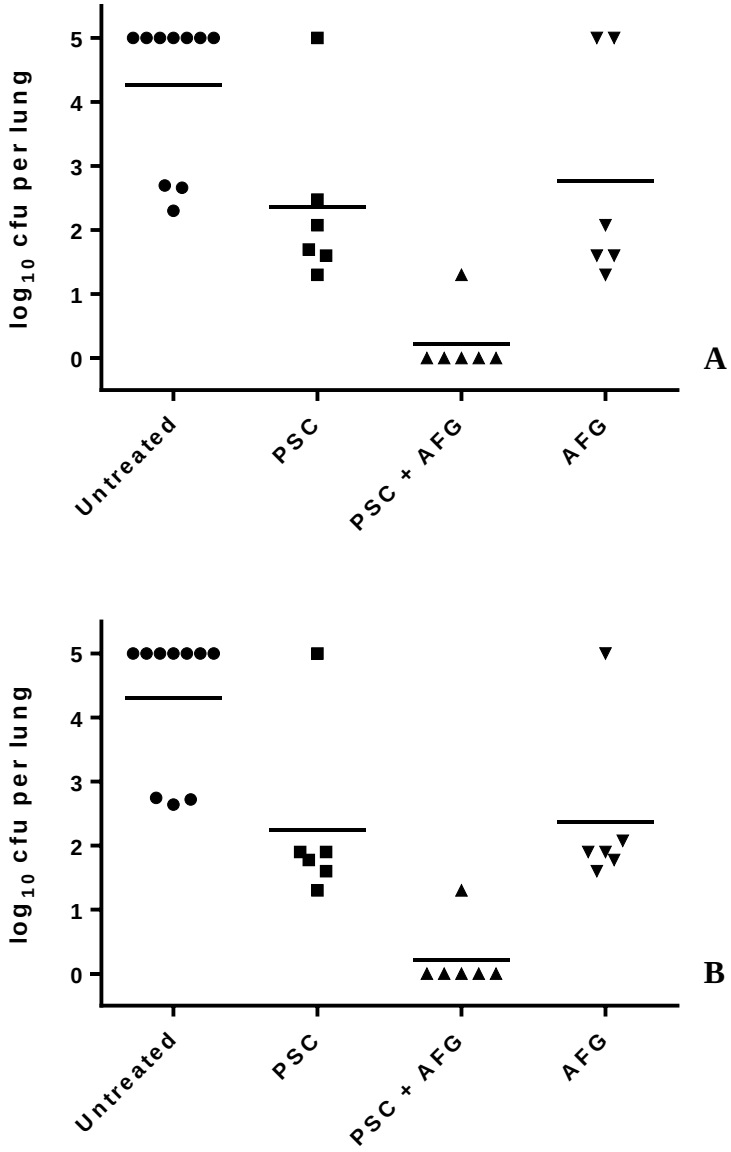

Figure 1: Effect of the combination treatment of anidulafungin (AFG) 10 mg/kg, and posaconazole (PCZ) 20 mg/kg, on the fungal burden of mouse lungs infected with *A. fumigatus* of clinical (A) and environmental (B) origins and comparison with antifungal treatment with a single agent. A horizontal line through the columns represents the median values.

For mice treated with PSC, one mouse infected with the AFU19 strain died on day 4 of treatment and one mouse infected with strain A07023 died on day 5. The fungal burdens ranged from $\log_{10}$ 1.301 to 2.478 CFU/lung and 1.031 to 1.903 CFU/lung in mice infected with strains AFU19 and A07023, respectively (Figure 1).

No significant difference in fungal burden reduction was observed between mice treated with AFG and mice treated with PSC. AFG-treated mice showed $\log_{10}$ values ranging from 1.031 to 2.079 CFU/lung, whereas PSC-treated mice showed $\log_{10}$ values ranging from 1.602 to 2.079 CFU/lung in mice infected with strains AFU19 and A07023, respectively (Figure 1). However, AFG was less effective in prolonging survival than PSC in the group infected with the clinical strain (AFU19) ($P < 0.0001$), as two deaths occurred in the group treated with AFG on days 5 and 6. In the group infected with the environmental strain (A07023), a death occurred on day 7 (Figure 2).

At the end of experiment (day 7), survival in the untreated groups was 20% and 30% for the mice infected with the AFU19 and A07023 strains, respectively. When the mice were treated with PSC or AFG, the survival rate was significantly higher than the untreated group (83.33% in PSC-treated mice that were infected with both strains or AFG-treated mice infected with A07023 strain; 66.66% in the AFG-treated mice that were infected with AFU19 strain). In the groups where PSC and AFG were administered in combination to mice infected with both AFU19 and A07023 strains, the survival rate was 100% (Figure 2).

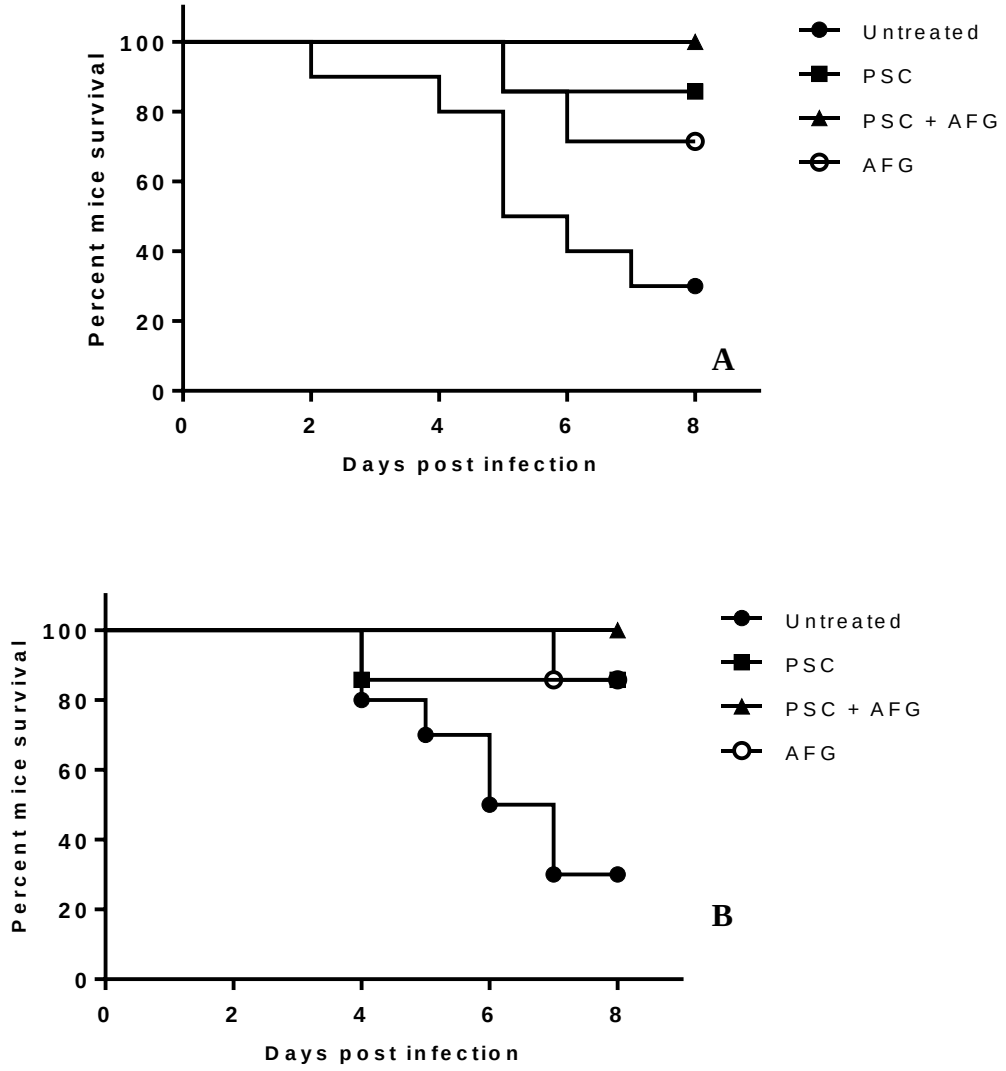

Figure 2. Kaplan-Meier survival plots of immunosuppressed mice infected by inhalation of *A. fumigatus* of clinical (A) and environmental (B) origins, and treated with anidulafungin (AFG), 10 mg/kg, and posaconazole (PSC), 20 mg/kg, combination therapy and monotherapy.

The residual fungal burden in mice treated with the combination therapy was significantly lower than those that received the monotherapy ($P < 0.0001$), with the fungal burden in some mice reduced to zero compared to that in untreated groups and groups receiving PSC or AFG monotherapy. For the groups infected with both strains, the median

log_{10} ranged from 0 to 1.301 CFU/lung in mice that received PSC and AFG concomitantly (Figure 1).

4 DISCUSSION

Azoles and echinocandins are two classes of antifungals that have different targets in the fungal cell, acting on the cell membrane and the cell wall, respectively. For this reason, the use of these antifungal agents has been shown more effective in the treatment of aspergillosis when used in combination than either class used as a monotherapy (Marr et al., 2015; Mavridou et al., 20015; Seydemousavi et al., 2013a).

In this study, we have demonstrated that the monotherapy with either PSC or AFG did reduce the fungal burden and increased the survival in relation to the untreated groups. However, the treatment was even more effective when administered in combination, significantly reducing the residual fungal burden and maintaining survival in 100% of the mice. Although we found the results were largely independent *in vitro* (or synergistic depending on the reading end-point), we obtained completely synergistic outcomes *in vivo*.

Recently, Martin-Vicente and colleagues demonstrated synergistic interaction between PSC and AFG in a systemic aspergillosis model by *A. fumigatus*. The combination increased survival and reduced fungal burden in mice kidneys as compared to the monotherapy with each drug or with voriconazole.

The synergistic interaction between PSC and AFG (and between other azoles and echinocandins) can be explained by the inhibition of ergosterol biosynthesis by PSC, changing the structure of the membrane and facilitating access of the AFG in the FKS1

enzyme, and by the alterations in the cell wall caused by AFG, which can facilitate the penetration of PSC into the fungal cell.

Synergistic interaction of PSC with other echinocandin, have already shown by Cacciapuoti and colleagues, who observed high efficacy with PSC and CAS as monotherapies and in combination using a systemic murine model of aspergillosis. Marr and colleagues have also reported synergistic interaction of AFG with VCZ in a randomized study with patients who had invasive aspergillosis.

This study demonstrates a trend of synergistic interaction between PSC and AFG in this model of pulmonary aspergillosis, since significantly better results were observed when these two drugs were used in combination. More in-depth studies are needed to confirm this synergistic interaction and determine if it may be used as a therapeutic alternative in cases of difficult-to-treat aspergillosis.

5 ACKNOWLEDGMENTS

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6 DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest.

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CAPÍTULO 5

In vitro Interactions of Azoles and Echinocandins against Clinical Strains of *Aspergillus flavus*

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ABSTRACT

Combinations of an azole (itraconazole, voriconazole, or posaconazole) with an echinocandin (caspofungin, micafungin, or anidulafungin) were tested against 20 clinical isolates of *Aspergillus flavus* according to EUCAST guidelines. The interactions were determined using two endpoints—minimal effective concentration (MEC) and minimal inhibitory concentration (MIC)—via calculation of the fractional inhibitory concentration (FIC) index. A higher prevalence of synergistic interactions was observed for MIC, whereas indifference was the most frequent outcome according to MEC among the 20 strains. Combined treatment of *A. flavus* with these two important classes of antifungals should be explored further in *in vivo* studies.

Key-Words: *Aspergillus flavus*, echinocandin, azole, combination therapy

INTRODUCTION

Aspergillus flavus is the etiological agent of several forms of infection in humans, from hypersensitivity reactions to invasive fungal infection. This species is the most common cause of fungal sinusitis, cutaneous infections, and endophthalmitis in tropical countries, and after *A. fumigatus*, is the most frequently isolated agent among patients with invasive aspergillosis [1, 2].

Azole antifungals are the first line of treatment of *Aspergillus* spp. infections. Voriconazole (VCZ) as the first choice, posaconazole (PCZ) mainly as prophylaxis in patients at risk, and itraconazole (ITZ) and isavuconazole are used in specific cases such as primary resistance to VCZ [3, 4]. At the same time, many cases of azole resistance have been linked to *A. fumigatus*, and a smaller number to *A. flavus* [5, 6, 7].

Echinocandins are also available for the treatment of aspergillosis and have been used in association with azoles in cases of complicated treatment [8, 9, 10]. Because both classes of antifungal agents have different therapeutic targets in the fungal cell, with echinocandins inhibiting glucan synthesis (essential for fungal cell wall formation) and azoles inhibiting the biosynthesis of ergosterol (an essential component of the fungal cell membrane), their use in combination seems to be promising [11, 12].

Because most cases of invasive aspergillosis are caused by *A. fumigatus*, most studies showing an interaction between azoles and echinocandins have been dealing with this species, and a few studies have explored interactions toward *A. flavus*. The main objective of the present study was to determine the *in vitro* profile of azole–echinocandin interactions against clinical isolates of *A. flavus*.

MATERIALS AND METHODS

A. flavus strains

Twenty *A. flavus* strains were studied, which were recovered from patients with different forms of aspergillosis, including sinusitis and invasive and cutaneous aspergillosis, in the Laboratory of Mycological Research of the Federal University of Santa Maria, Brazil. The strains were identified by micro- and macromorphology and by sequencing of internal transcribed spacer (ITS) regions. The MasterPure yeast DNA purification kit (Epicentre®, Lucigen Corporation, Wisconsin, USA) was used for DNA extraction, whereas amplification of the DNA fragment spanning the ITS region was performed by PCR using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) as described previously [13]. The isolates were stored at –80°C in 10% glycerol. For antifungal sensitivity testing, they were subcultured on Potato Dextrose Agar (PDA) at 35°C until sporulation. In all the assays, *A. fumigatus* (ATCC 204305) and *A. flavus* (ATCC 204304) served as internal control strains.

Antifungals

Azole antifungals ITZ, PCZ, and VCZ were acquired from Sigma-Aldrich, São Paulo, SP, Brazil, as standard powders. Echinocandins, anidulafungin (ANF; Pharmacia & Upjohn Co. Kalamazoo, MI, USA), caspofungin (CAS; Laboratories Merck Sharp & Dohme-Chibret, Clermont Ferrand, France), and micafungin (MFG; Astellas Pharma Tech Co., Takaoka, Toyama, Japan) were purchased from their respective manufacturers. Stock solutions of all the antifungal drugs were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, São Paulo, SP, Brazil) and stored at –70°C. Working solutions were made in the RPMI 1640 medium (Sigma-Aldrich, São Paulo, SP, Brazil).

Antifungal Sensitivity Testing

Antifungal susceptibility of *A. flavus* strains was evaluated by the microdilution method following the EUCAST (European Committee on Antimicrobial Susceptibility

Testing) recommendations [14]. The strains were cultivated in PDA at 35°C until sporulation (3 to 5 days). Conidial suspensions were prepared by covering the colonies with 5 mL of distilled sterile water supplemented with 0.1% of Tween 20, and then the conidia were rubbed with a cotton swab and transferred to a sterile tube. The conidial suspensions were adjusted to a cell concentration of 2 to 5 × 10^6 conidia / mL. A 1:10 dilution was prepared in sterile water to obtain a final suspension of 2 to 5 × 10^5 conidia / mL; 100 µL of the suspension was placed into each well of 96-well microtiter plates that contained 100 µL of antifungal drugs diluted in RPMI 1640 at the final concentrations ranging from 0.063 to 32.00 mg / L for ITZ and VCZ, 0.008 to 4.00 mg / L for PCZ, and 0.001 to 8.00 mg / L for CAS, MFG, and ANF. As sterility and growth control, columns 11 and 12 of each plate were filled with RPMI plus water and RPMI plus the inoculum, respectively. The inoculum concentration was confirmed by colony counting on Sabouraud Dextrose Agar. The inoculated plates were incubated at 37°C. The minimal effective concentration (MEC) of echinocandins was determined after 24 h and the minimal inhibitory concentration (MIC) of azoles after 48 h of incubation [14].

Antifungal Interaction Assays

Testing of the antifungal-drug interactions was performed in accordance with the EUCAST broth microdilution checkerboard method [14]. The final concentrations of the antifungal drugs ranged from 0.001 to 0.125 mg / L for echinocandins, 0.016 to 8.00 mg / L for ITZ, and 0.004 to 2.00 mg / L for VCZ and PCZ. Aliquots (50 µL) of the antifungal drugs four times more concentrated than the final concentration were dispensed into the microtiter plates. After that, 100 µL of the conidial suspension at the concentration of 2 to 5 × 10^5 conidia / mL, prepared as described above, was added into each well. The microplates were incubated at 37°C. MEC and MIC readings were carried out within 24 and 48 h of incubation, respectively. All the experiments were

conducted in triplicate. To assess the nature of *in vitro* interactions between azoles and echinocandins, the fractional inhibitory concentration (FIC) index (FICI) was calculated as follows: $\text{FICI} = FIC_A + FIC_B = \frac{C_A^{comb}}{C_A^{alone}} + \frac{C_B^{comb}}{C_B^{alone}}$, where $C_A{}^{alone}$ and $C_B{}^{alone}$ are the concentrations of drugs *A* and *B* alone, respectively, and $C_A{}^{comb}$ and $C_B{}^{comb}$ are the concentrations of drugs *A* and *B* in combination, respectively. FICIs were calculated for all the wells that were intended for MEC analysis at 24 h or for MIC analysis at 48 h, for each isolate and the triplicates. FICI averages were calculated from the triplicates to determine drug–drug interactions, and the results from the three replicates were considered one outcome. The interactions were defined as synergistic if the FICI was ≤ 0.5; nonsignificant interactions (indifference) if 0.5 < FICI < 4.0; and antagonistic if FICI was ≥ 4.0 [15].

RESULTS

The FICI results and the interaction percentages of each azole–echinocandin combination are summarized in Table 1 and Figures 1, 2, and 3, respectively. Among the ITZ–echinocandin combinations, we observed more frequent indexes showing indifference according to the MEC endpoint (FICI ranges: 0.078–8.125 for combination ITZ-MFG and 0.067–31.00 for ITZ–ANF), except for the ITZ–CAS combination, which showed a higher frequency of synergy (53.54%) toward the 20 clinical isolates. In contrast, for the MIC endpoint, the ITZ–ANF and ITZ–CAS combinations (FICI ranges: 0.002–0.502 and 0.004–0.502, respectively) showed higher frequency of synergistic activity, while the ITZ–MFG combination showed higher prevalence of indifference, as seen for the MEC reading endpoint.

For all combinations of VCZ with echinocandins, indifference was the prevalent result according to MEC, whereas according to MIC, higher prevalence of synergistic interactions was observed. For combinations VCZ–CAS, VCZ–MFG, and VCZ–ANF,

the FICI ranges were 0.064–8.125, 0.039–8.125, and 0.075–31.00, respectively, according to the MEC readings, and 0.005–2.00, 0.005–1.001, and 0.004–1.002, respectively, according to MIC.

The PCZ combinations with all echinocandins also showed indifference as the most prevalent result in terms of the MEC endpoint. On the other hand, synergy was the main result when MIC was used for calculations. The FICI ranges according to the MEC endpoint were 0.038–9.625, 0.078–8.625, and 0.009–32.50 for combinations PCZ–CAS, PCZ–MFG, and PCZ–ANF, respectively. In terms of MIC, the FICI range was 0.008–2.001 for all PCZ–echinocandin combinations.

For all azole–echinocandin combinations, low frequency of antagonism was observed in terms of MEC readings, whereas according to the MIC endpoint, there was no antagonism.

DISCUSSION

Although *A. flavus* is less prevalent than *A. fumigatus* among cases of invasive aspergillosis, when this species is the pathogen of this form of infection, high mortality rates are observed; furthermore, *A. flavus* is more virulent in terms of the ability to cause noninvasive aspergillosis [16].

Drug sensitivity studies have shown good activity of azole antifungals against clinical and environmental *A. flavus* strains [17, 18], and echinocandins show good or moderate activity too against this species [17, 19]. Nevertheless, some authors have reported resistance of *A. flavus* to both azoles and echinocandins [7, 20, 21].

Azole–echinocandin combinations against *A. flavus* have been almost unexplored. In this study, we evaluated *in vitro* interactions of ITZ, VCZ, or PCZ with each of the echinocandins: CAS, MFG, or ANF. For all the combinations, depending on the

endpoint used for calculations, the results showed synergistic or indifferent interactions with infrequent or no antagonism, when tested against 20 clinical isolates.

For the MIC endpoint, the combinations of VCZ with the three echinocandins revealed high prevalence of synergistic activity. Successful therapy with the combination of this azole and caspofungin has been reported in cases of pulmonary infection associated with paranasal sinusitis and scleritis caused by *A. flavus* [22, 23]. Similarly, the combined treatment with VCZ and ANF yielded prolonged survival and reduced fungal load as compared to ANF alone in a murine model of *A. flavus* aspergillosis [24].

The most diverse results were obtained for the combinations of echinocandins with ITZ. The ITZ–ANF combination proved to be almost exclusively synergic among the 20 clinical isolates according to MIC; the same outcome has already been reported by other authors [25]. In contrast, for MEC readings, this interaction was indifferent and revealed a significant frequency of antagonism. The combination of ITZ with CAS was mostly synergistic for both endpoints, whereas the ITZ-MFG combination was indifferent toward the majority of clinical isolates in terms of both endpoints. Shalit et al.[26] reported cases of synergy (according to MIC) and some cases of indifference (according to MEC) for the *in vitro* combination of ITZ and CAS against *A. flavus* isolates. The combination of ITZ with echinocandins should be explored further to clarify their interaction.

PCZ–echinocandin combinations were synergistic for MIC readings and indifferent for MEC readings. Synergistic results have been demonstrated when PCZ is combined with CAS or ANF against *A. fumigatus* [27, 28, 29, 30]; however, to our knowledge, the combinations of this azole with echinocandins against *A. flavus* are

explored for the first time in this study, showing promising results for future *in vivo* experiments.

The synergistic *in vitro* effect of azole - echinocandin combinations against *A. flavus* isolates depended on the endpoint. More combinations were classified as synergistic when the MIC endpoint was used rather than the MEC. This fact can be explained by the fungistatic activity of the echinocandins, since they are capable of causing alterations in the morphology of *Aspergillus* hyphae in very low concentrations, however for total growth inhibition (MIC) a much higher concentration is required, so the calculation of the FICI tends to synergy.

Although limited by the different endpoints interpretations, these observations encourage future evaluations of these antifungal combinations in *in vivo* models of infection by *A. flavus*.

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DECLARATION OF INTEREST

The authors declare no conflicts of interest.

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Table 1 FICIs for azole–echinocandin combinations in terms of 100% inhibition of growth (MIC, minimal inhibitory concentration) or distinct morphology (MEC, minimal effective concentration) in broth microdilution assays of *Aspergillus flavus* strains (n = 20).

Drug Combinations	Geometric mean value (range)					
	MEC endpoint (24h)			MIC endpoint (48h)		
ITZ + CAS	MIC_{ITZ}	MEC_{CAS}	FIC index	MIC_{ITZ}	MIC_{CAS}	FIC index
	0.125 (1.00–2.00)	0.094 (0.008–0.250)	0.646 (0.019–8.125)	0.125 (1.00–2.00)	>8.00 (>8.00–>8.00)	0.193 (0.004–0.502)
ITZ + MFG	MIC_{ITZ}	MEC_{MFG}	FIC index	MIC_{ITZ}	MIC_{MFG}	FIC index
	0.125 (1.00–2.00)	0.028 (0.008–0.063)	1.421 (0.078–8.125)	0.125 (1.00–2.00)	>8.00 (>8.00–>8.00)	0.340 (0.004–0.502)
ITZ + ANF	MIC_{ITZ}	MEC_{ANF}	FIC index	MIC_{ITZ}	MIC_{MFG}	FIC index
	0.125 (1.00–2.00)	0.011 (0.002–0.031)	3.558 (0.067–31.0)	0.125 (1.00–2.00)	>8.00 (>8.00–>8.00)	0.372 (0.002–0.502)
VCZ + CAS	MIC_{VCZ}	MEC_{CAS}	FIC index	MIC_{VCZ}	MIC_{CAS}	FIC index
	0.90 (0.50–1.00)	0.094 (0.008–0.250)	0.779 (0.064–8.125)	0.90 (0.50–1.00)	>8.00 (>8.00–>8.00)	0.250 (0.005–2.00)
VCZ + MFG	MIC_{VCZ}	MEC_{MFG}	FIC index	MIC_{VCZ}	MIC_{MFG}	FIC index
	0.90 (0.50–1.00)	0.028 (0.008–0.063)	1.517 (0.039–8.125)	0.90 (0.50–1.00)	>8.00 (>8.00–>8.00)	0.151 (0.005–1.001)
VCZ + ANF	MIC_{VCZ}	MEC_{ANF}	FIC index	MIC_{VCZ}	MIC_{ANF}	FIC index
	0.90 (0.50–1.00)	0.011 (0.002–0.031)	4.522 (0.075–31.0)	0.90 (0.50–1.00)	>8.00 (>8.00–>8.00)	0.501 (0.004–1.002)
PCZ + CAS	MIC_{PCZ}	MEC_{CAS}	FIC index	MIC_{PCZ}	MIC_{CAS}	FIC index
	0.181 (0.125–0.250)	0.094 (0.008–0.250)	1.082 (0.038–9.625)	0.181 (0.125–0.250)	>8.00 (>8.00–>8.00)	0.397 (0.008–2.001)
PCZ + MFG	MIC_{PCZ}	MEC_{MFG}	FIC index	MIC_{PCZ}	MIC_{MFG}	FIC index
	0.181 (0.125–0.250)	0.028 (0.008–0.063)	1.464 (0.078–8.625)	0.181 (0.125–0.250)	>8.00 (>8.00–>8.00)	0.374 (8.0–2.001)
PCZ + ANF	MIC_{PCZ}	MEC_{ANF}	FIC index	MIC_{PCZ}	MIC_{ANF}	FIC index
	0.181 (0.125–0.250)	0.011 (0.002–0.031)	3.994 (0.009–32.5)	0.181 (0.125–0.250)	>8.00 (>8.00–>8.00)	0.383 (0.008–2.001)

ITZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole; ANF, anidulafungin; CAS, caspofungin; MFG, micafungin.

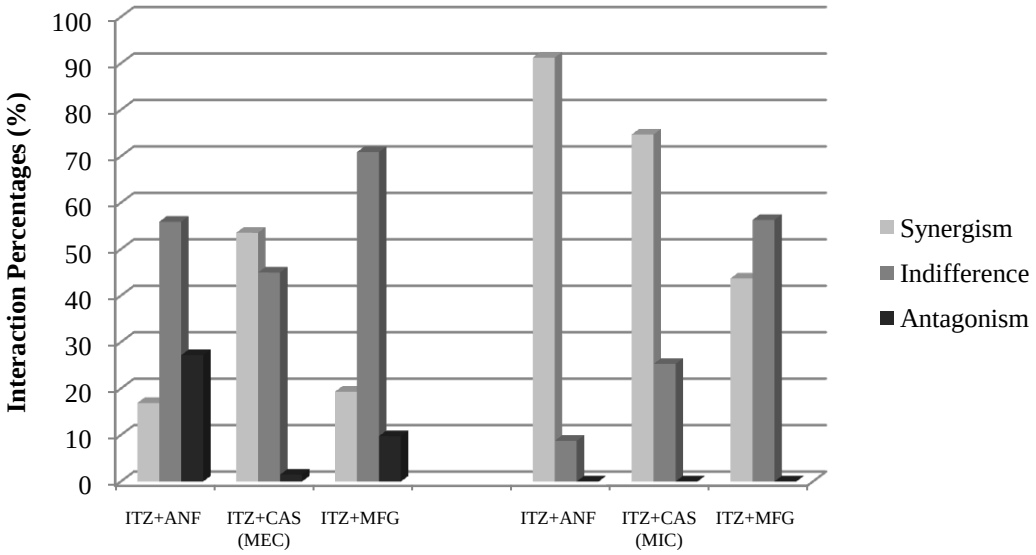

Figure 1 Frequencies of synergism, indifference, and antagonism toward clinical isolates of *A. flavus* (n = 20) as determined by MIC (minimal inhibitory concentration) and MEC (minimal effective concentration) for the combinations of itraconazole (ITZ) and each echinocandin: anidulafungin (ANF), caspofungin (CAS), or micafungin (MFG).

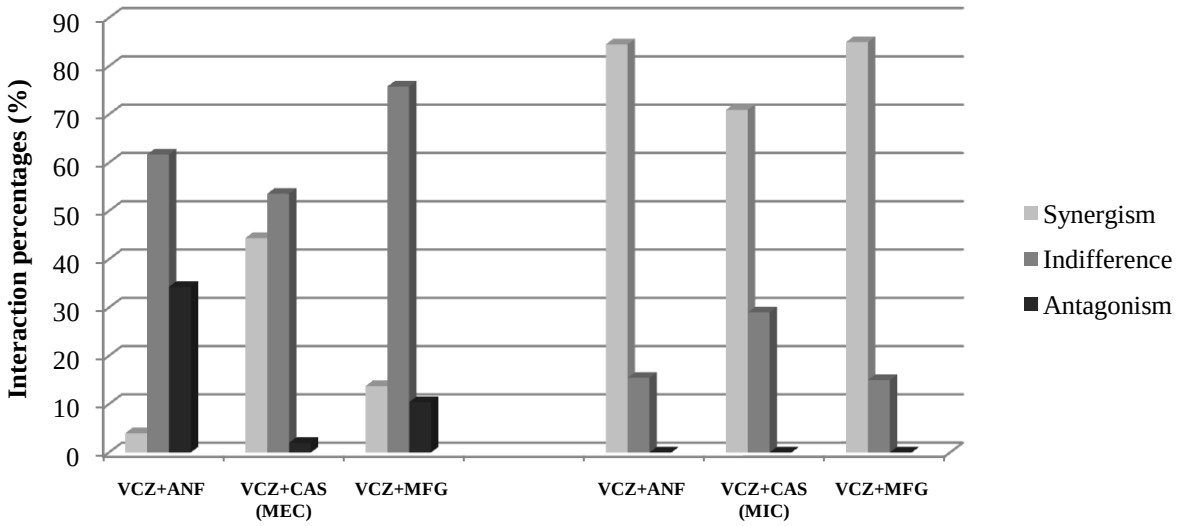

Figure 2 Frequencies of synergism, indifference, and antagonism toward clinical isolates of *A. flavus* (n = 20) as determined by MIC (minimal inhibitory concentration) and MEC (minimal effective concentration) for the combinations of voriconazole (VCZ) and each echinocandin: anidulafungin (ANF), caspofungin (CAS), or micafungin (MFG).

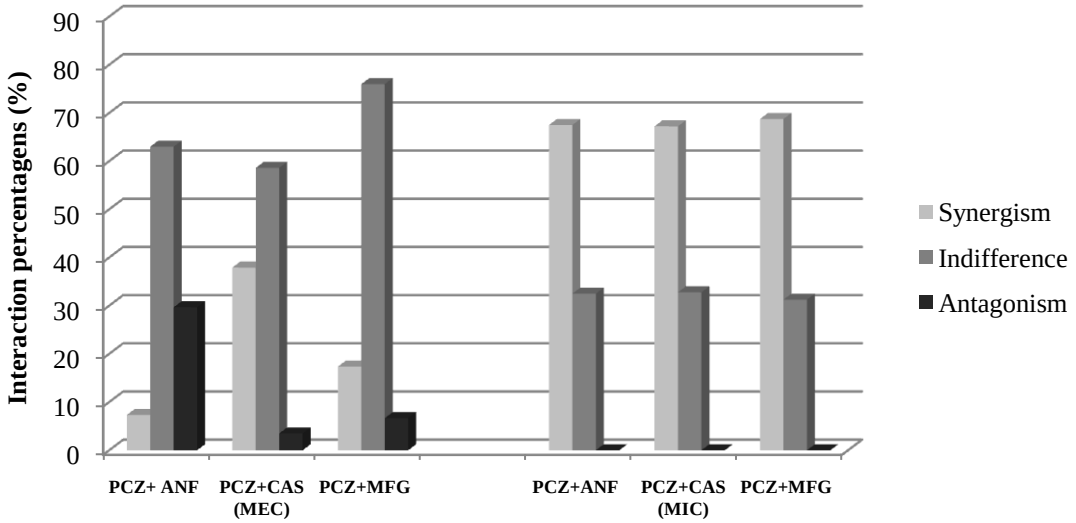

Figure 3 Frequencies of synergism, indifference, and antagonism toward clinical isolates of *A. flavus* (n = 20) as determined by MIC (minimal inhibitory concentration) and MEC (minimal effective concentration) for the combinations of posaconazole (PCZ) and each echinocandin: anidulafungin (ANF), caspofungin (CAS), or micafungin (MFG).

CAPÍTULO 6

Comparison between EUCAST-Microdilution, Etest and Agar-based detection of azole resistance methods on the susceptibility of *Aspergillus fumigatus* to Isavuconazole

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Abstract

Etest and agar-based detection of azole resistance methods were compared with the standard EUCAST microdilution for isavuconazole activity against 146 *Aspergillus fumigatus* clinical strains. Ninety-eight wild-type, 37 TR34/L98H and 11 TR46/Y121F/T289A mutant strains were tested. A 75.34 % agreement was found between Etest and microdilution and 99.32% and 91.78% were the agreement between agar-based method and microdilution using the concentrations of 2.0 and 1.0 mg/L of isavuconazole in the agar, respectively. The outcomes confirm the Etest and the agar-based detection of azole resistance as alternative and easier to perform tests for the determination of the minimal inhibitory concentration (MIC) and/or detection of isavuconazole resistance in *A. fumigatus*.

Key-Words**:** *Aspergillus fumigatus*, Etest, isavuconazole, azole resistance

Introduction

Isavuconazole is a second-generation triazole with pronounced efficacy in the treatment of *Aspergillus* caused infections. This antifungal has been used in clinical practice in Europe and the USA and has shown comparable action and lower adverse effects than voriconazole, which is the current treatment of choice for invasive aspergillosis (Horn et al. 2016; U.S. Food and Drug Administration, 2015; European Medicine Agency, 2015; Maertens et al. 2011).

The monitoring of the antifungal activity of isavuconazole through *in vitro* susceptibility tests is very important considering its short time of clinical use and the therapeutic problem of *A. fumigatus* azole-resistant. While broth microdilution remains the gold standard with respect to antifungal susceptibility, others easy-to-perform methodologies have been implemented in mycology laboratories in order to facilitate and obtaining faster results, as well as to perform a screening of the resistant strains (Arendrup et al. 2017; Buil et al. 2017; Lamoth et al. 2013)

Among these methodologies are the Etest, based in a predefined gradient of antifungal concentrations on a plastic strip used to determine the minimum inhibitory concentration (MIC) and the Agar-based method, developed by plates containing agar medium supplemented with a defined antifungal concentration and a growth control (van der Linden et al. 2012; Lass-Flörl, 2010; Guinea et al. 2007). We investigated the performance of each susceptibility test and compared the results obtained from each one with EUCAST microdilution for isavuconazole activity against *A. fumigatus* isolates.

Materials and Methods

A total of 146 *A. fumigatus* clinical isolates belonging to fungus culture collection of Department of Medical Microbiology of Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, were included. Of these, 98 were wild-type and 48

*CYP*51A mutant with hot-spot alterations involving TR34/L98H (n= 37) and TR46/Y121/ T289A (n=11). The isolates were maintained in glycerol broth (10%) at -80°C and were subcultured on Sabouraud dextrose agar (SDA) at 35 to 37 °C. After sporulation (3 to 5 days) the inoculum suspensions were prepared following the EUCAST recommendations. The suspensions were adjusted spectrophotometrically in sterile Milli-Q water to 0.5 McFarland (2 to 5 x 10^{6} CFU / mL) at 530 nm (80–83% transmittance).

Broth microdilution method was performed according to EUCAST guidelines for conidia forming moulds (E.DEF 9.3.1 document, EUCAST 2015). Isavuconazole was acquired as standard powder and solubilised in dimethyl sulfoxide at concentration of 3200 mg / L. This solution was storage at -80 ºC until use. Two-fold drug concentrations of 0.015–16.0 mg / L were prepared and distributed in the 96-well plates. After this step, 100 µL of the 1:10 diluted inoculum (final concentration of 2 to 5 x 10^{5} CFU / mL) were added and the plates were incubated at 35 ºC for 48 h. The MIC reading was defined as the minimal concentration that inhibited 100 % of the fungal growth. Quality control strains were used in all tests.

For Etest method RPMI 1640 (Sigma, St. Louis, MO.) agar supplemented with 2% of glucose and 0.165 M morpholinepropanesulfonic acid (MOPS) buffer and buffered to pH 7.0 was used. The 90-mm diameter plates contained RPMI were inoculated by submerging a sterile/nontoxic swab into the undiluted inoculum suspension and spread it across the agar surface in three directions. The agar was allowed to dry for +/-15 min at room temperature and Etest strips (Etest; AB Biodisk, Solna, Sweden) were applied. After 48 h of incubation at 35 ºC the MICs were defined as the lowest isavuconazole concentration at which the border of the inhibition ellipse intersected the scale on the Etest strip. The trailing growth was ignored.

The agar plates were prepared containing RPMI-1640 agar medium (buffered with MOPS, 2 % of glucose, pH 7.0) supplemented with 1 or 2 mg / L of isavuconazole and a drug-free growth control well. The plates were inoculated with 1 drop (25 µL) of inoculum (2 to 5 x 10^{6} CFU / mL) and incubated for 48 hours at 35 ºC. The readings assessing the absence or presence of growth were performed twice on 24 hours and 48 hours by three independent technicians. When the results were in discordance among the three readers, the test was done more than one time.

The percentage of agreement between both Etest and isavuconazole containing agar plate methods with EUCAST broth microdilution was calculated. Agreement between Etest and microdilution methods was concluded when the MIC values fell within ±2 dilutions of the twofold dilution scheme, considering 0 twofold dilutions when the MICs were above the major concentration tested for both methods (16 mg/L for EUCAST microdilution and 32 mg / L for Etest). Agreement between isavuconazole containing agar plate and EUCAST broth microdilution was considered when strains with MICs ≤ 1.0 or 2.0 mg/L did not grow on the agar at these two concentrations, respectively and strains with MIC > 1.0 or 2.0 mg / L grew on agar at these two concentrations, respectively.

Results

The essential agreement between the methods is described in the Tables 1 and 2. In the comparison of Etest with EUCAST microdilution 69.38% of concordance was observed in the wild-type strains, the majority of these strains showed variation of ± 2 MICs (73.52%). While in the mutant strains higher concordance index was found, 83.67% for those containing TR34/L98H and 100% for the strains with TR46/Y121F/T289A mutation. Considering all strains, the concordance index between both methods was above 75%.

Evaluating the agreement between isavuconazole containing agar plate and EUCAST microdilution methods, 100% concordance indices were found with both 1 mg/L and 2 mg/L isavuconazole agar concentrations for the strains containing mutations. For the wild-type *A. fumigatus* strains the percentage of concordance was higher on the concentration of 2 mg/L of isavuconazole (98.97%) while on the concentration of 1 mg / L the agreement obtained was 87.75%.

The Figure 1 shows the distribution of isavuconazole MICs for both Etest and EUCAST microdilution methods. We can observe a tendency to lower MICs by Etest compared to microdilution against all groups of strains. In the wild-type strains, whereas by Etest the most strains showed MICs of 0.25 or 0.5 mg / L and by microdilution the most strains had MICs of 1.0 or 2.0 mg / L. The same is observed in the TR34 / L98H mutant strains in which by Etest the most showed MICs of 2.0 or 4.0 mg / L and by microdilution the most MICs were 8.0 or 16.0 mg / L. The strains containing the TR46 / Y121F / T289A mutation had the most MICs of 16.0 mg / L by microdilution, whereas by Etest the most prevalent MIC was 32.0 mg / L, however 16 mg / L was the highest concentration of isavuconazole tested in microdilution and for data analysis purposes strains with MIC > 16 mg / L were included in this group (MIC = 16.0 mg/L).

Considering the clinical breakpoint (isavuconazole resistance MIC > 1 mg/ L) resistance was detected by the three methodologies in 100% of the strains with mutations. In the wild-type strains 6.1% showed MIC > 1.0 mg / L by microdilution and MIC < 1.0 mg/L by Etest. Whereas 11 strains showed MIC ≤ 1.0 mg/L in the microdilution but grew on agar containing 1 mg/L of isavuconazole. Also, 1 strain with MIC = 2 mg/L by microdilution did not grow on agar containing 1 mg/L and another strain with MIC = 2.0 mg/L grown on agar containing 2 mg/L of isavuconazole.

Discussion

In vitro susceptibility testing allows the early view of the antifungal effect and consequently provides more directed treatment to the patient; in addition, resistance monitoring provides data that helps in the choice of empirical treatment in each medical centre. We compared two easy-to-perform methodologies, Etest and agar-based detection of azole resistance, with EUCAST microdilution to test the susceptibility of wild-type and mutant *A. fumigatus* strains to isavuconazole.

The agar-based detection of azole resistance method is already used for detection of resistant *A. fumigatus* strains in diagnostic laboratories. The commercial agar plates, VIPcheck^{TM} (Nijmegen, the Netherlands) were developed by academic professionals from Radboud University Medical Centre and containing 4 wells with itraconazole (4 mg/L), voriconazole (2 mg/ L), posaconazole (0.5 mg/L) and a growth control. This method was validated as a sensitive and specific screening for differentiation between azole susceptible and resistant *A. fumigatus* strains. (Buil et al, 2017). Here we describe that resistance to isavuconazole can also be measured using this methodology, and the concentration of 2 mg / L showed greater agreement with EUCAST microdilution. The inclusion of isavuconazole in VIPcheck^{TM} plates for screening is highly recommended since resistance to this azole is highly related to resistance to voriconazole; and isavuconazole is often used as a treatment alternative when resistance to first-line treatment is present (Gregson et al. 2013).

Good correlation between Etest and microdilution based on CLSI or EUCAST have already been described by other authors who evaluated the susceptibility of *Aspergillus* spp. to azoles (itraconazole, voriconazole and posaconazole) and amphotericin B by both methodologies (Lamoth et al. 2015; Guinea et al. 2007; Pfaller et al. 2003; Espinel-Ingroff et al. 2002; Pfaller et al. 2000). A few studies involving

isavuconazole have been described, Guinea et al. (2008) found 96% agreement between the MICs of isavuconazole obtained by Etest and CLSI M38-A against *Aspergillus*. More recently, Arendrup et al. (2017) showed good correlation between isavuconazole Etest *versus* EUCAST method against wild-type and CYP51A mutant *A. fumigatus* strains.

Our outcomes are confirmatory and agree with the observations of the authors described above. In addition to the relevant concordance indices, a fact that reinforces the Etest as an alternative method for determining the antifungal activity of isavuconazole against *Aspergillus*, another important aspect that we can highlight in this study is that the detection of resistance by Etest was 100% in the strains tested.

In conclusion, both methodologies have proven to be reliable and can be used as alternative, faster and easier methods compared to the standard microdilution to distinguish isavuconazole resistant and sensitive *A. fumigatus* strains, however they should be used with caution. Also, multicenter studies with a greater number of strains are necessary to prove the reliability of the use of these methodologies.

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Declaration of Interest

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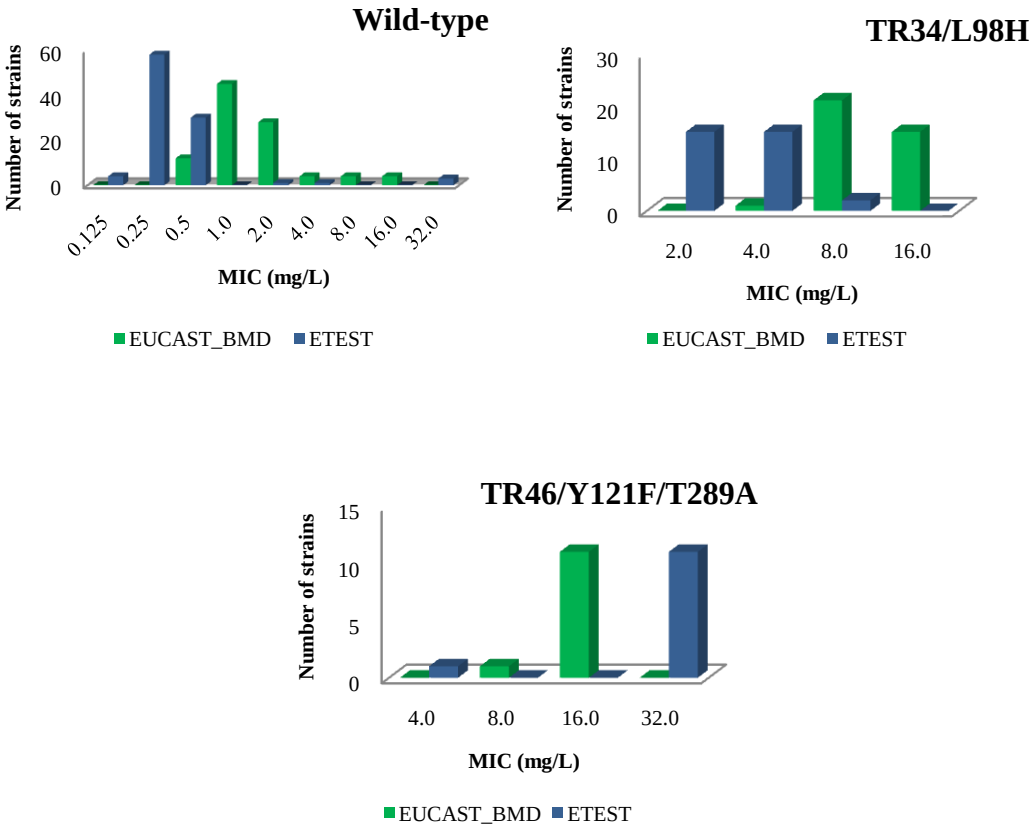
Table 1 Essential agreement (%) between isavuconazole - Etest Strip *versus* EUCAST broth microdilution

***CYP*51A profile**	**± 0 MIC**	**± 1 MIC**	**± 2 MIC**	**Total Agreement**
Wild-type (n= 98)	13.23	13.23	73.52	69.38
TR34/L98H (n= 37)	21.62	29.72	32.43	83.67
TR46/Y121F/T289A (n= 11)	90.91	9.09	0	100
All isolates (n = 146)	24.54	19.09	56.36	75.34

Table 2 Essential agreement (%) between isavuconazole (ISC) containing agar plates *versus* EUCAST broth microdilution

CYP51 Profile	**EUCAST x Agar ISC 1.0 mg/L**	**EUCAST x Agar ISC 2.0 mg/L**
Wild-type (n= 98)	87.75	98.97
TR34/L98H (n= 37)	100	100
TR46/Y121F/T289A (n= 11)	100	100
All isolates (n = 146)	91.78	99.32

Figure1 Isavuconazole MICs distribution by EUCAST broth microdilution and Etest methods for *Aspergillus fumigatus* wild-type and TR34/L98H or TR46/Y121F/T289A mutant strains.



10 DISCUSSÃO GERAL

A introdução de novos agentes antifúngicos e recentes relatos de resistência emergente durante o tratamento de infecções por *Aspergillus* evidenciaram maior necessidade de monitorização com testes de susceptibilidade *in vitro*. Microdiluição em caldo (MC) continua sendo o método padrão ouro para determinação da atividade antifúngica, enquanto que outros testes estão sendo propostos a fim de aperfeiçoar a rotina laboratorial.

Em geral os isolados brasileiros clínicos e ambientais de *A. fumigatus* e *A. flavus* mostram-se sensíveis frente aos antifúngicos azólicos, às equinocandinas e à anfotericina B. No entanto, *A. fumigatus* é menos suscetível ao itraconazol, enquanto que *A. flavus* mostra menor sensibilidade à anfotericina B. Muitos estudos relatam resistência de *A. fumigatus* a antifúngicos azólicos, especialmente ao itraconazol e voriconazol, com taxas de até 38% em países europeus, como Holanda e Reino Unido (BUEID et al, 2010; van INGEN et al, 2015), onde a resistência é atribuída tanto à terapia com azólicos em longo prazo em pacientes com aspergilose crônica, tanto à seleção no ambiente de forma cruzada com os triazólicos de uso agrícola (SNELDERS et al, 2008; MORTENSEN et al, 2011).

No Brasil, os dados epidemiológicos sobre a suscetibilidade dos isolados de *Aspergillus* recuperados de ambos pacientes e meio ambiente são bastante escassos. Embora a resistência clínica seja observada em pacientes tratados com azólicos, os testes de susceptibilidade não são comuns na rotina laboratorial. Além disso, Negri e colaboradores (2017) e van der Linden e colaboradores (2015) testaram cepas de *A. fumigatus* brasileiras frente a azólicos e encontraram somente CIMs intermediárias, sem presença de resistência. No entanto, no primeiro capítulo desta tese foi relatado 20 e 25% de resistência ao itraconazol em isolados clínicos e ambientais de *A. fumigatus*, respectivamente, o que é alarmante considerando que os triazólicos são a primeira linha de tratamento para a aspergilose e que os fungicidas da mesma classe são amplamente utilizados na agricultura brasileira.

Resistência aos triazólicos já foi descrita em outros países da América Latina. Na Colômbia, 19 cepas ambientais de *A. fumigatus*, coletadas de campos de flores e outros locais na cidade de Bogotá, mostraram ser azol-resistentes e conter mutações no gene *cyp51A*, principalmente TR de 46 pares de bases combinada com pontos de mutação nos códons Y121F e T289A (TR46/Y121F/T289A) (LE PAPPE et al, 2016). Já na

Argentina, uma cepa de *A. fumigatus*, isolada de paciente com queratite, também mostrou resistência ao itraconazol, sendo que a mutação encontrada em *cyp51A* foi no códon G54E. Dentre as cepas brasileiras estudadas nesta tese encontramos as mutações TR34/L98H em um isolado clínico e M220R em um isolado ambiental, ambos com CIM > 16 mg/L para o itraconazol, descrevendo pela primeira vez cepas brasileiras de *A. fumigatus* apresentando mutações em *cyp51*A.

A mutação TR34/L98H já foi descrita tanto em cepas clínicas quanto ambientais de *A. fumigatus*. Acredita-se que o desenvolvimento deste mecanismo de resistência ocorra no ambiente sob pressão seletiva de fungicidas azólicos, uma vez que cepas contendo essa mutação são com freqüência isoladas de pacientes que nunca foram expostos a antifúngicos azólicos (VERWEIJ et al, 2009; SNELDERS et al, 2008).

Segundo um estudo realizado por Camps e colaboradores (2012), isolados de *A. fumigatus* contendo essa mutação possivelmente possuem um ancestral em comum, uma vez que, foram encontradas relações genéticas elevadas entre diferentes isolados contendo essa mutação em comparação com aqueles do tipo selvagem ou contendo outras mutações. No entanto, três hipóteses sobre a origem desse mecanismo foram abordadas, incluindo migração geográfica de conídios resistentes ou desenvolvimento local independente e seleção subsequente da mutação, ou ambos estão ocorrendo ao mesmo tempo (VERMEULEN et al, 2013). Embora ocorra uma maior correlação genética entre os isolados que contenham a mutação TR34/L98H em relação àqueles que não têm ou abriguem outros tipos de mutações, a detecção deste mecanismo em cepas de regiões diferentes da Europa reforça que a origem pode ocorrer por ambas às hipóteses, tanto a migração de conídios resistentes quanto o desenvolvimento local independente.

As mutações no códon 220 de *cyp51A* estão correlacionadas com a resistência ao itraconazol e diferentes padrões de CIMs para os outros azólicos. Até agora, a mutação neste códon somente foi descrita em cepas clínicas e sua origem foi atribuída ao tratamento prolongado de pacientes com antifúngicos azólicos (MELLADO et al, 2004; GARCIA-EFFRON et al, 2005). No entanto, a cepa que descrevemos neste estudo contendo a mutação M220R é de origem ambiental e apresentou CIM elevada para itraconazol, mas não para os outros azólicos, mostrando que as mutações no códon 220 também podem se desenvolver no ambiente.

Frente a este cenário de emergência de resistência de *Aspergillus* aos azólicos, ensaios avaliando a suscetibilidade a combinações de fármacos com diferentes alvos na

célula fúngica ganham importância. A combinação de antifúngicos azólicos e equinocandinas tem sido avaliada por diferentes metodologias incluindo testes *in vitro*, *in vivo* e tratamento de pacientes com aspergilose (MAAR et al, 2012; SEYEDMOUSAVI et al, 2013; ELEFANTI et al, 2013; LEPAK et al, 2013). Em geral efeitos favoráveis ou indiferentes relativo a essas combinações têm sido encontrados em grande parte dos estudos, dependendo dos fármacos associados, da metodologia usada e da suscetibilidade das cepas de *Aspergillus* testadas.

O modo de ação das equinocandinas implica em dificuldades de leitura nos testes *in vitro*, uma vez que atuam causando alterações nas hifas mas não inibição total do crescimento. O Capítulo 3 desta tese descreve a combinação das três equinocandinas com os antifúngicos azólicos itraconazol, posaconazol e voriconazol pelas metodologias de microdiluição em caldo - *checkerboard* (MCC) e Etest frente a isolados clínicos de *A. fumigatus* que apresentaram CIMs elevadas ao itraconazol. As leituras foram feitas baseadas em dois tempos de incubação, 24 e 48 horas, e diferentes interpretações, através da CIM e da CEM. Diferentes graus de interações foram encontrados com base no método utilizado e nas condições de leitura. Em geral, as combinações avaliadas via Etest, em todas as condições, apresentaram porcentagens maiores de sinergismo do que as avaliadas usando a MCC.

Muitos autores relatam interação sinérgica entre voriconazol e anidulafungina (LEPAK et al, 2013; ELEFANTI et al. 2013; SIOPI et al, 2016), no entanto, na maioria dos estudos, os resultados sinérgicos dependeram do método, da dose antifúngica, do ponto final de leitura e da suscetibilidade das cepas de *Aspergillus* aos azólicos. No presente estudo, encontrou-se resultados diferentes para esta combinação, que foram completamente sinérgicas por Etest pela leitura da CIM em 24 horas, até a falta de sinergismo na leitura da CIM e da CEM através da MCC.

Maior porcentagem de sinergismo também foi encontrada no Etest em comparação com a MCC na combinação de voriconazol com micafungina, que nesta última mostrou somente resultados indiferentes. Em um estudo conduzido por Chandrasekar e colaboradores (2004) utilizando modelo *in vivo* de aspergilose invasiva em porcos, os autores relatam que a terapia combinada não foi superior à monoterapia, o que vai de acordo com os resultados obtidos através da MCC neste estudo. Por outro lado, o estudo *in vitro* realizado por Lewis e Kontoyannis (2005) testou essa combinação através da MCC utilizando MTT (3-[4,5-dimetiltiazol-2-il]-2,5-difenil-

tetrazóleo brometo) como revelador e mostrou que a atividade de voriconazol aumentou pela adição de micafungina.

Na combinação de voriconazol com caspofungina, observamos um menor índice de sinergismo e resultados mais concordantes entre ambas as metodologias. Resultados conflitantes foram mostrados por outros autores, enquanto Zhang e colaboradores (2014) demonstraram total indiferença em um modelo murino de aspergilose invasiva por *A. fumigatus*, Kirkpatrick e colaboradores (2002) demonstraram alta eficácia desta combinação em modelo de aspergilose invasiva induzida em porcos.

Nas combinações de posaconazol com as equinocandinas foi observada porcentagem significativa de sinergismo através de Etest, especialmente com anidulafungina e micafungina, e resultados indiferentes através da MCC. Com a caspofungina ambos os métodos mostraram maiores percentuais de indiferença. As combinações de posaconazol com equinocandinas foram pouco exploradas até o momento. Manavathu e colaboradores (2003) relataram interações sinérgicas *in vitro* através da MCC para 20 isolados clínicos de *A. fumigatus* sensíveis ao itraconazol. Lepak e colaboradores (2013) também mostraram resultados sinérgicos em um modelo *in vivo* utilizando duas cepas de *A. fumigatus* resistentes ao posaconazol, uma resistente à caspofungina e outra sensível a ambos. Os autores observaram que a monoterapia com posaconazol reduziu significativamente a carga fúngica e a caspofungina sozinha não teve boa ação para todos os isolados, enquanto que a terapia combinada não foi superior à monoterapia com posaconazol nos isolados sensíveis ao mesmo, mas naqueles resistentes a combinação teve resultados sinérgicos.

Nas combinações de itraconazol com as equinocandinas encontramos resultados consistentes entre os dois métodos, com exceção da combinação com anidulafungina em que na leitura da CEM na MCC mostrou resultados contrastantes com outras condições experimentais. Diferentes tipos de interação têm sido descritas dependendo das condições experimentais. Cuenca-Estrella e colaboradores (2005) mostraram 64,33% de sinergismo na combinação de itraconazol com caspofungina pela leitura da CEM, enquanto que na MCC nenhum sinergismo foi observado na leitura da CIM. Shalit e colaboradores (2003) também encontraram resultados controversos. Os autores demonstraram 100% de sinergismo na leitura da CEM e 100% de indiferença na leitura da CIM. Por outro lado, estudos *in vivo* mostraram excelente interação entre itraconazol e caspofungina ou micafungina (OGAWA et al, 2015; OKAMOTO et al, 2006; FURUGEN et al, 2005).

A leitura da CEM no Etest mostrou ter melhor correlação com a MCC na maioria das combinações, tanto nas leituras em 24 horas quanto naquelas em 48 horas, no entanto, dependendo da combinação azol-equinocandina testada, outras condições experimentais mostraram maior concordância entre si, inferindo que o Etest pode ser uma alternativa confiável, de fácil execução e leitura na avaliação de combinações entre antifúngicos frente a *A. fumigatus*. Entretanto, as condições experimentais, tais como concentração do inóculo, meio de cultura, tempo de incubação, necessitam ser mais exploradas a fim de obter-se maior uniformidade.

Embora os testes *in vitro* forneçam orientações relevantes à escolha de uma terapia, esses resultados nem sempre se traduzem em eficácia *in vivo*, podendo muitos outros fatores relacionados à infecção no hospedeiro influenciar no sucesso do tratamento. No capítulo 4 foi demonstrada a interação de posaconazol e anidulafungina em um modelo *in vivo* de aspergilose pulmonar por *A. fumigatus*. Neste estudo a monoterapia com posaconazol ou anidulafungina reduziu a carga fúngica e aumentou a sobrevida em relação aos grupos não tratados. No entanto, o tratamento foi ainda mais eficaz quando administrado em combinação, reduzindo significativamente a carga fúngica residual e mantendo a sobrevivência em 100%. Embora os resultados *in vitro* destas combinações tenham sido amplamente independentes, apresentando sinergismo ou indiferença dependendo do tipo de método e da condição de leitura, esta combinação mostrou-se totalmente sinérgica *in vivo*.

Martin-Vicente e colaboradores (2017) também demonstraram interação sinérgica entre posaconazol e anidulafungina em modelo de aspergilose sistêmica por *A. fumigatus*. A combinação aumentou a sobrevivência e reduziu a carga fúngica nos rins de camundongos em comparação com a monoterapia com cada antifúngico ou com voriconazol. O sinergismo encontrado pode ser explicado pela inibição da biossíntese de ergosterol feita pelo posaconazol, alterando a estrutura da membrana e facilitando o acesso da anidulafungina na enzima ß-(1,3)-D-glucana sintase, bem como pelas alterações causadas na parede celular, o que pode facilitar a penetração do posaconazol na célula fúngica.

Enquanto que vários estudos já avaliaram as interações entre azólicos e equinocandinas frente a *A. fumigatus*, poucos são os estudos frente a *A. flavus*. Esta última espécie, embora menos prevalente em casos de aspergilose invasiva, implica em altas taxas de mortalidade. Além disso, *A. flavus* mostra-se mais virulento nas infecções não-invasivas. No capítulo 5 estão descritas as interações *in vitro* através de MCC entre

itraconazol, posaconazol e voriconazol com cada uma das equinocandinas frente a 20 isolados clínicos de *A. flavus*. Sinergismo ou indiferença foram os principais resultados encontrados na maioria das combinações, dependendo do ponto final de leitura, enquanto pouco ou nenhum antagonismo foi observado.

Com base nas CIMs observadas, as combinações de voriconazol com as três equinocandinas revelaram alta prevalência de atividade sinérgica. Terapia bem-sucedida com a combinação deste azólico com caspofungina em casos de infecção pulmonar associada à sinusite paranasal e esclerite causada por *A. flavus* já foi relatada (SCHUSTER et al, 2005; HOWELL et al, 2005). De forma similar, o tratamento combinado de voriconazol e anidulafungina produziu sobrevida prolongada e carga fúngica reduzida em comparação com a monoterapia em um modelo murino de aspergilose por *A. flavus* (CALVO et al, 2012).

Os resultados mais diversos foram obtidos para as combinações de equinocandinas com itraconazol, dependendo do ponto final de leitura. Shalit e colaboradores (2003) relataram também diferenças no resultado das interações, com sinergismo de acordo com a CIM, mas indiferença pela leitura da CEM para a combinação *in vitro* de itraconazol e caspofungina frente a *A. flavus*.

Igualmente nas combinações das equinocandinas com o posaconazol, interações sinérgicas foram observadas pela leitura da CIM, mas registrou-se indiferença pela leitura da CEM. Embora resultados sinérgicos destas combinações tenham sido descritos frente a *A. fumigatus* nesta tese bem como por outros autores (MARTIN-VICENTE et al, 2017; MAVRIDOU et al, 2015) frente a *A. flavus* elas foram exploradas pela primeira vez, mostrando resultados favoráveis para futuros experimentos *in vivo.* Maiores porcentagens de sinergismos nas leituras das CIMs podem ser devido à atividade fungistática das equinocandinas, uma vez que as mesmas causam alterações na morfologia das hifas de *A. flavus* em concentrações muito baixas. No entanto, para a inibição do crescimento total (CIM) é necessária uma concentração muito maior, de modo que o cálculo do FICI tende a classificar a interação como sinérgica.

Outra alternativa que tem sido utilizada no tratamento de pacientes com aspergilose invasiva é o isavuconazol, um antifúngico azólico de amplo espectro que foi aprovado em 2015 para este fim. Entretanto, em locais onde a taxa de resistência aos azólicos é alta, já há estudos evidenciando cepas de *A. fumigatus* com altas CIMs frente ao isavuconazol. Neste sentido o último capítulo desta tese buscou relacionar os

principais métodos de suscetibilidade disponíveis para determinação da CIM, bem como detecção de resistência ao isavuconazol frente a cepas isoladas de pacientes em um hospital holandês contendo ou não mutações relacionadas à azol-resistência.

Comparamos duas metodologias de fácil execução, Etest e crescimento em ágar contendo azólicos, com a técnica padrão de MCC. Ambas as metodologias mostraram concordância com a MCC, confirmando a viabilidade das mesmas como métodos alternativos. Boa correlação entre Etest e MCC já foi descrita por outros autores que avaliaram a suscetibilidade de *Aspergillus* spp. frente a outros antifúngicos azólicos e anfotericina B por ambas as metodologias (PFALLER et al, 2000; ESPINEL-INGROFF et al, 2002; PFALLER et al, 2003; GUINEA et al, 2007; LAMOTH et al, 2015). Também já foram realizados estudos comparando técnicas de suscetibilidade onde o isavuconazol foi testado. Assim, Guinea e colaboradores (2008) encontraram 96% de concordância entre as CIMs de isavuconazol obtidas por Etest e MCC frente a *Aspergillus* spp.; Arendrup e colaboradores (2017) também mostraram concordância entre Etest e MCC frente a cepas de *A. fumigatus* selvagens e contendo mutações em *cyp51A.*

A metodologia de crescimento em ágar contendo azólicos já é validada e utilizada em alguns centros clínicos para triagem de resistência em *Aspergillus* spp, no entanto, isavuconazol ainda não está incluído nas placas comercializadas (VIPCheck ^{TM}) as quais contêm somente itraconazol, voriconazol e posaconazol (BUIL et al, 2017). Neste estudo mostramos que a concentração de 2 mg/L de isavuconazol no ágar (*cutoff* epidemiológico) mostrou maior concordância com a MC e que é possível detectar resistência também a este azólico utilizando-se a metodologia de crescimento em ágar, onde a inclusão do isavuconazol nas placas VIPCheck^{TM} é uma recomendação atual.

11 CONCLUSÕES

Significativa porcentagem de cepas brasileiras de origem clínica e ambiental de *A. fumigatus* evidenciaram resistência a um ou mais azólicos, bem como foram descritas as mutações TR34/L98H ou M220R presentes no gene CYP51A em isolados de *A. fumigatus* azol-resistentes isolados no Brasil.

O efeito sinérgico *in vitro* das combinações dos azólicos com as equinocandinas frente aos isolados de *A. fumigatus* e *A. flavus* foi dependente da metodologia utilizada e do ponto final de leitura. Frente a *A. fumigatus*, na maioria das combinações a leitura da

CEM no método de Etest teve uma melhor correlação com a MCC na definição do tipo de interação entre os fármacos. No entanto, outras condições experimentais foram mais concordantes entre si dependendo dos fármacos combinados, inferindo que o Etest pode ser uma alternativa confiável, de fácil execução e leitura na avaliação de combinações entre antifúngicos frente a *A. fumigatus*. Já frente a *A. flavus* a maioria combinações foram classificadas como sinérgicas nas leituras da CIM e indiferentes na leitura da CEM. Embora sejam limitadas pelas diferentes interpretações, essas observações incentivam avaliações futuras dessas combinações antifúngicas em modelos *in vivo* de infecção por *A. flavus*.

A interação sinérgica entre posaconazol e anidulafungina, confirmada no modelo de aspergilose pulmonar por *A. fumigatus* pode ser introduzida como alternativa no tratamento da aspergilose após a realização de estudos clínicos.

Finalmente, as metodologias de Etest e crescimento em ágar contendo antifúngico provaram ser confiáveis e podem ser usadas como métodos alternativos, mais rápidos e mais fáceis em comparação com a técnica padrão de MCC para distinguir cepas de *A. fumigatus* resistentes ao isavuconazol, no entanto, devem ser interpretadas com cautela.

12 PERSPECTIVAS

Estudos mais amplos com cepas de diferentes regiões do Brasil são urgentemente necessários, não só para aumentar a conscientização sobre a suscetibilidade antifúngica deste importante gênero fúngico em nosso país, mas também para implementar um melhor manejo da aspergilose em nossos centros médicos, evitando assim desenvolvimento e/ou aumento de resistência antifúngica, bem como alertar para uso racional de fungicidas triazólicos.

São necessários estudos mais aprofundados para confirmar os resultados obtidos através das diferentes metodologias nas combinações de azólicos e equinocandinas frente a *A. flavus* e *A. fumigatus* a fim de determinar se estas associações podem ser utilizadas como alternativa terapêutica em casos de aspergilose de difícil tratamento, bem como qual a associação mais vantajosa nestes casos. Além disso, são necessários estudos multicêntricos com maior número de cepas para comprovar a confiabilidade do uso de metodologias alternativas como o Etest e o crescimento em ágar contendo antifúngico.

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ANEXOS

Tabela 1. Suscetibilidade de isolados clínicos de *Aspergillus fumigatus* frente a antifúngicos azólicos, equinocandinas e anfotericina B através de microdiluição em caldo - EUCAST.

A. fumigatus **(isolados clínicos)**	**ITZ**	**VCZ**	**PCZ**	**AMB**	**MFG**	**ANF**	**CAS**
01	1,00	0,50	0,06	0,50	0,001	0,001	0,06
02	1,00	1,00	0,06	0,50	0,001	0,001	0,06
03	1,00	1,00	0,06	0,50	0,001	0,001	0,06
04	1,00	1,00	0,125	0,50	0,002	0,001	0,06
05	1,00	1,00	0,125	0,50	0,002	0,002	0,125
06	1,00	1,00	0,125	0,50	0,002	0,002	0,125
07	1,00	1,00	0,125	1,00	0,002	0,002	0,125
08	1,00	1,00	0,125	1,00	0,002	0,004	0,125
09	1,00	1,00	0,125	1,00	0,004	0,004	0,125
10	1,00	1,00	0,125	1,00	0,004	0,004	0,125
11	1,00	1,00	0,125	1,00	0,008	0,004	0,125
12	1,00	1,00	0,125	1,00	0,008	0,004	0,125
13	1,00	1,00	0,125	1,00	0,008	0,004	0,125
14	2,00	1,00	0,125	1,00	0,008	0,008	0,125
15	2,00	2,00	0,125	1,00	0,008	0,008	0,125
16	2,00	2,00	0,125	1,00	0,008	0,008	0,250
17	2,00	2,00	0,250	1,00	0,015	0,008	0,250
18	2,00	2,00	0,250	1,00	0,016	0,015	0,250
19	2,00	2,00	0,250	1,00	0,03	0,03	0,250
20	2,00	2,00	0,250	1,00	0,031	0,031	0,50
21	8,00	2,00	0,50	1,00	0,031	0,06	0,50
22	8,00	2,00	0,50	1,00	0,031	0,063	0,50
23	16,00	2,00	0,50	1,00	0,031	0,063	1,00
24	16,00	2,00	0,50	1,00	0,06	0,063	1,00
25	16,00	4,00	0,50	1,00	0,125	0,125	1,00

ITZ, itraconazol; VCZ, voriconazol; PCZ, posaconazol; AMB, anfotericina B; MFG, micafungina; ANF, anidulafungina; CAS, caspofungina.

Tabela 2. Suscetibilidade de isolados ambientais de *Aspergillus fumigatus* frente a antifúngicos azólicos, equinocandinas e anfotericina B através de microdiluição em caldo - EUCAST.

***A. fumigatus* (isolados ambientais)**	**ITZ**	**VCZ**	**PCZ**	**AMB**	**MFG**	**ANF**	**CAS**
100	1,00	0,25	0,03	0,06	0,002	0,001	0,03
101	1,00	0,25	0,03	0,25	0,002	0,001	0,03
102	1,00	0,50	0,03	0,25	0,004	0,002	0,03
103	1,00	0,50	0,03	0,25	0,004	0,004	0,03
104	1,00	0,50	0,03	0,25	0,015	0,004	0,03
105	1,00	0,50	0,03	0,25	0,03	0,004	0,06
106	1,00	0,50	0,03	0,25	0,03	0,004	0,06
107	1,00	0,50	0,03	0,25	0,03	0,008	0,06
108	1,00	0,50	0,06	0,25	0,03	0,008	0,06
109	1,00	0,50	0,06	0,25	0,03	0,008	0,06
110	2,00	0,50	0,06	0,25	0,03	0,008	0,06
111	2,00	1,00	0,06	0,50	0,03	0,008	0,06
112	2,00	1,00	0,06	0,50	0,06	0,008	0,06
113	2,00	1,00	0,125	0,50	0,06	0,008	0,06
114	2,00	1,00	0,125	0,50	0,06	0,008	0,125
115	4,00	1,00	0,125	0,50	0,06	0,015	0,125
116	4,00	1,00	0,125	0,50	0,125	0,015	0,125
117	4,00	2,00	0,125	0,50	0,125	0,015	0,125
118	4,00	2,00	0,25	0,50	0,125	0,03	0,125
119	16,00	2,00	0,50	1,00	1,00	0,06	1,00

ITZ, itraconazol; VCZ, voriconazol; PCZ, posaconazol; AMB, anfotericina B; MFG, micafungina; ANF, anidulafungina; CAS, caspofungina.

Tabela 3. Suscetibilidade de isolados clínicos de *Aspergillus flavus* frente a antifúngicos azólicos, equinocandinas e anfotericina B através de microdiluição em caldo - EUCAST.

***A. flavus* (isolados clínicos)**	**ITZ**	**VCZ**	**PCZ**	**AMB**	**MFG**	**ANF**	**CAS**
1	1,00	0,50	0,125	0,50	0,008	0,002	0,008
2	1,00	0,50	0,125	0,50	0,015	0,002	0,03
3	1,00	0,50	0,125	0,50	0,015	0,004	0,06
4	1,00	0,50	0,125	1,00	0,015	0,004	0,06
5	1,00	1,00	0,125	1,00	0,015	0,008	0,06
6	1,00	1,00	0,125	1,00	0,015	0,008	0,06
7	1,00	1,00	0,125	1,00	0,015	0,008	0,06
8	1,00	1,00	0,125	1,00	0,015	0,008	0,06
9	1,00	1,00	0,125	1,00	0,03	0,008	0,06
10	1,00	1,00	0,125	1,00	0,03	0,008	0,06
11	1,00	1,00	0,125	1,00	0,03	0,008	0,125
12	1,00	1,00	0,25	1,00	0,03	0,008	0,125
13	1,00	1,00	0,25	1,00	0,03	0,008	0,125
14	1,00	1,00	0,25	1,00	0,03	0,015	0,125
15	1,00	1,00	0,25	1,00	0,03	0,015	0,125
16	2,00	1,00	0,25	1,00	0,03	0,015	0,125
17	2,00	1,00	0,25	1,00	0,03	0,015	0,125
18	2,00	1,00	0,25	1,00	0,06	0,015	0,125
19	2,00	1,00	0,25	2,00	0,06	0,03	0,125
20	2,00	1,00	0,25	2,00	0,06	0,03	0,25

ITZ, itraconazol; VCZ, voriconazol; PCZ, posaconazol; AMB, anfotericina B; MFG, micafungina; ANF, anidulafungina; CAS, caspofungina.

Tabela 4. Suscetibilidade de isolados ambientais de *Aspergillus flavus* frente a antifúngicos azólicos, equinocandinas e anfotericina B através de microdiluição em caldo - EUCAST.

***A. flavus* (isolados ambientais)**	**ITZ**	**VCZ**	**PCZ**	**AMB**	**MFG**	**ANF**	**CAS**
201	0,50	0,50	0,03	0,50	0,004	0,002	0,03
202	0,50	0,50	0,06	0,50	0,015	0,008	0,06
203	1,00	0,50	0,06	0,50	0,015	0,008	0,06
204	1,00	0,50	0,06	0,50	0,015	0,008	0,06
205	1,00	1,00	0,06	0,50	0,03	0,008	0,06
206	1,00	1,00	0,125	0,50	0,06	0,008	0,06
207	1,00	1,00	0,125	0,50	0,06	0,008	0,06
208	1,00	1,00	0,125	0,50	0,06	0,008	0,06
209	1,00	1,00	0,125	0,50	0,06	0,015	0,06
210	1,00	1,00	0,125	0,50	0,06	0,015	0,06
211	1,00	1,00	0,125	0,50	0,06	0,015	0,06
212	1,00	1,00	0,125	1,00	0,06	0,015	0,125
213	1,00	1,00	0,125	1,00	0,06	0,015	0,125
214	1,00	1,00	0,125	1,00	0,06	0,015	0,125
215	1,00	1,00	0,125	1,00	0,06	0,015	0,125
216	1,00	1,00	0,125	1,00	0,06	0,015	0,125
217	1,00	1,00	0,125	1,00	0,06	0,015	0,125
218	1,00	1,00	0,125	1,00	0,06	0,015	0,125
219	1,00	1,00	0,125	1,00	0,125	0,015	0,125
220	1,00	1,00	0,125	1,00	0,125	0,015	0,125
221	1,00	1,00	0,125	1,00	0,125	0,015	0,125
222	1,00	1,00	0,125	1,00	0,125	0,015	0,125
223	2,00	1,00	0,125	1,00	0,125	0,015	0,125
224	2,00	1,00	0,125	1,00	0,125	0,015	0,125
225	2,00	1,00	0,125	1,00	0,125	0,03	0,125
226	2,00	1,00	0,125	1,00	0,125	0,03	0,125
227	2,00	1,00	0,125	1,00	0,125	0,03	0,125
228	2,00	1,00	0,125	1,00	0,125	0,03	0,125
229	2,00	1,00	0,125	1,00	0,125	0,03	0,125
230	2,00	1,00	0,125	1,00	0,125	0,03	0,125
231	2,00	1,00	0,125	1,00	0,125	0,03	0,125
232	2,00	1,00	0,125	1,00	0,125	0,03	0,125
233	2,00	1,00	0,125	1,00	0,25	0,03	0,125
234	2,00	1,00	0,125	1,00	0,25	0,03	0,25
235	2,00	1,00	0,125	1,00	0,25	0,06	0,25
236	2,00	2,00	0,125	1,00	0,25	0,06	0,25
237	2,00	2,00	0,125	2,00	0,25	0,06	0,25
238	4,00	2,00	0,25	2,00	0,25	0,06	0,25
239	4,00	2,00	0,25	2,00	0,25	0,06	0,25
240	8,00	2,00	0,25	8,00	0,25	0,125	0,25

ITZ, itraconazol; VCZ, voriconazol; PCZ, posaconazol; AMB, anfotericina B; MFG, micafungina; ANF, anidulafungina; CAS, caspofungina.

Tabela 5. Concentração Inibitória Mínima, Concentração Efetiva Mínima e Índice de Concentração Inibitória Fracionária frente a *Aspergillus fumigatus* resistentes ao itraconazol, para as combinações caspofungina (CAS), micafungina (MFG) e anidulafungina (ANF) com voriconazol (VCZ) por Etest® com leitura de 24 h .

		CASPOFUNGINA-VORICONAZOL						**MICAFUNGINA-VORICONAZOL**						**ANIDULAFUNGINA-VORICONAZOL**					
Cepa	Ponto Final	CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)					
		CAS	CAS+VCZ	VCZ	VCZ+CAS	ICIF	RES	MFG	MFG+VCZ	VCZ	VCZ+MFG	ICIF	RES	ANF	ANF+VCZ	VCZ	VCZ+ANF	ICIF	RES
01	MIC	>32,0	0,190	1,000	0,190	-15/-5	S	>32,0	0,380	1,000	0,380	-13/-3	S	>32,0	0,380	1,000	0,380	-11/-3	S
	MEC	0,190	0,094	1,000	0,094	-2/-7	S	0,023	0,006	1,000	0,006	-4/-15	S	0,006	0,125	1,000	0,125	+9/-6	I
02	MIC	>32,0	0,380	0,380	0,380	-13/0	I	>32,0	0,023	0,380	0,023	-21/-8	S	>32,0	0,012	0,380	0,012	-21/-10	S
	MEC	0,380	0,250	0,380	0,250	-1/-1	I	0,016	0,012	0,380	0,012	-1/-10	I	0,032	0,006	0,380	0,006	-5/-12	S
03	MIC	>32,0	0,250	1,000	0,250	-14/-4	S	>32,0	0,032	1,000	0,032	-20/-10	S	>32,0	0,016	1,000	0,016	-20/-12	S
	MEC	0,250	0,125	1,000	0,125	-2/-6	I	0,032	0,012	1,000	0,012	-3/-13	S	0,023	0,008	1,000	0,008	-3/-14	S
04	MIC	>32,0	0,750	0,750	0,750	-11/0	I	>32,0	0,190	0,750	0,190	-13/-4	S	>32,0	0,250	0,750	0,250	-12/-3	S
	MEC	0,380	0,190	0,750	0,190	-2/-4	I	0,012	0,004	0,750	0,004	-3/-15	S	0,006	0,006	0,750	0,006	0/-14	I
05	MIC	>32,0	0,094	6,000	0,094	-17/-12	S	>32,0	0,008	6,000	0,008	-22/-19	S	>32,0	0,023	6,000	0,023	-19/-16	S
	MEC	0,380	0,094	6,000	0,094	-4/-12	S	0,094	0,004	6,000	0,004	-9/-20	S	0,032	0,012	6,000	0,012	-3/-18	S
06	MIC	>32,0	0,250	0,500	0,250	-14/-2	I	>32,0	0,190	0,500	0,190	-13/-3	S	>32,0	0,190	0,500	0,190	-13/-3	S
	MEC	0,250	0,190	0,500	0,190	-1/-3	I	0,012	0,064	0,500	0,064	+5/-6	I	0,016	0,064	0,500	0,064	+4/-6	I
07	MIC	>32,0	0,125	0,250	0,125	-15/-2	I	>32,0	0,023	0,250	0,023	-19/-7	S	>32,0	0,016	0,250	0,016	-20/-8	S
	MEC	0,250	0,064	0,250	0,064	-4/4	S	0,016	0,016	0,250	0,016	0/-8	I	0,008	0,006	0,250	0,006	-1/-11	I
08	MIC	>32,0	0,380	1,000	0,380	-13/-3	S	>32,0	0,012	1,000	0,012	-21/-13	S	>32,0	0,008	1,000	0,008	-22/-14	S
	MEC	0,380	0,190	1,000	0,190	-2/-5	I	0,023	0,008	1,000	0,008	-3/-14	S	0,023	0,003	1,000	0,003	-6/-17	S
09	MIC	>32,0	0,500	1,500	0,500	-12/-3	S	>32,0	0,125	1,500	0,125	-14/-7	S	>32,0	0,190	1,500	0,190	-13/-6	S
	MEC	0,380	0,125	1,500	0,125	-3/-7	S	0,016	0,064	1,500	0,064	+4/-9	I	0,016	0,064	1,500	0,064	+4/-9	I
10	MIC	>32,0	0,380	0,750	0,380	-13/-2	I	>32,0	0,064	0,750	0,064	-16/-7	S	>32,0	0,190	0,750	0,190	-13/-4	S
	MEC	0,500	0,250	0,750	0,250	-2/-3	I	0,006	0,023	0,750	0,023	+4/-10	I	0,002	0,002	0,750	0,002	0/-17	I
11	MIC	>32,0	0,125	0,250	0,125	-16/-2	I	>32,0	0,094	0,250	0,094	-15/-3	S	>32,0	0,094	0,250	0,094	-15/-3	S
	MEC	0,250	0,016	0,250	0,016	-8/-8	S	0,008	0,006	0,250	0,006	-1/-11	I	0,002	0,008	0,250	0,008	+4/-10	I
12	MIC	>32,0	0,094	0,380	0,094	-17/-4	S	>32,0	0,047	0,380	0,047	-17/-6	S	>32,0	0,125	0,380	0,125	-14/-3	S
	MEC	0,250	0,047	0,380	0,047	-5/-6	S	0,012	0,016	0,380	0,016	+1/-9	I	0,008	0,006	0,380	0,006	-1/-12	I
13	MIC	>32,0	0,500	1,500	0,500	-12/-3	S	>32,0	0,190	1,500	0,190	-13/-6	S	>32,0	0,250	1,500	0,250	-12/-5	S
	MEC	0,380	0,125	1,500	0,125	-3/-7	S	0,016	0,032	1,500	0,032	+2/-11	I	0,032	0,016	1,500	0,016	-2/-13	I
14	MIC	>32,0	0,380	2,000	0,380	-13/-5	S	>32,0	0,190	2,000	0,190	-13/-7	S	>32,0	0,190	2,000	0,190	-13/-7	S
	MEC	0,380	0,125	2,000	0,125	-3/-8	S	0,016	0,023	2,000	0,023	+1/-13	I	0,012	0,016	2,000	0,016	-1/-14	I
15	MIC	>32,0	0,500	3,000	0,500	-12/-5	S	>32,0	0,250	3,000	0,250	-12/-7	S	>32,0	0,250	3,000	0,250	-12/-7	S
	MEC	0,500	0,125	3,000	0,125	-4/-9	S	0,125	0,094	3,000	0,094	-1/-10	I	0,047	0,047	3,000	0,047	0/-12	I

Tabela 6. Concentração Inibitória Mínima, Concentração Efetiva Mínima e Índice de Concentração Inibitória Fracionária frente a *Aspergillus fumigatus* resistentes ao itraconazol (ITZ), para as combinações caspofungina (CAS), micafungina (MFG) e anidulafungina (ANF) com voriconazol (VCZ) por Etest® com leitura de 48 h .

		CASPOFUNGINA-VORICONAZOL						**MICAFUNGINA-VORICONAZOL**						**ANIDULAFUNGINA-VORICONAZOL**					
Cepa	Ponto Final	CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)					
		CAS	CAS+VCZ	VCZ	VCZ+CAS	ICIF	RES	MFG	MFG+VCZ	VCZ	VCZ+MFG	ICIF	RES	ANF	ANF+VCZ	VCZ	VCZ+ANF	ICIF	RES
01	MIC	>32,0	0,750	1,000	0,750	-9/-1	I	>32,0	0,380	1,000	0,380	-11/-3	S	>32,0	0,380	1,000	0,380	-11/-3	S
	MEC	0,190	0,250	1,000	0,250	+1/-4	I	0,023	0,006	1,000	0,006	-4/-15	S	0,006	0,094	1,000	0,094	+8/-7	S
02	MIC	>32,0	0,250	0,380	0,250	-12/-1	I	>32,0	0,125	0,380	0,125	-14-3	S	>32,0	0,125	0,380	0,125	-14/-3	S
	MEC	0,380	0,125	0,380	0,125	-3/-3	S	0,016	0,047	0,380	0,047	+3/-6	I	0,032	0,016	0,380	0,016	-2/-9	I
03	MIC	>32,0	0,500	1,000	0,500	-10/-2	I	>32,0	0,500	1,000	0,500	-10/-2	I	>32,0	0,380	1,000	0,380	-11/-3	S
	MEC	0,380	0,190	1,000	0,190	-2/-5	I	0,016	0,125	1,000	0,125	+6/-6	I	0,032	0,064	1,000	0,064	+2/-8	I
04	MIC	>32,0	0,750	0,750	0,750	-9/0	I	>32,0	0,250	0,750	0,250	-12/-3	S	>32,0	0,250	0,750	0,250	-12/-3	S
	MEC	0,380	0,250	0,750	0,250	-1/-3	I	0,023	0,008	0,750	0,008	-3/-13	S	0,008	0,023	0,750	0,023	+3/-10	I
05	MIC	>32,0	1,500	6,000	1,500	-7/-4	S	>32,0	0,750	6,000	0,750	-9/-6	S	>32,0	1,000	6,000	1,000	-8/-5	S
	MEC	0,380	0,500	6,000	0,500	+1/-7	I	0,012	0,008	6,000	0,008	-1/-19	I	0,008	0,008	6,000	0,008	0/-19	I
06	MIC	>32,0	0,250	0,500	0,250	-12/-2	I	>32,0	0,190	0,500	0,190	-13/-3	S	>32,0	0,380	0,500	0,380	-11/-1	I
	MEC	0,500	0,190	0,500	0,190	-3/-3	S	0,016	0,032	0,500	0,032	+2/-8	I	0,016	0,008	0,500	0,008	-2/-12	I
07	MIC	>32,0	0,125	0,250	0,125	-14/-2	I	>32,0	0,032	0,250	0,032	-18/-6	S	>32,0	0,094	0,250	0,094	-15/-3	S
	MEC	0,250	0,064	0,250	0,064	-4/-4	S	0,023	0,016	0,250	0,016	-1/-8	I	0,008	0,012	0,250	0,012	+1/-9	I
08	MIC	>32,0	0,380	1,000	0,380	-11/-3	S	>32,0	0,750	1,000	0,750	-9/-1	I	>32,0	0,380	1,000	0,380	-11/-3	S
	MEC	0,380	0,190	1,000	0,190	-2/-5	I	0,023	0,008	1,000	0,008	-3/-14	S	0,023	0,004	1,000	0,004	-5/-16	S
09	MIC	>32,0	0,750	1,500	0,750	-9/-2	I	>32,0	0,125	1,500	0,125	-14/-7	S	>32,0	0,190	1,500	0,190	-13/-6	S
	MEC	0,500	0,250	1,500	0,250	-2/-5	I	0,032	0,032	1,500	0,032	0/-11	I	0,016	0,016	1,500	0,016	0/-13	I
10	MIC	>32,0	0,380	0,750	0,380	-11/-2	I	>32,0	0,064	0,750	0,064	-16/-7	S	>32,0	0,094	0,750	0,094	-15/-6	S
	MEC	0,500	0,190	0,750	0,190	-3/-4	S	0,008	0,016	0,750	0,016	+2/-11	I	0,002	0,047	0,750	0,047	+9/-8	I
11	MIC	>32,0	0,016	0,250	0,016	-20/-8	S	>32,0	0,125	0,250	0,125	-14/-2	I	>32,0	0,125	0,250	0,125	-14/-2	I
	MEC	0,250	0,190	0,250	0,190	-1/-1	I	0,008	0,012	0,250	0,012	+1/-9	I	0,002	0,012	0,250	0,012	+5/-9	I
12	MIC	>32,0	0,190	0,380	0,190	-13/-2	I	>32,0	0,125	0,380	0,125	-14/-3	S	>32,0	0,125	0,380	0,125	-14/-3	S
	MEC	0,250	0,094	0,380	0,094	-3/-4	S	0,012	0,008	0,380	0,008	-1/-11	I	0,008	0,006	0,380	0,006	-1/-12	I
13	MIC	>32,0	0,500	1,500	0,500	-10/-3	S	>32,0	0,250	1,500	0,250	-12/-5	S	>32,0	0,250	1,500	0,250	-12/-5	S
	MEC	0,380	0,125	1,500	0,125	-3/-7	S	0,016	0,012	1,500	0,012	-1/-14	I	0,032	0,003	1,500	0,003	-7/-15	S
14	MIC	>32,0	0,380	2,000	0,380	-11/-5	S	>32,0	0,190	2,000	0,190	-13/-7	S	>32,0	0,250	2,000	0,250	-12/-6	S
	MEC	0,380	0,190	2,000	0,190	-2/-7	I	0,016	0,008	2,000	0,008	-2/-16	I	0,016	0,016	2,000	0,016	0/-14	I
15	MIC	>32,0	0,750	3,000	0,750	-9/-4	S	>32,0	0,380	3,000	0,380	-11/-6	S	>32,0	0,250	3,000	0,250	-12/-7	S
	MEC	0,500	0,250	3,000	0,250	-2/-7	I	0,500	0,023	3,000	0,023	-9/-14	S	0,250	0,032	3,000	0,032	-6/-13	S

Tabela 7. Concentração Inibitória Mínima, Concentração Efetiva Mínima e Índice de Concentração Inibitória Fracionária frente a *Aspergillus fumigatus* resistentes ao itraconazol, para as combinações caspofungina (CAS), micafungina (MFG) e anidulafungina (ANF) com posaconazol (PCZ) por Etest® com leitura de 24 h .

		CASPOFUNGINA-POSACONAZOL						**MICAFUNGINA-POSACONAZOL**						**ANIDULAFUNGINA-POSACONAZOL**					
Cepa	Ponto Final	CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)					
		CAS	CAS+PCZ	PCZ	PCZ+CAS	ICIF	RES	MFG	MFG+PCZ	VCZ	PCZ+MFG	ICIF	RES	ANF	ANF+PCZ	PCZ	PCZ+ANF	ICIF	RES
01	MIC	>32,0	0,750	0,750	0,750	-9/0	I	>32,0	0,250	0,750	0,250	-12/-3	S	>32,0	0,250	0,750	0,250	-12/-3	S
	MEC	0,190	0,094	0,750	0,094	-2/-6	I	0,023	0,016	0,750	0,016	-1/-11	I	0,006	0,008	0,750	0,008	+1/13	I
02	MIC	>32,0	0,125	0,190	0,125	-14/+1	I	>32,0	0,047	0,190	0,047	-17/-4	S	>32,0	0,032	0,190	0,032	-18/-5	S
	MEC	0,380	0,125	0,190	0,125	-3/-1	I	0,016	0,016	0,190	0,016	0/-7	I	0,032	0,008	0,190	0,008	-4/-9	S
03	MIC	>32,0	0,125	0,250	0,125	-14/-2	I	>32,0	0,032	0,250	0,032	-18/-6	S	>32,0	0,016	0,250	0,016	-20/-8	S
	MEC	0,250	0,125	0,250	0,125	-2/-2	I	0,032	0,008	0,250	0,008	-4/-10	S	0,023	0,002	0,250	0,002	-7/-14	S
04	MIC	>32,0	0,380	0,380	0,380	-11/0	I	>32,0	0,190	0,380	0,190	-13/-2	I	>32,0	0,250	0,380	0,250	-12/-1	I
	MEC	0,380	0,125	0,380	0,125	-3/-3	S	0,012	0,016	0,380	0,016	+1/-9	I	0,006	0,004	0,380	0,004	-1/-13	I
05	MIC	>32,0	0,064	0,250	0,064	-16/-4	S	>32,0	0,032	0,250	0,032	-18/-6	S	>32,0	0,380	0,250	0,380	-11/+1	I
	MEC	0,380	0,032	0,250	0,032	-7/-6	S	0,094	0,016	0,250	0,016	-5/-8	S	0,032	0,004	0,250	0,004	-6/-12	S
06	MIC	>32,0	0,250	0,380	0,250	-12/-1	I	>32,0	0,094	0,380	0,094	-15/-4	S	>32,0	0,002	0,380	0,002	-26/-15	S
	MEC	0,250	0,190	0,380	0,190	-1/-2	I	0,012	0,023	0,380	0,023	+2/-8	I	0,016	0,002	0,380	0,002	-6/-15	S
07	MIC	>32,0	0,047	0,125	0,047	-17/-3	S	>32,0	0,012	0,125	0,012	-21/-7	S	>32,0	0,002	0,125	0,002	-26/-12	S
	MEC	0,250	0,047	0,125	0,047	-5/-3	S	0,016	0,002	0,125	0,002	-6/-12	S	0,008	0,002	0,125	0,002	-4/-12	S
08	MIC	>32,0	0,094	0,500	0,094	-15/-5	S	>32,0	0,064	0,500	0,064	-16/-10	S	>32,0	0,032	0,500	0,032	-18/-8	S
	MEC	0,380	0,094	0,500	0,094	-4/-5	S	0,023	0,008	0,500	0,008	-3/-12	S	0,023	0,004	0,500	0,004	-5/-14	S
09	MIC	>32,0	0,380	0,750	0,380	-11/-2	I	>32,0	0,190	0,750	0,190	-13/-4	S	>32,0	0,125	0,750	0,125	-14/-5	S
	MEC	0,380	0,380	0,750	0,380	0/-2	I	0,016	0,023	0,750	0,023	+1/-10	I	0,016	0,008	0,750	0,008	-2/-13	I
10	MIC	>32,0	0,190	0,023	0,190	-13/+6	I	>32,0	0,064	0,023	0,064	-16/+3	I	>32,0	0,004	0,023	0,004	-24/-5	S
	MEC	0,500	0,190	0,023	0,190	-3/+6	I	0,006	0,012	0,023	0,012	+2/+2	I	0,002	0,002	0,023	0,002	0/-7	I
11	MIC	>32,0	0,094	0,094	0,094	-15/0	I	>32,0	0,047	0,094	0,047	-17/-2	I	>32,0	0,032	0,094	0,032	-18/-3	S
	MEC	0,250	0,016	0,094	0,016	-8/-5	S	0,008	0,004	0,094	0,004	-2/-9	I	0,002	0,004	0,094	0,004	+1/-9	I
12	MIC	>32,0	0,064	0,125	0,064	-16/-2	I	>32,0	0,047	0,125	0,047	-17/-3	S	>32,0	0,032	0,125	0,032	-18/-4	S
	MEC	0,250	0,064	0,125	0,064	-4/-2	I	0,012	0,003	0,125	0,003	-4/-11	S	0,008	0,002	0,125	0,002	-4/-12	S
13	MIC	>32,0	0,380	0,380	0,380	-11/0	I	>32,0	0,190	0,380	0,190	-13/-2	I	>32,0	0,032	0,380	0,032	-18/-7	S
	MEC	0,380	0,380	0,380	0,380	0/0	I	0,016	0,023	0,380	0,023	+1/-8	I	0,032	0,004	0,380	0,004	-6/-13	S
14	MIC	>32,0	0,250	0,500	0,250	-12/-2	I	>32,0	0,125	0,500	0,125	-14/-4	S	>32,0	0,125	0,500	0,125	-14/-4	S
	MEC	0,380	0,032	0,500	0,032	-8/-8	S	0,016	0,008	0,500	0,008	-2/-12	I	0,012	0,008	0,500	0,008	-1/-12	I
15	MIC	>32,0	0,380	0,380	0,380	-11/0	I	>32,0	0,125	0,380	0,125	-14/-3	S	>32,0	0,190	0,380	0,190	-13/-2	I
	MEC	0,500	0,380	0,380	0,380	-1/0	I	0,125	0,012	0,380	0,012	-7/-10	S	0,047	0,032	0,380	0,032	-1/-7	I

Tabela 8. Concentração Inibitória Mínima, Concentração Efetiva Mínima e Índice de Concentração Inibitória Fracionária frente a *Aspergillus fumigatus* resistentes ao itraconazol, para as combinações caspofungina (CAS), micafungina (MFG) e anidulafungina (ANF) com posaconazol (PCZ) por Etest® com leitura de 48 h .

		CASPOFUNGINA-POSACONAZOL						**MICAFUNGINA-POSACONAZOL**						**ANIDULAFUNGINA-POSACONAZOL**					
Cepa	Ponto Final	CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)					
		CAS	CAS+PCZ	PCZ	PCZ+CAS	ICIF	RES	MFG	MFG+PCZ	VCZ	VCZ+MFG	ICIF	RES	ANF	ANF+PCZ	PCZ	PCZ+ANF	ICIF	RES
01	MIC	>32,0	0,750	0,750	0,750	-9/0	I	>32,0	0,250	0,750	0,250	-12/-3	S	>32,0	0,250	0,750	0,250	-12/-3	S
	MEC	0,190	0,094	0,750	0,094	-2/-6	I	0,023	0,016	0,750	0,016	-1/-11	I	0,006	0,008	0,750	0,008	+1/-13	I
02	MIC	>32,0	0,190	0,250	0,190	-13/-1	I	>32,0	0,125	0,250	0,125	-14/-2	I	>32,0	0,094	0,250	0,094	-15/-3	S
	MEC	0,380	0,032	0,250	0,032	-7/-6	S	0,016	0,064	0,250	0,064	+4/-4	I	0,032	0,008	0,250	0,008	-4/-10	S
03	MIC	>32,0	0,380	0,500	0,380	-11/-1	I	>32,0	0,250	0,500	0,250	-12/-2	I	>32,0	0,250	0,500	0,250	-12/-2	I
	MEC	0,380	0,094	0,500	0,094	-4/-5	S	0,016	0,125	0,500	0,125	+6/-4	I	0,032	0,064	0,500	0,064	+2/-6	I
04	MIC	>32,0	0,380	0,500	0,380	-11/-1	I	>32,0	0,250	0,500	0,250	-12/-2	I	>32,0	0,250	0,500	0,250	-12/-2	I
	MEC	0,380	0,125	0,500	0,125	-3/-4	S	0,023	0,016	0,500	0,016	-1/-10	I	0,008	0,006	0,500	0,006	-1/-13	I
05	MIC	>32,0	0,500	2,000	0,500	-10/-4	S	>32,0	0,500	2,000	0,500	-10/-4	S	>32,0	0,380	2,000	0,380	-11/-5	S
	MEC	0,380	0,190	2,000	0,190	-2/-7	I	0,012	0,023	2,000	0,023	+2/-13	I	0,008	0,047	2,000	0,047	+5/-11	I
06	MIC	>32,0	0,250	0,380	0,250	-12/-1	I	>32,0	0,094	0,380	0,094	-15/-4	S	>32,0	0,064	0,380	0,064	-16/-5	S
	MEC	0,500	0,008	0,380	0,008	-12/-11	S	0,016	0,016	0,380	0,016	0/-9	I	0,016	0,002	0,380	0,002	-6/-15	S
07	MIC	>32,0	0,125	0,125	0,125	-14/0	I	>32,0	0,023	0,125	0,023	-19/-5	S	>32,0	0,032	0,125	0,032	-18/-4	S
	MEC	0,250	0,016	0,125	0,016	-8/-6	S	0,023	0,008	0,125	0,008	-3/-8	S	0,008	0,002	0,125	0,002	-4/-12	S
08	MIC	>32,0	0,500	0,500	0,500	-10/0	I	>32,0	0,380	0,500	0,380	-11/-1	I	>32,0	0,380	0,500	0,380	-11/-1	I
	MEC	0,380	0,125	0,500	0,125	-3/-4	S	0,023	0,023	0,500	0,023	0/-9	I	0,023	0,008	0,500	0,008	-3/-12	S
09	MIC	>32,0	0,500	0,750	0,500	-10/-1	I	>32,0	0,190	0,750	0,190	-13/-4	S	>32,0	0,125	0,750	0,125	-14/-5	S
	MEC	0,500	0,125	0,750	0,125	-4/-5	S	0,032	0,012	0,750	0,012	-3/-12	S	0,016	0,004	0,750	0,004	-4/-15	S
10	MIC	>32,0	0,250	0,190	0,250	-12/+1	I	>32,0	0,064	0,190	0,064	-16/-3	S	>32,0	0,012	0,190	0,012	-21/-8	S
	MEC	0,500	0,094	0,190	0,094	-5/-2	I	0,008	0,008	0,190	0,008	0/-9	I	0,002	0,002	0,190	0,002	0/-13	I
11	MIC	>32,0	0,125	0,190	0,125	-14/-1	I	>32,0	0,064	0,190	0,064	-16/-3	S	>32,0	0,047	0,190	0,047	-17/-4	S
	MEC	0,250	0,016	0,190	0,016	-8/-7	S	0,008	0,006	0,190	0,006	-1/-10	I	0,002	0,002	0,190	0,002	0/-13	I
12	MIC	>32,0	0,190	0,250	0,190	-13/-1	I	>32,0	0,064	0,250	0,064	-16/-4	S	>32,0	0,094	0,250	0,094	-15/-3	S
	MEC	0,250	0,016	0,250	0,016	-8/-8	S	0,012	0,004	0,250	0,004	-3/-12	S	0,008	0,006	0,250	0,006	-1/-11	I
13	MIC	>32,0	0,380	0,380	0,380	-11/0	I	>32,0	0,190	0,380	0,190	-13/-2	I	>32,0	0,032	0,380	0,032	-18/-7	S
	MEC	0,380	0,094	0,380	0,094	-4/-4	S	0,016	0,016	0,380	0,016	0/-9	I	0,032	0,004	0,380	0,004	-6/-13	S
14	MIC	>32,0	0,380	0,750	0,380	-11/-2	I	>32,0	0,190	0,750	0,190	-13/-4	S	>32,0	0,250	0,750	0,250	-12/-3	S
	MEC	0,380	0,047	0,750	0,047	-6/-8	S	0,016	0,008	0,750	0,008	-2/-13	I	0,016	0,004	0,750	0,004	-4/-15	S
15	MIC	>32,0	0,380	1,500	0,380	-11/-4	S	>32,0	0,016	1,500	0,016	-20/-13	S	>32,0	0,190	1,500	0,190	-13/-6	S
	MEC	0,500	0,064	1,500	0,064	-6/-9	S	0,500	0,094	1,500	0,094	-5/-8	S	0,250	0,094	1,500	0,094	-3/-8	S

Tabela 9. Concentração Inibitória Mínima, Concentração Efetiva Mínima e Índice de Concentração Inibitória Fracionária frente a *Aspergillus fumigatus* resistentes ao itraconazol, para as combinações caspofungina (CAS), micafungina (MFG) e anidulafungina (ANF) com itraconazol (ITZ) por Etest® com leitura de 24 h.

		CASPOFUNGINA-ITRACONAZOL						**MICAFUNGINA-ITRACONAZOL**						**ANIDULAFUNGINA-ITRACONAZOL**					
Cepa	Ponto Final	CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)					
		CAS	CAS+ITZ	ITZ	ITZ+CAS	ICIF	RES	MFG	MFG+ITZ	ITZ	ITZ+MFG	ICIF	RES	ANF	ANF+ITZ	ITZ	ITZ+ANF	ICIF	RES
01	MIC	>32,0	0,125	32,00	0,125	-16/-16	S	>32,0	0,016	32,00	0,016	-22/-22	S	>32,0	0,016	32,00	0,016	-22/-22	S
	MEC	0,190	0,008	32,00	0,008	-9/-24	S	0,023	0,012	32,00	0,012	-2/-23	I	0,006	0,002	32,00	0,002	-3/-28	S
02	MIC	>32,0	2,00	4,00	2,00	-8/-2	I	>32,0	1,00	4,00	1,00	-10/-4	S	>32,0	0,016	4,00	0,016	-22/-16	S
	MEC	0,380	0,125	4,00	0,125	-3/-10	S	0,016	0,002	4,00	0,002	-6/-22	S	0,032	0,002	4,00	0,002	-8/-21	S
03	MIC	>32,0	1,50	32,00	1,50	-9/-9	S	>32,0	1,50	32,00	1,50	-9/-9	S	>32,0	0,750	32,00	0,750	-11/-11	S
	MEC	0,250	0,125	32,00	0,125	-2/-16	I	0,032	0,032	32,00	0,032	0/-20	I	0,023	0,006	32,00	0,006	-4/-25	S
04	MIC	>32,0	0,380	32,00	0,380	-13/-13	S	>32,0	0,750	32,00	0,750	-11/-11	S	>32,0	0,008	32,00	0,008	-24/-24	S
	MEC	0,380	0,008	32,00	0,008	-11/-24	S	0,012	0,004	32,00	0,004	-3/-26	S	0,006	0,002	32,00	0,002	-3/-28	S
05	MIC	>32,0	0,50	4,00	0,50	-12/-6	S	>32,0	3,00	4,00	3,00	-7/-1	I	>32,0	2,00	4,00	2,00	-8/-2	I
	MEC	0,380	0,125	4,00	0,125	-3/-10	S	0,094	0,047	4,00	0,047	-2/-3	I	0,032	0,004	4,00	0,004	-6/-20	S
06	MIC	>32,0	0,380	8,00	0,380	-13/-9	S	>32,0	1,500	8,00	1,500	-9/-5	S	>32,0	0,016	8,00	0,016	-22/-18	S
	MEC	0,250	0,125	8,00	0,125	-2/-12	I	0,012	0,012	8,00	0,012	0/-19	I	0,016	0,002	8,00	0,002	-6/-24	S
07	MIC	>32,0	0,125	4,00	0,125	-16/-10	S	>32,0	2,00	4,00	2,00	-8/-2	I	>32,0	2,00	4,00	2,00	-8/-2	I
	MEC	0,250	0,008	4,00	0,008	-10/-18	S	0,016	0,012	4,00	0,012	-1/-17	I	0,008	0,002	4,00	0,002	-4/-22	S
08	MIC	>32,0	1,00	4,00	1,00	-10/-4	S	>32,0	1,00	4,00	1,00	-10/-4	S	>32,0	0,125	4,00	0,125	-16/-10	S
	MEC	0,380	0,250	4,00	0,250	-1/-8	I	0,023	0,012	4,00	0,012	-2/-17	I	0,023	0,002	4,00	0,002	-7/-23	S
09	MIC	>32,0	1,00	32,00	1,00	-10/-10	S	>32,0	0,190	32,00	0,190	-15/-15	S	>32,0	0,016	32,00	0,016	-22/-22	S
	MEC	0,380	0,380	32,00	0,380	0/-13	I	0,016	0,006	32,00	0,006	-3/-25	S	0,016	0,002	32,00	0,002	-6/-28	S
10	MIC	>32,0	0,380	16,00	0,380	-13/-12	S	>32,0	0,380	16,00	0,380	-13/-11	S	>32,0	0,250	16,00	0,250	-14/-12	S
	MEC	0,500	0,125	16,00	0,125	-5/-14	S	0,006	0,008	16,00	0,008	+1/-22	I	0,002	0,032	16,00	0,032	+8/-18	I
11	MIC	>32,0	0,380	8,00	0,380	-13/-9	S	>32,0	0,250	8,00	0,250	-14/-10	S	>32,0	0,250	8,00	0,250	-14/-10	S
	MEC	0,250	0,125	8,00	0,125	-2/-12	I	0,008	0,004	8,00	0,004	-2/-22	I	0,002	0,002	8,00	0,002	0/-5	I
12	MIC	>32,0	0,750	4,00	0,750	-11/-5	S	>32,0	3,00	4,00	3,00	-7/-1	I	>32,0	3,00	4,00	3,00	-8/-1	I
	MEC	0,250	0,250	4,00	0,250	0/-8	I	0,012	0,002	4,00	0,002	-5/-22	S	0,008	0,002	4,00	0,002	-4/-23	S
13	MIC	>32,0	2,00	32,00	2,00	-8/-8	S	>32,0	1,00	32,00	1,00	-10/-10	S	>32,0	0,032	32,00	0,032	-20/-20	S
	MEC	0,380	0,380	32,00	0,380	0/-13	I	0,016	0,016	32,00	0,016	0/-22	I	0,032	0,002	32,00	0,002	-8/-28	S
14	MIC	>32,0	1,50	32,00	1,50	-9/-9	S	>32,0	0,50	32,00	0,50	-12/-12	S	>32,0	0,380	32,00	0,380	-13/-13	S
	MEC	0,380	0,190	32,00	0,190	-2/-15	I	0,016	0,016	32,00	0,016	0/-22	I	0,012	0,004	32,00	0,004	-3/-26	S
15	MIC	>32,0	8,00	32,00	8,00	-4/-4	S	>32,0	0,380	32,00	0,380	-13/-13	S	>32,0	0,250	32,00	0,250	-14/-14	S
	MEC	0,500	0,500	32,00	0,500	0/-12	I	0,125	0,012	32,00	0,012	-7/-23	S	0,047	0,004	32,00	0,004	-7-26	S

Tabela 10. Concentração Inibitória Mínima, Concentração Efetiva Mínima e Índice de Concentração Inibitória Fracionária frente a *Aspergillus fumigatus* resistentes ao itraconazol, para as combinações caspofungina (CAS), micafungina (MFG) e anidulafungina (ANF) com itraconazol (ITZ) por Etest® com leitura de 48 h.

		CASPOFUNGINA-ITRACONAZOL						**MICAFUNGINA-ITRACONAZOL**						**ANIDULAFUNGINA-ITRACONAZOL**					
Cepa	Ponto Final	CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)						CONCENTRAÇÃO (µg/mL)					
		CAS	CAS+ITZ	ITZ	ITZ+CAS	ICIF	RES	MFG	MFG+ITZ	ITZ	ITZ+MFG	ICIF	RES	ANF	ANF+ITZ	ITZ	ITZ+ANF	ICIF	RES
01	MIC	>32,0	0,190	32,00	0,190	-15/-15	S	>32,0	0,023	32,00	0,023	-21/-21	S	>32,0	0,016	32,00	0,016	-22/-22	S
	MEC	0,190	0,008	32,00	0,008	-9/-24	S	0,023	0,002	32,00	0,002	-7/-28	S	0,006	0,002	32,00	0,002	-3/-28	S
02	MIC	>32,0	3,00	4,00	3,00	-7/-1	I	>32,0	1,00	4,00	1,00	-10/-4	S	>32,0	0,023	4,00	0,023	-21/-15	S
	MEC	0,380	0,125	4,00	0,125	-1/-10	I	0,016	0,002	4,00	0,002	-6/-22	S	0,032	0,002	4,00	0,002	-8/-22	S
03	MIC	>32,0	1,50	32,00	1,50	-9/-9	S	>32,0	1,50	32,00	1,50	-9/-9	S	>32,0	0,750	32,00	0,750	-11/-11	S
	MEC	0,380	1,00	32,00	1,00	+3/-10	I	0,016	0,004	32,00	0,004	-4/-26	S	0,032	0,006	32,00	0,006	-5/-25	S
04	MIC	>32,0	0,500	32,00	0,500	-12/-12	S	>32,0	0,125	32,00	0,125	-16/-16	S	>32,0	0,008	32,00	0,008	-24/-24	S
	MEC	0,380	0,008	32,00	0,008	-11/-24	S	0,023	0,008	32,00	0,008	-3/-24	S	0,008	0,002	32,00	0,002	-4/-28	S
05	MIC	>32,0	3,00	4,00	3,00	-7/-1	I	>32,0	4,00	4,00	4,00	-7/0	I	>32,0	2,00	4,00	2,00	-8/-2	I
	MEC	0,380	0,125	4,00	0,125	-1/-10	I	0,012	0,008	4,00	0,008	-1/-18	I	0,008	0,008	4,00	0,008	0/-18	I
06	MIC	>32,0	0,500	8,00	0,500	-12/-8	S	>32,0	1,500	8,00	1,500	-9/-5	S	>32,0	0,016	8,00	0,016	-22/-18	S
	MEC	0,500	0,125	8,00	0,125	-4/-12	S	0,016	0,190	8,00	0,190	+7/-11	I	0,016	0,008	8,00	0,008	-2/-20	I
07	MIC	>32,0	0,380	4,00	0,380	-13/-7	S	>32,0	3,00	4,00	3,00	-7/-1	I	>32,0	3,00	4,00	3,00	-7/-1	I
	MEC	0,250	0,012	4,00	0,012	-9/-17	S	0,023	0,002	4,00	0,002	-7/-22	S	0,008	0,008	4,00	0,008	0/-20	I
08	MIC	>32,0	1,000	4,00	1,000	-10/-4	S	>32,0	1,00	4,00	1,00	-10/-4	S	>32,0	0,190	4,00	0,190	-15/-9	S
	MEC	0,380	0,750	4,00	0,750	+2/-5	I	0,023	0,023	4,00	0,023	0/-15	I	0,023	0,004	4,00	0,004	-5/-20	S
09	MIC	>32,0	1,000	32,00	1,000	-10/-10	S	>32,0	0,023	32,00	0,023	-21/-21	S	>32,0	0,023	32,00	0,023	-21/-21	S
	MEC	0,500	0,032	32,00	0,032	-8/-20	S	0,032	0,008	32,00	0,008	-4/-24	S	0,016	0,016	32,00	0,016	0/-22	I
10	MIC	>32,0	0,500	16,00	0,500	-12/-10	S	>32,0	0,380	16,00	0,380	-13/-11	S	>32,0	0,380	16,00	0,380	-13/-11	S
	MEC	0,500	0,032	16,00	0,032	-8/-18	S	0,008	0,008	16,00	0,008	0/-22	I	0,002	0,032	16,00	0,032	+8/-18	I
11	MIC	>32,0	0,380	8,00	0,380	-13/-9	S	>32,0	0,500	8,00	0,500	-12/-8	S	>32,0	0,500	8,00	0,500	-12/-8	S
	MEC	0,250	0,125	8,00	0,125	-2/-12	I	0,008	0,008	8,00	0,008	0/-20	I	0,002	0,008	8,00	0,008	+4/-20	I
12	MIC	>32,0	0,750	4,00	0,750	-11/-5	S	>32,0	4,00	4,00	4,00	-6/0	I	>32,0	4,00	4,00	4,00	-6/0	I
	MEC	0,250	0,250	4,00	0,250	0/-8	I	0,012	0,004	4,00	0,004	-3/-20	S	0,008	0,008	4,00	0,008	0/-18	I
13	MIC	>32,0	2,00	32,00	2,00	-8/-8	S	>32,0	1,00	32,00	1,00	-10/-10	S	>32,0	0,047	32,00	0,047	-19/-19	S
	MEC	0,380	0,380	32,00	0,380	0/-13	I	0,016	0,032	32,00	0,032	+2/-20	I	0,032	0,002	32,00	0,002	-8/-28	S
14	MIC	>32,0	1,50	32,00	1,50	-9/-9	S	>32,0	1,00	32,00	1,00	-10/-10	S	>32,0	1,00	32,00	1,00	-10/-10	S
	MEC	0,380	0,250	32,00	0,250	-1/-14	I	0,016	0,016	32,00	0,016	0/-22	I	0,016	0,004	32,00	0,004	-4/-26	S
15	MIC	>32,0	8,00	32,00	8,00	-4/-4	S	>32,0	16,00	32,00	16,00	-2/-2	I	>32,0	0,500	32,00	0,500	-12/-12	S
	MEC	0,500	1,00	32,00	1,00	+2/-10	I	0,500	0,032	32,00	0,032	-8/-20	S	0,250	0,008	32,00	0,008	-10/-24	S

Tabela 11. Suscetibilidade de *Aspergillus fumigatus*, sensíveis e resistentes aos antifúngicos azólicos, ao isavuconazol através das metodologias de microdiluição em caldo, Etest e crescimento em ágar contendo antifúngico.

Cepas	Metodologia				Presença de mutações no gene *CYP*51A
	Microdiluição em caldo -EUCAST	Etest	Crescimento em ágar contendo 1 mg/L	Crescimento em ágar contendo 2 mg/L	
V203-77	1,00	0,25	no	no	Wild-type
V203-75	2,00	0,25	yes	no	Wild-type
V203-76	2,00	0,25	yes	no	Wild-type
V203-58	2,00	0,25	yes	no	Wild-type
V203-59	16,00	2,00	yes	yes	TR34/L98H
V203-71	1,00	0,25	no	no	Wild-type
V203-80	16,00	2,00	yes	yes	TR34/L98H
V204-14	2,00	0,50	yes	no	Wild-type
V204-02	2,00	0,25	yes	no	Wild-type
V204-03	> 16,00	> 32	yes	yes	TR34/L98H
V204-13	2,00	0,25	no	no	Wild-type
V203-57	16,00	2,00	yes	yes	TR34/L98H
V203-56	2,00	0,25	yes	no	Wild-type
V204-01	4,00	0,50	yes	yes	Wild-type
V203-81	2,00	0,25	yes	no	Wild-type
V203-67	2,00	0,25	yes	no	Wild-type
V203-61	2,00	0,125	yes	no	Wild-type
V204-20	1,00	0,25	no	no	Wild-type
V204-18	1,00	0,25	no	no	Wild-type
V204-24	2,00	0,5	yes	no	Wild-type
V204-21	1,00	0,25	no	no	Wild-type
V198-17	1,00	0,25	no	no	Wild-type
V197-06	8,00	0,25	yes	yes	Wild-type
V203-79	2,00	0,5	yes	no	Wild-type
V207-60	1,00	0,125	yes	no	Wild-type
V207-61	1,00	0,25	yes	no	Wild-type
V207-64	1,00	0,25	yes	no	Wild-type
V207-65	0,50	0,19	no	no	Wild-type
V207-62	1,00	0,38	yes	no	Wild-type
V207-59	0,50	0,25	yes	no	Wild-type
V207-58	0,50	0,38	yes	no	Wild-type
V208-07	1,00	0,094	no	no	Wild-type
V208-10	8,00	1,5	yes	yes	TR34/L98H
V208-12	2,00	0,25	yes	no	Wild-type
V208-08	0,50	0,25	no	no	Wild-type
V208-09	0,50	0,25	yes	no	Wild-type
V208-21	8,00	0,38	yes	yes	Wild-type
V208-22	1,00	0,25	yes	no	Wild-type
V208-46	1,00	0,38	no	no	Wild-type
V208-45	8,00	2,00	yes	yes	TR34/L98H
V208-38	2,00	0,25	yes	yes	Wild-type
V208-39	0,50	0,25	no	no	Wild-type
V208-40	1,00	0,25	no	no	Wild-type

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V208-44	1,00	0,38	yes	no	Wild-type
V208-50	1,00	0,25	yes	no	Wild-type
V208-52	2,00	0,25	yes	no	Wild-type
V208-54	>8,00	2,00	yes	yes	TR34/L98H
V208-60	1,00	0,19	no	no	Wild-type
V208-34	8,00	2,00	yes	yes	TR34/L98H
V208-35	1,00	0,25	no	no	Wild-type
V208-73	4,00	0,25	yes	yes	Wild-type
V208-62	2,00	0,25	yes	no	Wild-type
V208-63	2,00	0,38	yes	no	Wild-type
V208-64	2,00	0,25	yes	no	Wild-type
V208-70	> 8,00	>32,00	yes	yes	TR46/Y121F/T289A
V209-05	1,00	0,25	no	no	Wild-type
V209-08	> 8,00	2,00	yes	yes	TR34,L98H
V209-09	>8,00	>32,00	yes	yes	TR46/Y121F/T289A
V209-14	1,00	0,25	yes	no	Wild-type
V209-15	1,00	0,25	yes	no	Wild-type
V209-17	8,00	4,00	yes	yes	TR34,L98H
V209-31	1,00	0,25	yes	no	Wild-type
V209-32	>8,00	>32,00	yes	yes	TR46/Y121F/T289A
V209-34	8,00	1,50	yes	yes	TR34,L98H
V209-46	2,00	0,25	yes	no	Wild-type
V209-47	0,50	0,25	no	no	Wild-type
V209-49	8,00	4,00	yes	yes	TR46/Y121F/T289A
V209-50	>8,00	>32,00	yes	yes	Wild-type
V209-55	2,00	0,25	no	no	Wild-type
V209-56	8,00	2,00	yes	yes	TR34/L98H
V207-63	1,00	0,38	yes	no	Wild-type
V207-66	1,00	0,25	no	no	Wild-type
V202-77	8,00	0,25	yes	yes	Wild-type
V196-20	>8,00	>32,00	yes	yes	Wild-type
V213-09	1,00	0,25	no	no	Wild-type
V213-14	>8,00	>32,00	yes	yes	TR46/Y121F/T289A
V213-15	>8,00	1,50	yes	yes	TR34/L98H
V213-29	1,00	0,38	no	no	Wild-type
V213-30	2,00	0,38	yes	no	Wild-type
V212-57	8,00	2,00	yes	yes	TR34/L98H
V212-64	4,00	0,50	yes	yes	Wild-type
V212-68	8,00	4,00	yes	yes	TR34/L98H
V212-69	>8,00	>32.00	yes	yes	TR46/Y121F/T289A
V212-70	0,50	0,50	no	no	Wild-type
V212-71	4,00	4,00	yes	yes	TR34/L98H
V212-77	1,00	0,38	no	no	Wild-type
V211-16	>8,00	>32,00	yes	yes	TR46/Y121F/T289A
V211-17	1,00	0,38	no	no	Wild-type
V211-18	>8,00	>32,00	yes	yes	TR46/Y121F/T289A
V211-19	8,00	4,00	yes	yes	TR34/L98H

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V211-30	8,00	4,00	yes	yes	TR34/L98H
V211-31	1,00	0,38	no	no	Wild-type
V211-32	8,00	3,00	yes	yes	TR34/L98H
V211-33	8,00	4,00	yes	yes	TR34/L98H
V211-39	1,00	0,25	no	no	Wild-type
V211-40	>8,00	>32,00	yes	yes	TR46/Y121F/T289A
V211-54	1,00	0,25	no	no	Wild-type
V211-55	8,00	2,00	yes	yes	TR34/L98H
V211-63	0,50	0,25	no	no	Wild-type
V211-73	8,00	2,00	yes	yes	TR34/L98H
V211-74	8,00	4,00	yes	yes	TR34/L98H
V210-05	1,00	0,25	no	no	Wild-type
V210-06	>8,00	>32	yes	yes	TR46/Y121F/T289A
V210-08	8,00	4,00	yes	yes	TR34/L98H
V210-09	2,00	0,38	yes	no	Wild-type
V210-13	8,00	4,00	yes	yes	TR34/L98H
V210-14	>8,00	>32	yes	yes	TR34/L98H
V210-30	1,00	0,38	no	no	Wild-type
V210-32	1,00	0,25	no	no	Wild-type
V210-33	1,00	0,38	no	no	Wild-type
V210-34	1,00	0,25	no	no	Wild-type
V210-35	8,00	2,00	yes	yes	TR34/L98H
V210-36	1,00	0,25	no	no	Wild-type
V210-46	1,00	0,25	no	no	Wild-type
V210-48	8,00	8,00	yes	yes	TR34/L98H
V210-52	1,00	0,38	no	no	Wild-type
V210-57	1,00	0,25	no	no	Wild-type
V210-58	1,00	0,25	no	no	Wild-type
V210-64	>8,00	>32	yes	yes	Wild-type
V210-68	0,50	0,38	no	no	Wild-type
V209-58	0,50	0,38	no	no	Wild-type
V209-61	1,00	0,38	no	no	Wild-type
V209-62	0,50	0,125	no	no	Wild-type
V209-63	1,00	0,25	no	no	Wild-type
V209-64	8,00	4,00	yes	yes	TR34/L98H
V209-72	4,00	4,00	yes	yes	Wild-type
V200-03	2,00	0,38	yes	no	Wild-type
V200-04	2,00	0,38	yes	no	Wild-type
V200-05	2,00	0,38	yes	no	Wild-type
V200-08	2,00	0,38	yes	no	Wild-type
V200-09	8,00	4,00	yes	yes	TR34/L98H
V199-22	>8,00	8,00	yes	yes	TR34/L98H
V199-23	>8,00	>32,00	yes	yes	TR34/L98H
V199-25	>8,00	4,00	yes	yes	TR34/L98H
V199-36	1,00	0,25	no	no	Wild-type
V199-73	>8,00	>32,00	yes	yes	TR46/Y121F/T289A
V199-70	>8,00	1,50	yes	yes	Wild-type

continuação

V199-37	>8,00	>32,00	yes	yes	TR34/L98H
V199-28	>8,00	4,00	yes	yes	TR34/L98H
V199-31	1,00	0,25	no	no	Wild-type
V199-35	8,00	0,25	yes	yes	Wild-type
V199-32	2,00	0,38	yes	no	Wild-type
V199-71	>8,00	4	yes	yes	TR34/L98H
V199-24	>8,00	>32	yes	yes	TR34/L98H
V204-25	2,00	0,25	yes	no	Wild-type
V204-19	2,00	0,25	yes	no	Wild-type