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Tese

Aspergillus no contexto "*One Health*": Epidemiologia molecular, resistência a azóis e novos compostos com atividade antifúngica frente a isolados clínicos de humanos e de aves aquáticas

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Pelotas, 2020

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RESUMO

MELO, Aryse Martins. ***Aspergillus* no contexto *"One Health":* Epidemiologia molecular, resistência a azóis e novos compostos com atividade antifúngica frente a isolados clínicos de humanos e de aves aquáticas.** 2020. 206f. Tese (Doutorado em Microbiologia e Parasitologia) – Programa de Pós-Graduação em Microbiologia e Parasitologia, Instituto de Biologia, Universidade Federal de Pelotas, Pelotas, 2020.

O contexto "*One health"* considera a saúde em três pilares: saúde animal, saúde humana e saúde do ecossistema. Nesta abordagem, evidencia-se o papel das mudanças ambientais na emergência de doenças crônicas e infecciosas, e, como forte aliada no desenvolvimento de estratégias de prevenção de doenças. Fungos do gênero *Aspergillus* se enquadram substancialmente nesta abordagem, tendo em vista sua ubiquidade, bem como sua importância como potenciais patógenos animais e humanos. Além da resistência intrínseca apresentada por algumas espécies, a emergência da resistência a azóis adquirida por isolados de *Aspergillus fumigatus sensu stricto* é uma preocupação da comunidade científica a nível global. Este trabalho teve como objetivo a avaliação da epidemiologia molecular de isolados clínicos de *Aspergillus* de aves e humanos do sul do Rio Grande do Sul, Brasil, e avaliar perfil de suscetibilidade e mecanismos de resistência aos azóis. Como resultados, realizamos uma ampla revisão de literatura no contexto *One Health*, com enfoque no papel da aves como indicador ambiental e na dispersão de cepas de *Aspergillus;* descrevemos pela primeira vez a aspergilose em albatrozes durante a reabilitação; e descrevemos casos de aspergilose em aves aquáticas de vida livre. Também obtivemos importantes resultados *in vitro* sobre a atividade antifúngica do composto disseleneto de difenila sozinho e em combinação com antifúngicos clássicos. Em relação a pesquisa de resistências aos azóis em *A. fumigatus*, não foram detectadas mutações relacionadas ao contexto ambiental da emergência da resistência nessa espécie fúngica na região estudada no Brasil. Além disso, a pesquisa de diversidade genética demonstrou elevada diversidade nos isolados estudados, pelo que se conclui que a técnica utilizada é de grande valia para o acompanhamento clínico dos pacientes, além de permitir confirmar que o ambiente

de recuperação de animais é uma potencial fonte de infecção para aves (no caso estudado, pinguins) durante reabilitação. A detecção de um mesmo genótipo em estirpe de *A*. *fumigatus* isolado em um paciente humano e um pinguim de vida livre demonstraram a importância de uma abordagem *One Health* do *Aspergillus* e da aspergilose, uma vez que uma mesma estirpe possui potencial de colonização/infecção tanto em animais como em humanos. Por fim, como resultado da parceria internacional estabelecida durante o desenvolvimento desse trabalho, tivemos o primeiro reporte da mutação de resistência TR34/L98H em ambiente *indoor* em Portugal, e o resultado da vigilância em *Aspergillus* realizada por um laboratório de referência em Portugal, no qual encontrou-se grande prevalência de espécies crípticas de *Aspergillus* em isolados clínicos e ambientais, além de resistência pan-azol em estirpes de *A. fumigatus*, confirmando-se os mecanismos moleculares envolvidos com a resistência antifúngica no gene *cyp51A*. Os resultados obtidos neste trabalho mostram a importância da abordagem multidisciplinar do *Aspergillus* e da aspergilose, e a necessidade de ampliar a vigilância de *Aspergillus* no contexto *One Health*, com objetivo de entender melhor os mecanismos envolvidos na dispersão de estirpes, e na adoção de medidas mais efetivas de monitoramento da emergência da resistência, bem como para adoção de estratégias de controle do crescimento desse problema global.

Palavras-chave: *Aspergillus fumigatus*, identificação molecular; susceptibilidade antifúngica, genotipagem, microssatélites.

ABSTRACT

MELO, Aryse Martins. ***Aspergillus* in the *"One Health" context*: Molecular epidemiology, resistance to azoles and new compounds with antifungal activity against clinical isolates from humans and avian species.** 2020. 206f. Thesis (PhD degree in Microbiology and Parasitology) – Programa de Pós-Graduação em Microbiologia e Parasitologia, Instituto de Biologia, Universidade Federal de Pelotas, Pelotas, 2020.

The "One health" context considers health on three pillars: animal health, human health and ecosystem health. In this approach, the role of environmental changes in the emergence of chronic and infectious diseases is highlighted, and as a strong ally in the development of disease prevention strategies. Fungi of the *Aspergillus* genus fit substantially in this approach, in view of their ubiquity, as well as their importance as potential animal and human pathogens. In addition to the intrinsic resistance presented by some species, the emergence of azole resistance acquired by isolates of *Aspergillus fumigatus* sensu stricto is a concern of the scientific community at a global level. This study aimed to evaluate the molecular epidemiology of clinical isolates of *Aspergillus* from birds and humans from the south of Rio Grande do Sul, Brazil, and to evaluate the susceptibility profile and mechanisms of azole resistance. As a result, we carried out a wide literature review in the context of *One Health*, focusing on the role of birds as an environmental indicator and on the dispersion of *Aspergillus* strains; we first described aspergillosis in albatrosses during rehabilitation; and we describe cases of aspergillosis in free-living aquatic birds. We also obtained important in vitro results on the antifungal activity of the diphenyl diselenide compound alone and in combination with classic antifungals. Regarding the search for azole resistance in *A. fumigatus*, no mutations related to the environmental context of the emergence of resistance in this fungal species in the region studied in Brazil were detected. In addition, genetic diversity research has shown high diversity in the isolates studied, so it is concluded that the technique used is of great value for the clinical monitoring of patients, in addition to confirming that the animal recovery environment is a potential source of infection for birds (in this case, penguins) during rehabilitation. The detection

of the same genotype in *A. fumigatus* strain isolated in a human patient and a free-living penguin demonstrated the importance of an *Aspergillus* and aspergillosis *One Health* approach, since the same strain has both a potential for colonization / infection in animals as in humans. Finally, as a result of the international partnership established during the development of this work, we had the first report of the resistance mutation TR34 / L98H in an indoor environment in Portugal, and the result of surveillance in *Aspergillus* carried out by a reference laboratory in Portugal, in which a high prevalence of cryptic species of *Aspergillus* was found in clinical and environmental isolates, in addition to pan-azole resistance in *A. fumigatus* strains, confirming the molecular mechanisms involved with antifungal resistance in the cyp51A gene. The results obtained in this work show the importance of the multidisciplinary approach of *Aspergillus* and aspergillosis, and the need to expand the surveillance of *Aspergillus* in the context of *One Health*, in order to better understand the mechanisms involved in the dispersion of strains, and in the adoption of more effective measures to monitoring the emergence of resistance, as well as the adoption of strategies to control the growth of this global problem.

Keywords: cryptic species, *Aspergillus fumigatus*, *benA* sequencing; microdilution, birds, humans.

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1. INTRODUÇÃO

A aspergilose é uma doença que acomete humanos e diversos outros animais, apresentando distintas formas clínicas. Em humanos, a condição imunológica e/ou fator de risco do hospedeiro é determinante para a apresentação clínica, manifestando-se desde formas não invasivas, como a aspergilose broncopulmonar alérgica (ABPA), até a forma invasiva (CADENA; THOMPSON; PATTERSON, 2016). A aspergilose invasiva (AI) é a forma mais grave da doença, e apresenta uma taxa de mortalidade de acima 95% se não tratada e de cerca de 50% se tratada (DENNING; PENDLETON; PEARCE, 2015).

Em animais, a aspergilose tem destaque nas aves, tanto de produção, quanto silvestres e marinhas. Apesar de representar uma taxa de mortalidade proporcional de cerca de 50% em pinguins-de-Magalhães (*Spheniscus magellanicus*) em cativeiro, e densidade de incidência de 7,3 casos para cada 100 pinguins/mês durante a reabilitação (XAVIER et al., 2007; SILVA FILHO et al., 2015), pouco se sabe sobre a importância da doença nessas aves em vida livre, devido a escassez de estudos que abordem essa problemática (OBENDORF; MCCOLL, 1980; HOCKEN, 2000).

Essa doença é causada por fungos do gênero *Aspergillus*, um microrganismo filamentoso ubíquo e anemófilo, com formas anamórficas e teleomórficas conhecidas, com colônias de crescimento rápido, que variam morfologicamente de acordo com a espécie em questão. Microscopicamente, apresentam hifas hialinas septadas, bifurcadas em ângulo de 45º, e fiálides sustentadas numa vesícula, a dilatação terminal do conidióforo. As fiálides dão origem aos conídios, que são liberados em grande quantidade e facilmente dispersos no ambiente (RAPER; FENNELL, 1965).

Atualmente esse fungo pode ser classificado em seções. *Aspergillus* seção *Fumigati* é a principal seção envolvida na manifestação da aspergilose e abrange diversas espécies, denominadas espécies crípticas, as quais são fenotipicamente muito semelhantes a *Aspergillus fumigatus sensu stricto*. As espécies pertencentes a uma mesma seção podem ser diferenciadas por técnicas moleculares (BALAJEE et al., 2007; SAMSON et al., 2014). A classificação taxonômica de fungos desse gênero

baseada em biologia molecular é datada de 2005 em diante (BALAJEE et al., 2005). Estudos apontam para a espécie *Aspergillus fumigatus sensu stricto* como o principal agente etiológico de casos de infeção em humanos, porém *A. lentulus*, *A. udagawae, A. pseudofischeri e A. thermomutatus* também são descritas como espécies causadoras de aspergilose invasiva nesse hospedeiro (BARRS et al., 2013; ESCRIBANO et al., 2013; HOWARD, 2014; LAMOTH, 2016), inclusive no Brasil (NEGRI et al., 2014; BASTOS et al., 2015). Em aves, somente *A. fumigatus sensu stricto* foi identificado até o momento dentro da seção *Fumigati* (BURCO et al., 2012a; TALBOT et al., 2018; SABINO et al., 2019).

As espécies de *Aspergillus* podem apresentar variações nos genes codificadores da calmodulina, actina e ß-tubulina (BALAJEE et al., 2007), bem como diferenças com relação aos fatores de virulência, como por exemplo, a alta produção de gliotoxina por *A. fumigatus sensu stricto,* uma micotoxina que pode modular o sistema imune do hospedeiro, e causar apoptose de diversos tipos celulares (TELL, 2005; LARSEN et al., 2007; HOF KUPFAHL, 2009). Esta micotoxina é produzida pelos conídios quando começam a filamentar no tecido pulmonar, facilitando a penetração das hifas no tecido . Em adição, diferenças de suscetibilidade antifúngica entre espécies da secção *Fumigati* já foram descritas, sendo *A. lentulus* intrinsicamente resistente ao itraconazol, diferentemente de *A. fumigatus sensu stricto* considerado intrinsicamente sensível a este fármaco (ESCRIBANO et al., 2013; ALASTRUEY-IZQUIERDO; ALCAZAR-FUOLI; CUENCA-ESTRELLA, 2014; HOWARD, 2014).

Além da resistência intrínseca apresentada por algumas espécies crípticas, o aumento da resistência adquirida a azóis em isolados de *A. fumigatus sensu stricto*, tanto em cepas isoladas do ambiente, como em pacientes previamente tratados com esses fármacos vem sendo reportadas. Dados recentes demonstram que defensivos agrícolas a base de azóis, os quais são amplamente utilizados na produção agrícola, podem desempenhar importante função na seleção de cepas ambientais com resistência cruzada ao itraconazol (DUNNE et al., 2017; LAVERGNE et al., 2017; REN et al., 2017; CHOWDHARY; MEIS, 2018). Os principais mecanismos de resistência descritos até o momento para *Aspergillus* são mutações no gene *cyp51A*, que possui função vital na síntese do ergosterol, um dos principais componentes da membrana celular fúngica, e local de ação dos compostos azólicos (HOWARD et al., 2009; SHARPE et al., 2018; TSITSOPOULOU et al., 2018).

Estudos de epidemiologia molecular e resistência a azóis em isolados de *Aspergillus* ambientais e clínicos de humanos e de aves no Brasil são escassos, e apesar de os poucos estudos existentes não demonstrarem ainda uma preocupação com a resistência antifúngica no nosso país, a vigilância nesse tema deve ser constante (NEGRI et al., 2014, 2017; SPANAMBERG et al., 2016; GONÇALVES, 2017; DENARDI et al., 2018). Considerando que nosso grupo de pesquisa está geograficamente localizado no estado do Rio Grande do Sul, o qual ocupa posição estratégica para a oferta nacional de diversos produtos agrícolas (arroz, trigo, aveia) e está entre os principais exportadores de fumo, soja e arroz (FEE-RS, 2019), a confirmação da influência de fungicidas agrícolas na seleção de isolados ambientais de *Aspergillus* resistentes aos azóis (ALVAREZ-MORENO et al., 2017; DUNNE et al., 2017; REN et al., 2017), associada a importância da doença na região evidenciada por estudos prévios (XAVIER et al., 2007; SILVA FILHO et al., 2015), enfatiza a necessidade de uma abordagem interdisciplinar.

Assim, justifica-se a abordagem "*One health"* deste estudo, envolvendo identificação molecular das principais espécies de *Aspergillus* de isolados clínicos de aves e de humanos, e dados sobre a taxa de resistência a antifúngicos e seus mecanismos de resistência aos azóis.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar a epidemiologia molecular e o perfil de resistência a azóis bem como os mecanismos de resistência envolvidos em estirpes resistentes, de isolados clínicos de *Aspergillus* de aves e de humanos do sul do Rio Grande do Sul, Brasil, bem como avaliar o potencial antifúngico do disseleneto de difenila $(PhSe)_2$ frente a isolados clínicos de *Aspergillus.*

2.2. Objetivos específicos

- Identificar a nível de espécie os isolados clínicos de *Aspergillus* provenientes de casos de aspergilose em aves aquáticas de cativeiro e de vida livre;
- Avaliar a diversidade genética dos isolados clínicos de *A. fumigatus* de humanos e aves;
- Avaliar o perfil de susceptibilidade de *A. fumigatus* ao itraconazol, ao voriconazol e ao posaconazol, e o papel da profilaxia no desenvolvimento de uma resistência adquirida;
- Detectar mutações no gene *cyp51A* (e seu promotor) e sua relação com mecanismos de resistência aos azóis nos isolados clínicos de *Aspergillus fumigatus* sensu strico com resistência fenotípica aos azóis testados.
- Determinar a atividade antifúngica da molécula disseleneto de difenila $(PhSe)_2$ frente a isolados de *Aspergillus* spp.

3. REVISÃO DE LITERATURA

3.1. Aspergilose

A aspergilose é uma enfermidade fúngica causada por fungos do gênero *Aspergillus* que pode acometer humanos, cães, gatos, cavalos, mamíferos marinhos, aves selvagens e domésticas, e até mesmo corais (SEYEDMOUSAVI et al., 2015). A infecção geralmente ocorre via inalação dos conídios presentes no ambiente, e o trato respiratório inferior é o principal sítio anatômico da doença; entretanto outras formas como cutânea, ocular, sinonasal e cerebral são descritas em hospedeiros de diversas espécies (MARR; PATTERSON; DENNING, 2002; SEYEDMOUSAVI et al., 2015). Até ao momento, a aspergilose é considerada uma doença não contagiosa, porém estudos recentes levantam o alerta sobre a possibilidade de transmissão desse fungo entre pacientes hospitalizados (LEMAIRE et al., 2018; ENGEL et al., 2019a;), aumentando o alerta sobre necessidades de mais estudos e vigilância quanto ao papel do ambiente na doença.

3.1.1. Aspergilose em humanos

Estima-se que a aspergilose acometa mais de 15 milhões de pessoas no mundo, e seja responsável pela morte de mais de um milhão de pessoas por ano (GAFFI, 2019). No Brasil, um levantamento de dados no período de um ano (2011) estimou uma prevalência 8.664 casos de aspergilose invasiva (AI), 12.032 casos de aspergilose pulmonar crônica (APC) pós tuberculose (TB), e 390.486 casos de aspergilose broncopulmonar alérgica (ABPA), e 599.283 casos de asma grave com sensibilização fúngica, totalizando 1.010.465 casos de aspergilose (GIACOMAZZI et al., 2016).

Seguindo a tendência de diversas outras doenças infecciosas, o número de casos de aspergilose em humanos parece ter sofrido considerável aumento ao passar das décadas. O trabalho de Zilberberg e colaboradores demonstra que em comparação a um estudo da década de 90, ambos nos Estados Unidos, a incidência de aspergilose invasiva

em humanos quase quadruplicou, passando de 3 para 10 casos a cada 10.000 atendimentos hospitalares (ZILBERBERG et al., 2018).

As manifestações clínicas da aspergilose em humanos são classificadas de acordo com a extensão da colonização micelial ou invasão tecidual e influenciadas pela capacidade de resposta imune do hospedeiro. Reações alérgicas desencadeadas por antígenos dos conídios de *Aspergillus* inalados em pacientes com asma e sinusite podem ocorrer e, nestes casos, a simples redução da exposição do paciente aos ambientes massivamente contaminados geralmente resulta em melhora do quadro clínico. Por outro lado, as demais manifestações requerem maior atenção (MARR; PATTERSON; DENNING, 2002).

A ABPA ocorre na maioria dos casos em pacientes que possuem asma atópica (prevalência aproximada de 2%) ou fibrose cística (FC) (prevalência variando entre 7 e 15%) (LATGÉ, 1999; MARR; PATTERSON; DENNING, 2002; ENGEL et al., 2019b). Estudos sugerem que nesses pacientes, o *Aspergillus* pode formar biofilme nas vias aéreas dos pacientes com FC, com consequente aumento de falha terapêutica (KAUR; SINGH, 2015), sendo *A. fumigatus sensu stricto* o principal agente causal em pacientes com FC (SABINO et al., 2015).

O aspergiloma ou bola fúngica ocorre em cavidades pulmonares preexistentes, em pacientes com histórico de tuberculose ou outras desordens pulmonares. A prevalência da doença em pacientes após tratamento para tuberculose foi de 34% em estudo realizado recentemente (NAGEEB; NAGMOTI, 2019). Na mesma linha, um estudo realizado em pacientes da Uganda estima que 1 a cada 15 pacientes tratados para TB desenvolva aspergilose pulmonar crônica (APC), baseado em um acompanhamento de até 10 anos após o fim do tratamento para TB. Considerando que 1 a cada 4 pacientes com TB possuem lesão cicatricial cavitária após a doença, caracterizando este como o principal grupo de risco para APC dentre os pacientes tratados para TB e 7.7 milhões de pessoas sobrevivem a esta doença anualmente, o estudo estima que entre 112.000 e 160.000 pacientes por ano, no mundo, podem desenvolver a APC (PAGE et al., 2019).

A AI aguda possui altas taxas de mortalidade, podendo ser maior 95% se não tratada (DENNING; PENDLETON; PEARCE, 2015) e acomete principalmente pacientes imunocomprometidos, com neutropenia prolongada, especialmente transplantados de

órgãos sólidos e de células-tronco, pacientes hematológicos, pacientes HIV positivo, e em vigência de terapias imunossupressoras (BASSETTI et al., 2015).

Por fim, a aspergilose pulmonar crônica necrosante (APCN) é outra manifestação da AI, também conhecida como aspergilose invasiva subaguda. Fato que vem sendo observado é a relação dessa manifestação em pacientes com diabetes mellitus, alcoólatras, doença granulomatosa crônica, pacientes pós cirúrgicos, pacientes HIV+, pacientes em estado crítico de unidades de tratamento intensivo (UTI), demonstrando a importância de maior investigação da doença, e seus fatores de risco (STEVENS; MELIKIAN, 2011; BASSETTI et al., 2015). Um estudo conduzido por Meersseman e colaboradores relatou uma incidência de 5,8% e taxa de mortalidade de 50% atribuídos a AI em pacientes de UTI (MEERSSEMAN et al., 2007). Ainda em pacientes de UTI, preocupa a ocorrência de aspergilose associada a pacientes com síndromes respiratórias aguda grave causadas por *Influenza* (KU et al., 2017; SHAH et al., 2018; SCHWARTZ et al., 2020) e mais recentemente, associadas ao vírus SARS-CoV-2, causador da COVID-19 (ALANIO et al., 2020; BLAIZE et al., 2020; KOEHLER et al., 2020).

A definição de caso da aspergilose, categorizada como possível, provável, ou provado, segue diferentes diretrizes de acordo com o grupo de risco analisado e a manifestação clínica correspondente. A definição de CPA, por exemplo é sugerida considerando fatores como características clínicas, critérios radiológicos e critérios microbiológicos (Figura 1) (DENNING et al., 2018). Na mesma linha, a definição de caso de traqueobronquite por *Aspergillus*, bem como de aspergilose invasiva associada a influenza (IAPA) em pacientes de Unidades de tratamento Intensivo (UTI), foi proposto mais recentemente, uma vez que os critérios previamente definidos para definição de caso de AI, não incluía diversos fatores distintos, nos quais estes pacientes se encaixavam (Figura 2) (VERWEIJ et al., 2020). Apesar de não se encaixar totalmente no algoritmo clínico, este mesmo protocolo para determinação de IAPA vem sendo atualmente aplicado para a definição de caso de aspergilose invasiva associada ao coronavirus (CAPA), até que um consenso de especialista cheguem a um novo protocolo de definição de caso que se encaixe melhor ao quadro destes pacientes (MOHAMED; ROGERS; TALENTO, 2020; VAN ARKEL et al., 2020).

Table 3. Final consensus definition of CPA in resource-constrained settings, determined by Global Action Fund for Fungal Infections international expert panel*

Required criteria†	Details
Symptoms for ≥3 mo	Hemoptysis and/or persistent cough, and/or weight loss; other symptoms are common, but not required, notably fatigue, chest pain, dyspnea, and sputum production
Radiologic features	Progressive cavitation on chest imaging and/or intracavitary fungal ball and/or pleural thickening or pericavitary fibrosis or infiltrates all adjacent to cavities
Microbiological evidence of *Aspergillus* infection	Positive *Aspergillus*-specific IgG and/or sputum microscopy results showing hyphae consistent with *Aspergillus* and/or *Aspergillus* growth on ≥2 sputum or other respiratory samples
Mycobacterial infection ruled out with smear, GeneXpert, and/or mycobacterial culture‡	It is possible for mycobacterial infection and CPA to be present concurrently, but this diagnosis requires characteristic radiological findings on CT scan that are not present with pulmonary TB including pleural thickening, a fungal ball or other intracavitary material, or marked pericavitary infiltrates in addition to a positive *Aspergillus* IgG antibody test

*CPA, chronic pulmonary aspergillosis; CT, computed tomography; TB, tuberculosis.
†All 4 criteria are required.
‡GeneXpert (http://www.cepheid.com/us/cepheid-solutions/systems/genexpert-systems/genexpert-iv).

Figura 1 Critérios propostos para a definição de Aspergilose Pulmonar Crônica (APC), determinado pelo concelho internacional do Global Action Fund for Fungal Infections (GAFFI). Fonte: Denning et al., 2018.

Table 1 Proposed case definition for IAPA in ICU patients

Entry criteria: influenza-like illness + positive influenza PCR or antigen + temporally relationship		
	***Aspergillus* tracheobronchitis**	**IAPA in patients without documented *Aspergillus* tracheobronchitis**
Proven	Biopsy or brush specimen of airway plaque, pseudomembrane or ulcer showing hyphal elements and *Aspergillus* growth on culture or positive *Aspergillus* PCR in tissue	Lung biopsy showing invasive fungal elements and *Aspergillus* growth on culture or positive *Aspergillus* PCR in tissue
Probable	Airway plaque, pseudomembrane or ulcer and at least one of the following: Serum GM index > 0.5 *or* BAL GM index ≥ 1.0 *or* Positive BAL culture *or* Positive tracheal aspirate culture *or* Positive sputum culture *or* Hyphae consistent with *Aspergillus*	**A**: Pulmonary infiltrate and at least one of the following: Serum GM index > 0.5 *or* BAL GM index ≥ 1.0 *or* Positive BAL culture ***OR*** **B**: Cavitating infiltrate (not attributed to another cause) and at least one of the following: Positive sputum culture *or* Positive tracheal aspirate culture

Figura 2 Critérios propostos para definição de caso de aspergilose invasiva associada a pacientes com Influenza (IAPA). Fonte: Verweij et al., 2020.

Por fim, as diretrizes da *European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group* (EORTC/MSG) (PETER DONNELLY et al., 2020), estabelece um algoritmo para definição de caso de infecções fúngicas

invasivas, incluindo a AI, a qual se enquadra melhor para pacientes imunossuprimidos e neutropênicos. O diagnóstico definitivo (AI comprovada) é realizado a partir de biópsia tecidual, entretanto, muitos pacientes apresentam plaquetopenia, o que impede a realização da coleta; em adição, a taxa de isolamento do fungo em lavado bronco alveolar e escarro é considerada baixa. Dessa forma, a busca por métodos diagnósticos com maior sensibilidade e especificidade é necessária (CADENA; THOMPSON; PATTERSON, 2016), uma vez que o diagnóstico precoce é decisivo para maior chance de sobrevivência do paciente (VON EIFF et al., 1995). Dentre métodos diagnósticos da AI em humanos destacam-se a detecção da galactomanana (AQUINO; GOLDANI; PASQUALOTTO, 2007); e, mais recentemente, os testes moleculares comerciais, a partir de reação em cadeia da polimerase (qPCR) (AsperGenius, PathoNostics B.V., Netherlands), com opção não só de detecção do *Aspergillus*, mas também das principais mutações de resistência a azóis conhecidas até o momento, nomeadamente aquelas relacionadas à resistência ambiental, com sensibilidade de 78,6% e especificidade de 100% (WHITE; POSSO; BARNES, 2015; CADENA; THOMPSON; PATTERSON, 2016).

Os triazóis são os fármacos mais indicados para o tratamento da AI. O itraconazol foi considerado por um longo período o fármaco de escolha como terapia antifúngica, e atualmente, o voriconazol, é o principal fármaco indicado para a AI (LAT; THOMPSON, 2011). Além disso, isavuconazol e posaconazol são fármacos azóis de última geração registrados mais recentemente (BASSETTI et al., 2015). Outras opções menos utilizadas são a anfotericina B, principalmente na formulação lipossomal (LAmB), que, ainda assim possui maior toxicidade quando comparada aos azóis, e deve ser administrada com muita cautela, além de algumas espécies, como *A. terreus,* serem intrinsicamente resistentes a este fármaco (BLUM et al., 2008; NEWTON et al., 2016); e as equinocandinas, que possuem como principal alvo a síntese de 1-3 ß-D-glucana, um açúcar essencial da parede celular fúngica, a qual também deve ser administrada com muita cautela, desde que possui uma ação paradoxal, e o aumento das suas concentrações tendem a estimular o crescimento fúngico ao invés de inibí-lo (ARUANNO; GLAMPEDAKIS; LAMOTH, 2019).

Dentro da seção *Fumigati*, a principal seção envolvida nas infeções fúngicas em humanos, *A. fumigatus sensu stricto* se caracteriza como o principal agente etiológico da

aspergilose. Em adição, casos de aspergilose por *A. lentulus*, *A. thermomutatus,* e espécies do complexo *viridinutans* como *A. pseudofischeri e A. felis* já estão descritos tanto em pacientes CPA como de AI (BARRS et al., 2013; ESCRIBANO et al., 2013; HOWARD, 2014; LAMOTH, 2016).

No Brasil, espécies crípticas de *Aspergillus* já foram descritas causando aspergilose invasiva em humanos, como espécies pertencentes às seções *Flavi*, *Nigri* e *Nidillantes* (NEGRI et al., 2014), além das pertencentes a seção *Fumigati* como *A. thermomutatus* (NEGRI et al., 2014) e *A. lentulus* (BASTOS et al., 2015). No entanto, poucos estudos no país realizam a identificação molecular das espécies de *Aspergillus*, dessa forma, há uma grande carência de conhecimento sobre a prevalência das espécies responsáveis pela aspergilose em humanos e animais.

3.1.2. Aspergilose em animais

Na medicina veterinária, o panorama dessa micose é ainda mais amplo. A aspergilose acomete desde invertebrados até os mais diversos grupos vertebrados, incluindo animais ectotérmicos e, em maior escala, animais endotérmicos, tanto em ambientes aquáticos, apesar da característica hidrofóbica de *Aspergillus* spp., como em ambientes terrestres (SEYEDMOUSAVI et al., 2015).

Em invertebrados, a aspergilose ocasionada especialmente por *Aspergillus sydowwi*, seção *Versicolores* (recentemente integrada na seção *Nidulantes*), é uma doença que possui alta prevalência em corais do Caribe (*Gorgonia* spp.), variando entre 8 e 60% (KIM et al., 2000; JOLLES et al., 2002; KIM; HARVELL, 2004). Abelhas também são acometidas por esta doença fúngica, sendo os isolados de *Aspergillus* pertencentes à seção *Flavi*, seguida da seção *Fumigati* os principais agentes etiológicos. Tanto as larvas, a partir do contato fúngico com a cutícula do inseto, quanto adultos podem ser infectados, os últimos a partir da ingestão do conídio. O fungo é responsável pela petrificação das larvas, o que denomina a doença de “cria de pedra”. Apesar de ser considerada uma doença rara, em alguns países ela é de notificação obrigatória (SEYEDMOUSAVI et al., 2015).

Em peixes, os danos por *Aspergillus* spp estão associados à aquicultura, na forma de micotoxicoses. Em anfíbios, até onde se sabe, a doença nunca foi relatada. Apesar de já haver registros de aspergilose invasiva em algumas espécies de répteis como

serpentes em cativeiro (*Eunectes murinus*), as manifestações cutâneas, principalmente após lesões traumáticas, e as oftálmicas são as mais relatadas, tanto em serpentes, como em tartarugas (MILLER et al., 2004; SEYEDMOUSAVI et al., 2015).

Em mamíferos, poucos trabalhos epidemiológicos são descritos, sendo os relatos de caso mais comuns. Em ruminantes, a infecção por *Aspergillus* spp. pode ocasionar mastite, gastroenterite, pneumonia fúngica e alterações reprodutivas levando ao aborto (DEREJE et al., 2018). Em cavalos, o acometimento da bolsa gutural é o mais frequente, podendo levar à óbito devido a sua ruptura (LUDWIG et al., 2005). Em gatos, a forma sinonasal e sinoorbital são as mais observadas (BARRS; TALBOT, 2014), e em cães, a forma sinonasal é frequentemente reportada (STEWART; BIANCO, 2017). Além disso, pneumonia fúngica e encefalite micótica já foram reportados em mamíferos marinhos, principalmente cetáceos (SEYEDMOUSAVI et al., 2015).

Embora descrita em inúmeras espécies de vertebrados, as aves caracterizam-se como um dos principais grupos de risco para o desenvolvimento da doença. Nesse grupo em particular, a aspergilose é responsável por consideráveis prejuízos econômicos e ecológicos, justificando-se assim, os diversos estudos relacionados a esta doença que são conduzidos globalmente.

3.1.3. Aspergilose em aves

A aspergilose é uma das maiores causas de morbidade e mortalidade em aves, ocasionando prejuízo econômico e ecológico, sendo responsável por grandes perdas em produção aviária e por baixas em plantel de zoológicos e animais selvagens durante a reabilitação (XAVIER et al., 2007; BEERNAERT et al., 2010; ARNÉ et al., 2011).

As aves são especialmente suscetíveis a infecção por *Aspergillus* spp., devido a características anatômicas e fisiológicas. Dentre as anatômicas destaca-se a ausência de epiglote, que se caracteriza como uma barreira mecânica para entrada de partículas exógenas no trato respiratório; ausência de diafragma, impossibilitando o reflexo de tosse; ausência do epitélio pseudoestratificado ciliado na traquéia, dificultando a eliminação de propágulos inalados; e presença de sacos aéreos, que se caracterizam por estruturas anatômicas com presença de oxigenação e pouca vascularização, propiciando um ambiente ideal para crescimento fúngico, permitindo inclusive a formação

de estruturas reprodutivas, característica peculiar da aspergilose nesse grupo taxonômico. Quanto as características fisiológicas, destacam-se a ausência de macrófagos, responsáveis pela fagocitose dos conídios; a presença de heterófilos, ao invés de neutrófilos, com mecanismo de ação menos eficiente para eliminação das hifas no tecido; e a especial sensibilidade das aves à gliotoxina, um metabólito secundário liberado pelo *Aspergillus*, com propriedades imunossupressoras, incidindo: I) na redução da função mucociliar, promovendo maior chance do fungo alcançar as células epiteliais do hospedeiro e causar danos, iniciando a invasão tecidual, II) na inibição da fagocitose e na apoptose de células imunes, como macrófagos e linfócitos e III) na necrose tecidual, promovendo um ambiente rico em nutrientes para o crescimento fúngico (TELL, 2005; ARNÉ et al., 2011).

Aspergillus seção *Fumigati* é responsável por até 90% das mortes de aves por aspergilose (TELL, 2005; BEERNAERT et al., 2010; SILVA FILHO et al., 2015). Acredita-se que isso se deva às características de virulência dessa seção, principalmente de *A. fumigatus sensu stricto*, como capacidade de crescer em temperaturas mais elevadas, como a temperatura corporal das aves (38-45°C), o tamanho reduzido dos conídios, em comparação com outras seções do gênero, facilitando a penetração em trato respiratório inferior, e a grande produção de gliotoxina por esta espécie fúngica (HOF; KUPFAHL, 2009; BEERNAERT et al., 2010; FRISVAD; LARSEN, 2016).

Os sinais clínicos da aspergilose em aves são inespecíficos ou inexistentes, manifestando-se em muitos casos, no estágio final da doença. Dessa forma, o diagnóstico padrão-ouro é determinado por achados macroscópicos durante a necropsia, com isolamento do fungo em cultura e confirmação da invasão tecidual por histopatologia (BEERNAERT et al., 2010). A busca pelo diagnóstico precoce é essencial para a determinação de tratamentos. Exames de imagem, monitoramento sorológico, como a imunodifusão radial dupla; e detecção de galactomanana e de (1-3) ß-D glucana são algumas das ferramentas testadas para este propósito (CRAY; WATSON; ARHEART, 2009; BURCO et al., 2012b; CABANA et al., 2015, 2018).

3.1.3.1. Aspergilose em aves de produção

No setor aviário, a aspergilose é reportada em diferentes tipos de criações, como de frangos, perus, gansos, patos, pombos, emas e avestruzes, e aves jovens são as mais acometidas. Nessas criações, é comum observar surtos de aspergilose, com taxas de mortalidade variando entre 4,5 e 90% (ARNÉ et al., 2011). As perdas econômicas relacionadas a este grupo são referentes a condenação de carcaças em abatedouros devido a aerosaculite e pneumonia fúngica, redução da taxa de crescimento dos animais e mortalidade atribuída (SEYEDMOUSAVI et al., 2015).

Dentre os fatores de risco para aves domésticas, falta de controle da umidade do ambiente, acúmulo de matéria orgânica e uso de camas de materiais ricos em matéria orgânica são citados. Por exemplo, em uma criação de frangos de corte, dois lotes de animais de 15 e 30 dias, mantidos em locais com camas de casca de girassóis, apresentou uma mortalidade de 25%. Os demais lotes, que eram mantidos sobre camas com casca de arroz, não apresentaram perdas por aspergilose (ZAFRA et al., 2008).

A infecção por *Aspergillus* é identificada em aves aparentemente saudáveis, e sua detecção ocorre muitas vezes somente no momento da inspeção de carnes em abatedouros. No Brasil, em um estudo com 56 lotes de aves no estado do Rio Grande do Sul, 9.5% das aves abatidas tiveram resultado positivo para *Aspergillus* em cultura de amostras de pulmão. *A. fumigatus sensu stricto* foi a única espécie identificada, e a partir de estudos de microssatélites, mais de 40 genótipos diferentes foram encontrados, mostrando a grande diversidade de cepas infectantes nos lotes estudados (SPANAMBERG et al., 2016).

3.1.3.2. Aspergilose em aves selvagens

No enfoque ecológico, a aspergilose também é uma preocupação, tendo alta mortalidade em aves selvagens em cativeiro, como zoológicos e centros de recuperação (NAKEEB; BABUS; CLIFTON, 1981; XAVIER et al., 2007; BEERNAERT et al., 2010). No contexto de centros de recuperação, a aspergilose é considerada uma doença fúngica limitante para pinguins em reabilitação (XAVIER et al., 2007) representando uma taxa de mortalidade proporcional de cerca de 50% em pinguins-de-Magalhães (*Spheniscus magellanicus*) em cativeiro, e densidade de incidência de 7,3 casos para cada 100

pinguins/mês durante a reabilitação (SILVA FILHO et al., 2015). Por outro lado, pouco se sabe sobre a importância da doença em pinguins em vida livre, devido a escassez de estudos que abordem essa problemática (OBENDORF; MCCOLL, 1980; HOCKEN, 2000).

Apesar de fatores predisponentes para a aspergilose serem descritos em pinguins de vida livre, como a disponibilidade de conídios no ambiente, dieta inadequada (BEERNAERT et al., 2010), migração (YOUNG; CORNISH; LITTLE, 1998), e até mesmo estresse decorrente da atividade reprodutiva (JONES; OROSZ, 2000), ainda não se sabe a relevância dessa micose em aves em vida livre. Um estudo realizado em uma colônia de reprodução de pinguim-de-Magalhães no Chile, demonstrou uma taxa de 14% de mortalidade atribuída a aspergilose, instigando a realização de mais estudos que agreguem dados sobre a real importância dessa doença em pinguins também em vida livre (GODOY et al., 2013).

Um estudo envolvendo filhotes de cegonhas brancas (*Ciconia ciconia*) de vida livre, determinou 44,6% (45 casos) de mortalidade atribuída a pneumonia fúngica, dentre estes, *A. fumigatus sensu stricto* foi identificado como agente causal em 22 casos (OLIAS et al., 2010). Outro estudo realizado pelo mesmo grupo, encontrou grande diversidade genotípica em isolados clínicos de cegonhas brancas e em isolados ambientais, e nenhuma diferença significativa na virulência das cepas isoladas, concluindo que a maioria das cepas de *A. fumigatus sensu stricto* presentes no ambiente possuem potencial para infectar hospedeiros vertebrados (OLIAS et al., 2011). Nesta mesma linha, estudo realizado em um centro de recuperação de aves marinhas encontrou genótipos relacionados entre isolados clínicos e isolados do ambiente onde as aves eram mantidas, sugerindo que o ambiente de recuperação é uma potencial fonte de infecção das aves mantidas nestes locais (BURCO et al., 2012a).

Estudos envolvendo identificação molecular dos isolados em aves silvestres são limitados e, até o momento, realizados com aves de cativeiro, nos quais *A. fumigatus stricto sensu* foi a única espécie identificada dentro da seção *Fumigati*, a principal responsável pela doença nesse grupo, seguida pela seção *Flavi* (BURCO et al., 2012a; TALBOT et al., 2018; SABINO et al., 2019). Em relação a aspergilose em pinguins-de-Magalhães, não há dados na literatura sobre a identificação molecular dos isolados

clínicos, implicando em uma lacuna no conhecimento sobre a epidemiologia dessa doença nesses animais.

3.2. Aspergillus

Aspergillus é um gênero ubíquo saprófito, descrito pela primeira vez pelo botânico e padre Antônio Micheli, em 1729, sendo encontrado frequentemente em plantas em decomposição, desempenhando importante papel nesse processo e contribuindo para a reciclagem de carbono e nitrogênio (FANG; LATGÉ, 2018). Fungos desse gênero são utilizados amplamente na indústria para obtenção de enzimas, como as amilases; aditivos químicos, como o ácido cítrico; produção de molhos de soja; processos de biorremediação, entre outros (BENNETT, 2010).

É um fungo de fácil dispersão aérea devido ao tamanho reduzido de seus conídios, e produz ascósporos que permitem a sobrevivência do fungo por um longo período em ambientes desfavoráveis. Macroscopicamente, as colônias de *Aspergillus* variam quanto a topografia, textura e coloração. *Aspergillus* seção *Fumigati* possui crescimento rápido, colônia de reverso creme e superfície branca no início, passando a verde-azulada quando jovem e acinzentada com o passar do tempo, com textura aveludada a pulverulenta. Microscopicamente, possui hifas hialinas, septadas em ângulo agudo, conidióforos pequenos e organizados, e vesículas piriformes, onde se inserem as células conidiogênicas ou fiálides unisseriadas na parte superio da vesícula, as quais dão origem aos propágulos infectantes (conídios) (RAPER; FENNELL, 1965).

Existem diferenças significativas entre as espécies da seção *Fumigati*, e características típicas de *A. fumigatus sensu stricto* como o crescimento rápido, tolerância a amplitude térmica (crescendo entre 15 e 55ºC, podendo tolerar até 70º C), tolerância a amplitude de pH, baixa exigência nutricional, e produção de metabólitos secundários conferem a esta espécie vantagens para desempenhar um papel patogênico com sucesso (RHODES, 2006). Dentre os metabólitos produzidos por *A. fumigatus sensu stricto*, a gliotoxina é um dos mais conhecidos, com extensos estudos demonstrando a sua importância na patogenicidade do fungo. Tal como já referido anteriormente, este metabólito modula a resposta imune dos hospedeiros, e leva à apoptose de diferentes tipos celulares. A toxicidade desse metabólito tem sido atribuída a ponte disulfeto que

essa molécula apresenta, a qual tem papel crucial na atividade biológica da célula, atuando como reguladores das reações de oxirredução celulares, com deslocamentos entre os estados de oxidação (disulfeto) e redução (tiol) do enxofre. Ao mesmo tempo que essas reações são importantes para a detoxificação da célula fúngica, possuem caráter tóxico para as células dos seus hospedeiros, em especial as aves (CHAI; WARING, 2004; SCHARF et al., 2012).

3.2.1. Taxonomia

O gênero *Aspergillus* está incluso dentro da família Aspergillaceae, ordem Eurotiales, classe Eurotiomycetes, filo Ascomycota, no reino Fungi, domínio Eucariota (FANG; LATGÉ, 2018). O primeiro sequenciamento do genoma total de *Aspergillus* foi publicado em 2005, a partir da cepa Af 293 (NIERMAN et al., 2005). A partir de então, utilizando-se de ferramentas moleculares, descobriu-se que a identificação a nível de espécie dentro desse gênero não é mais possível utilizando-se somente métodos fenotípicos, sendo necessário empregar métodos moleculares para esta finalidade (BALAJEE et al., 2005). Isso porque, o que acreditava-se serem espécies distintas a partir da identificação morfológica clássica, hoje em dia entendem-se como seções, dentro das quais, existem inúmeras espécies, denominadas espécies crípticas, que vão sendo descobertas à medida que trabalhos nessa linha são desenvolvidos (SAMSON et al., 2007; BARRS et al., 2013; HUBKA et al., 2013; LAMOTH, 2016).

Com o advento da biologia molecular, outra questão taxonômica importante foi modificada na nomenclatura dos fungos do gênero *Aspergillus*. Além das questões das espécies crípticas já citadas, em 2011 a associação internacional para taxonomia de plantas adotou a abordagem "*One fungus, One name*", na qual a fase anamórfica e telemórfica do mesmo fungo possuem a mesma nomenclatura de gênero (SAMSON et al., 2014; TALBOT; BARRS, 2018).

Ainda, dentro de uma seção, existem os complexos, que são espécies mais intimamente relacionadas dentro de uma seção. Como exemplo, existe o complexo *viridinutans*, que possui 10 espécies até o momento, e está incluído dentro da seção *Fumigati*, que possui 63 espécies, das quais 19 são comprovadamente potenciais patogênicas (LAMOTH, 2016; TALBOT; BARRS, 2018). Dentro deste complexo,

Aspergillus felis é uma espécie de destaque, sendo inicialmente identificada como agente causal da aspergilose em gatos, e atualmente já descrita como agente causal da aspergilose também em humanos (BARRS et al., 2013; BARRS; TALBOT, 2014; LAMOTH, 2016).

A. lentulus foi a primeira espécie críptica descrita dentro da seção *Fumigati*. Dentre as diferenças observadas entre essa espécie e *A. fumigatus sensu stricto* cita-se a esporulação lenta, incapacidade de crescer a 48ºC, e resistência intrínseca ao itraconazol (BALAJEE et al., 2005; ALASTRUEY-IZQUIERDO; ALCAZAR-FUOLI; CUENCA-ESTRELLA, 2014). Além de *A. lentulus*, *A. udagawae, A. viridinutans, A. thermomutatus, A. novofumigatus* e *A. hiratsukae* são as espécies crípitcas mais frequentemente relatadas na clínica médica. Essas espécies crípticas, quando comparadas com *A. fumigatus sensu stricto*, parecem possuir patogenicidade limitada, uma vez que possuem termotolerância reduzida e menor produção de micotoxinas, como a gliotoxina. Por outro lado, em sua maioria, essas espécies possuem resistência intrínseca ao itraconazol, o que as torna refratárias ao tratamento de eleição para aspergilose (LAMOTH, 2016).

Para a identificação molecular de *Aspergillus*, a utilização da região espaçadora interna transcrita (ITS) é uma metodologia que permite a confirmação do gênero e seção, porém sua utilização não permite a identificação dos isolados em nível de espécie. Dessa forma, para estudos filogenéticos, dados combinados de regiões de genes codificadores da beta-tubulina (*benA*), calmodulina (*calM*) e actina (*rodA*) são utilizados. Entretanto, para a clínica médica, métodos mais rápidos são preferidos, e, dessa forma, a utilização do *benA* ou *caM* são indicadas para a identificação de *Aspergillus* ao nível da espécie.

3.2.2. Resistência

Além da resistência intrínseca, fator já conhecido em diferentes espécies ocultas de *Aspergillus* da seção *Fumigati*, o aumento na taxa de resistência a azóis por isolados de *A. fumigatus stricto sensu* tem sido documentado (HOWARD; ARENDRUP, 2011; REICHERT-LIMA et al., 2018) já sendo caracterizado como importante linha de investigação a ser conduzida pela comunidade científica globalmente (PERLIN; SHOR; ZHAO, 2015). Essa resistência é observada tanto em isolados ambientais, como em isolados clínicos de pacientes previamente tratados com esses fármacos, bem como de

aves com aspergilose (HOWARD et al., 2009; SHARPE et al., 2018; TSITSOPOULOU et al., 2018).

Para determinação de estirpes sensíveis ou resistentes, é importante que valores de referência de concentrações inibitórias mínimas sejam adotados, de forma a padronizar esse conceito globalmente. Nesse sentido, o valor de corte epidemiológico (ECV) para alguns azóis frente a *A. fumigatus* foi estabelecido a partir do protocolo do *Clinical and Laboratory Standards Institute (CLSI),* como ITC (1µg/ml), VRC (1µg/ml), POS (0.25µg/ml) e ISA (1µg/ml) para determinação de estirpes *wild type* (ESPINEL-INGROFF et al., 2010, 2013), enquanto o *breakpoint* clínico para consideração de estirpes resistentes foram estipulados baseado no protocolo do European Committee on Antimicrobial Susceptibility Testing (EUCAST) como ITC (>2µg/ml), VRC (>2µg/ml), e POS (>0.5µg/ml) (VERWEIJ et al., 2009).

Fármacos azóis atuam diretamente na inibição da biosíntese do ergosterol, por redução da atividade enzimática devido a competição por sítio ativo da lanosterol 14α-demetilase (PERLIN; SHOR; ZHAO, 2015). A lanosterol 14α- demetilase é codificada pelo gene *cyp51*, e, alterações na isoforma *CYP51A* estão diretamente relacionadas a emergência da resistência de *Aspergillus* spp. a azóis (HOWARD; ARENDRUP, 2011; SHARPE et al., 2018; BUIL et al., 2018) . Exemplos disto é a mutação TR34/L98H, que confere resistência pan-azol, e a mutação TR46/Y121F/T289A, aparentemente mais relacionada a alto nível de resistência ao voriconazol e susceptibilidade variável ao itraconazol (VAN DER LINDEN et al., 2013; SNELDERS et al., 2015; REN et al., 2017).

Enquanto mutações pontuais de apenas um nucleotídeo (SNPs), resultando em mudança no aminoácido em determinadas posições como G54 e M220, no *cyp51A* são frequentemente relatadas em pacientes com falha terapêutica após longo período de exposição a fármacos azóis para tratamento da aspergilose (HOWARD et al., 2009; CAMPS et al., 2012), outras mutações, incluindo a inserção de uma sequência de específica de nucleotídeos no promotor do *cyp51A,* denominadas *Tandem repeats* (TR), combinadas com SNPs que modificam determinados aminoácidos no gene *cyp51A,* como TR34/L98H e TR46/Y121F/T289A são mais observadas em pacientes que não tiveram contato prévio com estes antifúngicos, o que dá robustez à hipótese que essas mutações estão mais associadas a isolados que sofreram pressão de seleção ambiental,

principalmente devido ao vasto uso de defensivos agrícolas a base de azóis (DUNNE et al., 2017; LAVERGNE et al., 2017; SHARPE et al., 2018).

De fato, o uso de fungicidas selecionam positivamente *Aspergillus* resistentes aos triazóis agrícolas que apresentam também resistência a triazóis usados para tratamento da aspergilose(FIGURA 3) (SNELDERS et al., 2012; BERGER et al., 2017; REN et al., 2017) . E, na mesma linha, em países como a Colômbia e a Holanda, onde esses fungicidas são utilizados amplamente no cultivo de flores, demonstra-se uma alta taxa de isolados ambientais resistentes a azóis (ALVAREZ-MORENO et al., 2017; DUNNE et al., 2017; LAVERGNE et al., 2017).

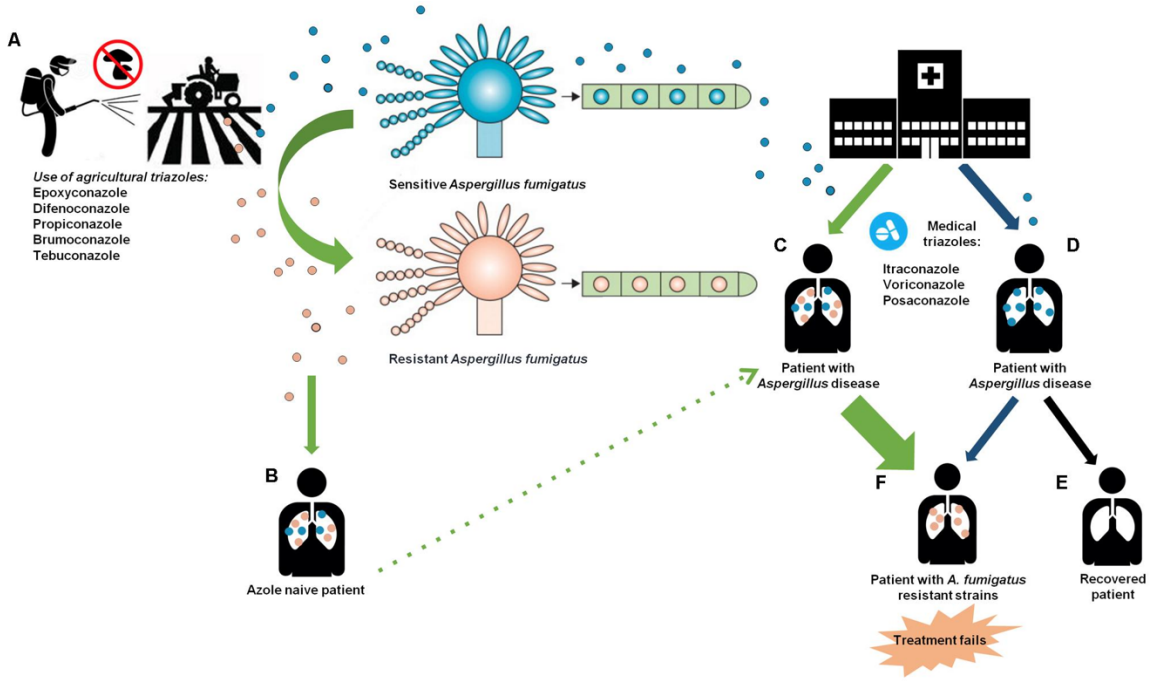

Figura 3 Esquema ilustrativo demonstrando a pressão de seleção de cepas resistentes de *Aspergillus* spp. no ambiente devido ao uso de fungicidas agrícolas azóis e seu papel na falha terapêutica de pacientes com aspergilose. Fonte: Berger et al., 2017.

Apesar de Negri e colaboradores (NEGRI et al., 2017) afirmarem que a resistência a triazóis ainda não é um problema emergente no Brasil, este e outros estudos alertam que esse tema deve ter uma vigilância constante (GONÇALVES, 2017; BEDIN DENARDI et al., 2018; REICHERT-LIMA et al., 2018). Estudos brasileiros com isolados clínicos e ambientais descrevem taxas de resistência *in vitro* a azóis de 5 a 25 % (BEDIN

DENARDI et al., 2018; REICHERT-LIMA et al., 2018). No país, os relatos de ocorrência das mutações no *cyp51A* relacionadas à resistência ambiental em isolados fenotipicamente resistentes é recente, com a mutação TR34/L98H em um isolado clínico humano e mutação M220R em um isolado ambiental (DENARDI et al., 2018), e mutação TR34/L98H/S297T/F495I em dois isolados clínicos (PONTES et al., 2020).

Em aves, embora não seja uma prática clínica comum, alguns estudos de susceptibilidade já foram realizados em isolados clínicos de *Aspergillus*. Um estudo recente, com isolados clínicos de aves em reabilitação e isolados do ambiente de reabilitação não encontrou nenhuma cepa resistente a azóis (SABINO et al., 2019). Por outro lado, um estudo com isolados clínicos de aves domésticas e selvagens na Bélgica e Holanda, detectou quatro isolados resistentes ao itraconazol e ao voriconazol, sendo que 2 destes de animais que receberam terapia antifúngica com itraconazol. Neste estudo, não houve busca por mecanismos de resistência dos isolados testados (BEERNAERT et al., 2009). Em outro estudo, com o objetivo de buscar mutações no *cyp51A* que conferem resistência a isolados de *Aspergillus* colhidos em aves em fazendas, nenhum isolado resistente foi encontrado. O sequenciamento do gene detectou nove alterações em amino ácidos, porém nenhuma relacionada diretamente a resistência (WANG et al., 2014). Dessa forma, ainda existem poucos dados sobre a resistência a azóis e seus mecanismos em aves. Em adição, apesar da importância da aspergilose em pinguins, e de já ter sido descrita a ocorrência de espécies crípticas da seção *Fumigati* em ambiente de praia (SABINO et al., 2019), não há dados na literatura sobre a identificação molecular dos isolados clínicos de pinguins, tampouco quanto a sua resistência a azóis.

3.2.3. Diversidade de *Aspergillus fumigatus sensu stricto*

Estudos de diversidade genética são importantes para determinação da epidemiologia molecular dos microrganismos, mas também para detecção de surtos, identificação de pacientes cronicamente colonizados, e até mesmo para acompanhamento da eficiência do tratamento antifúngico nos pacientes (ARAUJO et al., 2012). *A. fumigatus* é caracterizado por possuir grande variabilidade genética e, dessa forma, é essencial a utilização de técnicas de fácil reprodutibilidade entre diferentes

laboratórios, e com alto poder discriminatório para determinação da diversidade dos isolados estudados (DE VALK; KLAASSEN; MEIS, 2008; ASHU et al., 2017).

A amplificação aleatória de DNA polimórfico (RAPD) é uma técnica de tipagem genômica já descrita para *A. fumigatus*, na qual se utiliza um primer único em uma reação de PCR com baixas temperaturas de anelamento, resultando no anelamento do primer em múltiplas porções do DNA genômico. Na análise por eletroforese em gel de agarose, é observada a presença de múltiplos fragmentos, com tamanhos distintos, assim, isolados diferentes, tendem a apresentar diferentes padrões de bandas (AUFAUVRE-BROWN; COHEN; HOLDEN, 1992). O polimorfismo de comprimento de fragmento de restrição (RFLP) é outra técnica que pode ser utilizada para estudos de tipagem genética, na qual é utilizada uma enzima de restrição, que recorta o DNA previamente amplificado em determinadas regiões. A diferença no tamanho dos fragmentos obtidos reflete a diferença existente entre os isolados (DENNING et al., 1990).

A técnica de polimorfismo de comprimento de fragmento amplificado (AFLP) consiste na utilização de duas enzimas de restrição com frequência de corte diferentes. Após a clivagem do DNA, uma pequena sequência sintética de fita dupla DNA será acoplada a porção final dos fragmentos, e uma nova PCR com *primers* específicos será realizada. Os fragmentos resultantes poderão ser analisados em gel de poliacrilamida desnaturante ou ainda, os *primers* utilizados para se ligar aos fragmentos gerados a partir da uma das enzimas de restrição poderão ser marcados com fluorocromos para análise dos seus distintos tamanhos (SAVELKOUL et al., 1999; WARRIS et al., 2003).

A tipagem de sequência *multilocus* (MLST) é baseada na variação de sequencias de nucleotídeos entre os isolados. A técnica consiste em amplificação de regiões genômicas, e sequenciamento dessas regiões. Cada sequencia diferente recebe um número único. Cada alelo em cada *loci* recebe um número diferente. Em *loci* múltiplos, um tipo de sequência (ST) é definido como qualquer combinação única de alelos em todos os *loci* (KLAASSEN, 2009).

Por fim, a identificação de microssatélites ou repetições curtas em tandem (conhecidas como *short tandem repeats* - STRs) é uma técnica que cumpre com duas premissas importantes para a tipagem de organismos. A primeira diz respeito à reprodutibilidade inter laboratorial, sendo uma técnica de fácil padronização e aplicação

em diferentes laboratórios. Como consequência, é possível realizar a comparação dos resultados obtidos em cada laboratório com resultados de diferentes laboratórios a partir de um banco de dados compartilhado, facilitando, dessa forma a comparação da diversidade de *Aspergillus* a nível mundial. A segunda diz respeito ao alto poder discriminatório, o que permite uma alta resolução da genotipagem, com boa diferenciação entre isolados distintos (DE VALK; KLAASSEN; MEIS, 2008; ASHU et al., 2017; SEWELL et al., 2019). Essa técnica consiste em sequências curtas e repetitivas que estão abundantemente presentes nos genomas da maioria dos organismos superiores e, em menor extensão, em vários genomas de organismos procariontes também, e a diferenciação entre os isolados ocorre com base nas diferenças nos números de repetição de cada um (DE VALK; KLAASSEN; MEIS, 2008).

Em 2005, De Valk e colaboradores desenvolveram um painel de 9 marcadores para genotipagem de alta resolução de *A. fumigatus*, e de 99 isolados testados, foram obtidos 96 perfis genéticos distintos, resultando em um índice de diversidade de 0,9994 na escala de Simpson, com potencial de diferenciar $3x10^{13}$ alelos. A técnica é baseada em uma combinação multiplex para identificação de repetições de di-, tri-, e tetra-nucleotídeos, nomeadamente os conjuntos M2, M3 e M4, respectivamente, cada um com um conjunto de 3 *primers*. Analisando cada conjunto separadamente, o conjunto M3 foi o que apresentação maior poder discriminatório (0,9968) (Figura 4), sendo, dessa forma, este o conjunto recomendado para a realização de pesquisas de genotipagem, e então somente incluir os conjuntos M2 e M4 caso seja necessário, posteriormente (DE VALK et al., 2005).

TABLE 2. Diversity indices for all multiplex combinations

Combination of markers	No. of profiles[a]	*D* value[b]
M2	60	0.9879
M3	86	0.9968
M4	51	0.9784
M2 + M3	94	0.9988
M2 + M4	76	0.9928
M3 + M4	95	0.9992
M2 + M3 + M4	96	0.9994

[a] The total number of isolates is 99.
[b] The calculated *D* value is based on Simpson's index of diversity.

Figura 4 Poder discriminatório calculado por De Valk e colaboradores para 3 conjuntos de marcadores de microssatélites para genotipagem de *Aspergillus fumigatus*. Fonte: De Valk et al., 2005.

3.3. *Aspergillus* no Contexto "*One Health*"

O contexto "*One health*" considera a saúde em diversos extratos, abordando de forma interdisciplinar a saúde do indivíduo (animal e humano), a saúde da população ou grupo, e por fim, a saúde do ecossistema (LERNER; BERG, 2015). Na abordagem "*One health*", evidencia-se o papel das mudanças ambientais na emergência de doenças crônicas e infecciosas, e, como forte aliada no desenvolvimento de estratégias de prevenção de doenças por diversas agências internacionais como a *Food and Agriculture Organization of the United Nations*, a *World Organization for Animal Health* e a *World Health Organization* (RABINOWITZ et al., 2013).

Fungos do gênero *Aspergillus* se enquadram substancialmente nesta abordagem, tendo em vista sua ubiquidade, bem como sua importância como potenciais patógenos animais e humanos, podendo a infeção por este agente se manifestar de distintas formas clínicas (CHOWDHARY; MEIS, 2018). Essa abordagem em *Aspergillus* spp. é recente, e poucos trabalhos foram publicados até o momento neste contexto. Uma revisão do complexo *viridinutans*, pertencente à seção *Fumigati*, no contexto *One Health*, reuniu informações importantes sobre produção de metabólitos secundários, distribuição ambiental, síndromes clínicas, e susceptibilidade antifúngica apresentadas pelas 10 espécies, das quais seis são conhecidamente patogênicas. Essas informações utilizadas no contexto da micologia médica, humana e veterinária, permitem uma melhor estratégia,

abordagem e conduta clínica, como por exemplo, há evidências que a maioria das espécies patogênicas do complexo *viridinutans* parece possuir resistência intrínseca ao voriconazol (TALBOT; BARRS, 2018).

Somente a partir de uma abordagem *One Health* é possível compreender o emergente problema da resistência a azóis que já se tornou uma preocupação global, e o papel dos fungicidas agrícolas neste contexto. A exposição dos isolados aos defensivos agrícolas azólicos confere uma pressão de seleção ambiental às cepas de *Aspergillus* e consequente resistência cruzada aos azóis utilizados no tratamento das doenças fúngicas, seja na medicina humana ou veterinária (REN et al., 2017; CHOWDHARY; MEIS, 2018).

No Brasil, estima-se que o mercado de defensivos agrícolas movimentou 2.595 e 3.082 milhões de reais nos anos de 2012 e 2013, respectivamente. Desses, os fungicidas agrícolas foram responsáveis por 30% e 26% das movimentações nos respectivos anos, sendo as culturas de soja, trigo, milho e feijão as principais áreas de utilização desses defensivos. Além disso, as vendas acumuladas de defensivos agrícolas de janeiro a novembro de 2013 tiveram um crescimento de 30% comparado ao mesmo período de 2012 (SINDVEG, 2013). Em se tratando de área plantada, as culturas de arroz, soja, milho e trigo são as principais culturas agrícolas praticadas no Rio Grande do Sul, e a cultura de soja no estado foi a que teve maior crescimento, principalmente devido à demanda por exportação (FEE-RS, 2019). Esses dados caracterizam o nosso estado como um dos maiores produtores do país, e consequentemente, indicam as altas cargas de fungicidas agrícolas que são utilizados anualmente para a manutenção dessas culturas.

De acordo com Ministério da Agricultura, Pecuária e Abastecimento (MAPA), existem atualmente 16 fungicidas triazóis registrados para utilização agrícola: bromucazol, ciproconazol, difenoconazol, epoxiconazol, fluquiconazol, flutriafol, imibenconazol, ipconazol, metconazol, miclobutanil, paclotutrazol, propiconazol, tebuconazol, tetraxonazol, triadimenol e triticonazol. Um estudo conduzido recentemente, demonstrou que fungicidas agrícolas podem induzir cepas de *Aspergillus* ambientais à resistência (BERGER et al., 2017; REN et al., 2017). Dos fungicidas

testados, quatro podem ser encontrados na lista dos defensivos agrícolas de uso liberado no Brasil: epoxiconazol, metconazol, propiconazol e tebuconazol (AGROFIT, 2019).

Relacionando-se os dados apresentados, a nossa região possui importantes fatores que podem contribuir para o aumento da taxa de resistência em cepas de *Aspergillus* spp. no ambiente, principalmente devido ao importante papel do estado na agricultura. Dessa forma, assim como países com tradição no cultivo de flores como Colômbia e Holanda estão relatando emergência da resistência em *Aspergillus* spp. (ALVAREZ-MORENO et al., 2017; DUNNE et al., 2017), e considerando o potencial da região pelos fatores já relacionados ao forte desempenho na agricultura, a investigação da questão ambiental no contexto *One health* para a emergência da resistência a triazóis é de caráter urgente e a vigilância desse tema deve ser constante, devido às graves consequências que pode acarretar no contexto de saúde pública ao longo dos anos (CHOWDHARY; MEIS, 2018; SHARPE et al., 2018).

A gravidade da emergência da resistência a antifúngicos pode ser explicada, principalmente devido à limitação de fármacos antifúngicos que existem no mercado atualmente. Comparados aos fármacos antibacterianos, a diversidade de classes de antifúngicos é muito limitada. E, principalmente pelo fato de fungos serem organismos eucariotos, esbarra-se na dificuldade de descoberta de novas classes de antifúngicos que não sejam toxicas ao ser humano (ASHLEY et al., 2006). Por isso, o uso racional de fungicidas tanto na esfera ambiental como na clínica humana e veterinária deve ser incentivado, visando a saúde no contexto global.

A resistência antifúngica durante tratamentos clínicos também deve ser levantada. A detecção de mutações em forma de SNPs posterior ao início do tratamento em pacientes humanos infectados com cepas previamente suscetíveis é reportado a longa data (DENNING et al., 1997). Abandono de tratamento antes da alta médica, comum devido a longa duração da terapia antifúngica que varia de meses a anos, bem como profilaxia antifúngica para aspergilose nos pacientes de risco podem contribuir para essa problemática. No caso de reabilitação de aves como os pinguins, por exemplo, muitos centros utilizam profilaxia antifúngica, como é o caso do Centros de Recuperação de Animais Marinhos cujo tratamento preventivo com itraconazol na dose de 20mg/kg é

realizado durante 15 dias para animais considerados com potencial risco para desenvolvimento da doença (SILVA-FILHO; RUOPPOLO, 2014).

Essas questões implicam em uma reflexão sobre a utilização profilática dos fármacos azóis, bem como sobre o papel das doses subinibitórias na emergência da resistência. Na natureza, cepas selvagens tendem a ter vantagem sobre cepas com mutações, principalmente devido ao custo biológico de determinadas mutações. Quando expostas a doses subinibitórias, a população alvo pode sofrer algumas variações, dentre as quais destaca-se a pressão de seleção, onde as cepas suscetíveis estarão em desvantagem frente a cepas resistentes quando em contato com essas doses subinibitórias, tendendo a resultar em um aumento da população resistente frente a população sensível. (LI et al., 2017).

Como último ponto, estudos recentes demonstram que a possibilidade de transmissão horizontal da aspergilose em humanos deve ser revista (ENGEL et al., 2019a), e relacionando a forma clínica da aspergilose em aves, na qual há formação em abundância de estruturas fúngicas de reprodução em sacos aéreos (TELL, 2005; XAVIER et al., 2011), a hipótese de transmissão da aspergilose em aves confinadas, como é o caso de aviários, centros de recuperação e zoológicos deve ser levantada, ou então, ao menos, a possibilidade de contaminação ambiental pelos animais doentes. Dessa forma, a possibilidade seleção de cepas resistentes nos pacientes em tratamento e posterior chance de liberação ao ambiente, fecham o cenário de importância da resistência a antifúngicos no tratamento clínico da aspergilose (LELIÈVRE et al., 2013; PERLIN; SHOR; ZHAO, 2015; PERLIN; RAUTEMAA-RICHARDSON; ALASTRUEY-IZQUIERDO, 2017;

Associando todos os pontos levantados neste capítulo, comprova-se que fungos do gênero *Aspergillus* exercem uma interligação dos “três pilares” da saúde única (humanos, animais e ambiente) (LERNER; BERG, 2015; CHOWDHARY; MEIS, 2018;). Considerando que, ao contrário do raciocínio clássico em que a aspergilose no ser humano ou no animal é o ponto final, neste novo cenário, as cepas de *Aspergillus* que infectam esses hospedeiros podem retornar ao ambiente dando a esta questão um perfil cíclico e não mais linear (Figura 5).

Dessa forma, estudos multidisciplinares, envolvendo os três pilares do conceito *One health*, devem ser considerados na tentativa de contornar esse problema emergente, trazendo à luz novas soluções para o controle de pragas agrícolas, sem maiores interferências na saúde humana e animal, como vem acontecendo no caso da aspergilose (CHOWDHARY; MEIS, 2018).

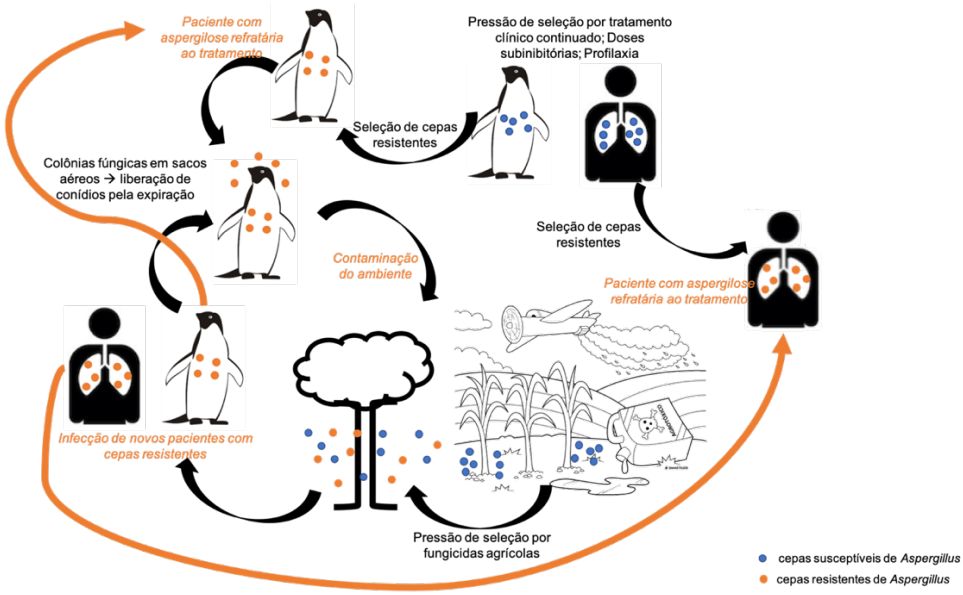

Figura 5 Esquema ilustrativo demonstrando a pressão de seleção de cepas resistentes de *Aspergillus* spp. a partir do tratamento clínico, juntamente com a hipótese de contaminação do ambiente e transmissão horizontal das cepas resistentes.

3.4. Busca por novas moléculas com potencial antifúngico

Dado a baixa variedade de moléculas antifúngicas disponíveis atualmente para aplicação clínica, e a preocupante emergência de estirpes de *Aspergillus* resistentes a azóis, é necessário investimento na busca novas moléculas com potencial antifúngico, com o objetivo de tentar mitigar o grande impacto da resistência antifúngica no contexto clínico. Além de ser necessário considerar fatores como toxicidade e interação com outras drogas, a busca por novas moléculas deve levar em conta a necessidade de descoberta de novos alvos, com diferentes mecanismos de ação dos antifúngicos já existentes (VAHEDI-SHAHANDASHTI; LASS-FLÖRL, 2020).

Nesse sentido, estudos têm demonstrado o potencial antifúngico de compostos inorgânicos e orgânicos à base de selênio frente a diferentes fungos de importância na

micologia médica, como *Fusarium* spp., *Candida* spp., *Pythium* spp., *Aspergillus* spp., *Cryptococcus* spp., *Sporothrix* spp., entre outras (LORETO et al., 2011, 2012; POESTER et al., 2019; ROSSATO et al., 2019). Os principais compostos de organosselênio descritos como potenciais novos antimicrobianos são ebselen (Eb) e disseleneto de difenila $(PhSe)_2$ (GIURG et al., 2017; MAY et al., 2018). Eb é um benzisoselenazol desenvolvido na década de 1980. É um medicamento aprovado pelo FDA dos EUA para doenças cardiovasculares, artrite, aterosclerose e câncer. De acordo com a importância da reciclagem de medicamentos, o Eb e seus análogos foram testados em outras aplicações, mostrando atividade antimicrobiana contra vírus, bactérias gram-positivas e gram-negativas, fungos e micobactérias (WÓJTOWICZ et al., 2003, 2004; JAROMIN et al., 2018). O $(PhSe)_2$ é outro composto orgânico, obtido pela oxidação do benzenosselenoato (ROSA et al., 2005). Esse composto carrega dois átomos de selênio em sua molécula, e possui maior estabilidade e menor peso molecular que o Eb. A atividade inibitória *in vitro* de $(PhSe)_2$ já foi demonstrada frente a diferentes spécies de fungos patogênicos (LORETO et al., 2011, 2012; DENARDI et al., 2013; POESTER et al., 2019; ROSSATO et al., 2019). Dessa forma, essa molécula parece ter um potencial promissor como alternativa antifúngica, justificando a necessidade de maiores estudos neste contexto.

3.4.1. Disseleneto de difenila $(PhSe)_2$

O selênio é um micronutriente essencial para humanos e animais, adquirido nas formas inorgânicas e orgânicas da ingestão de alimentos, estando presente em vegetais, carnes, frutos do mar, nozes e outros (HARIHARAN; DHARMARAJ, 2020). Esse elemento é essencial nos processos antioxidantes e imunológicos, como constituinte, ou promovendo a função de proteínas e enzimas, chamadas selenoproteínas. No corpo humano, as selenoproteínas desempenham um papel essencial nos hormônios da tireoide, nas respostas imunes não específicas, nos processos inflamatórios e na resposta ao estresse oxidativo (SCHOMBURG, 2012; CHAN et al., 2016; HARIHARAN; DHARMARAJ, 2020).

O grupo de selenoproteínas está principalmente envolvido na resposta ao estresse oxidativos (LABUNSKYY; HATFIELD; GLADYSHEV, 2014; HARIHARAN; DHARMARAJ,

2020). O estresse oxidativo é causado por altos níveis de espécies reativas de oxigênio (ROS), que são produtos de vários tipos de funções celulares que aumentam as respostas a danos ou estresse celular. Nesse sentido, a estrutura semelhante dos compostos de organosselênio às selenoproteínas permite que sua atividade em células de mamíferos diminua as ROS, melhorando a atividade antioxidante (HARIHARAN; DHARMARAJ, 2020).

O $(PhSe)_2$ é capaz de mimetizar a atividade das selenoproteínas glutationa peroxidases (GPX) e também podem ser um excelente substrato para outras selenoproteínas, as tioredoxina redutases (TR), em mamíferos, o que estimularia uma resposta rápida ao estresse oxidativos (HARIHARAN; DHARMARAJ, 2020). Diversas aplicações farmacológicas desse composto em mamíferos como os efeitos antiinflamatório, hepatoprotetor, citoprotetor, anticâncer e neuroprotetor, são todas baseadas nesta ação antioxidante (MÜLLER et al., 1984; ZHAO; MASAYASU; HOLMGREN, 2002; BRITO et al., 2009; PRIGOL; LUCHESE; NOGUEIRA, 2009; WILHELM et al., 2009; MENEZES et al., 2016; HARIHARAN; DHARMARAJ, 2020).

Com relação às propriedades antifúngicas do $(PhSe)_2$, sugere-se que o principal mecanismo de ação desse composto em células fúngicas é, ao contrário do observado em células de mamíferos, proporcionar uma atividade pró-oxidante e, consequentemente, um acúmulo intracelular de espécies reativas de oxigênio (ROS) (MÜLLER et al., 1984; YANG; SHEN; ONG, 2000; NOGUEIRA et al., 2003b; THANGAMANI et al., 2017).

Ainda, a favor desse composto, pode-se citar a sua baixa toxicidade. Esse composto tende a se acumular no tecido adiposo, fígado, rins, pulmões e cérebro, em ratos e camundongos (PRIGOL et al., 2012). A dose letal aguda (LD50) de $(PhSe)_2$ em ratos pela via intraperitoneal é de 1200 μmol / kg, (NOGUEIRA; ROCHA, 2010) e para uma dose única administrada por via oral em camundongos é estimado em > 312 mg / kg (SAVEGNAGO et al., 2007). Em coelhos, um estudo de longo prazo revelou que sua administração oral produziu efeitos toxicológicos mínimos (DE BEM et al., 2007).

Por fim, estudos prévios também demonstram bons resultados na interação dessa molécula com antifúngicos clássicos (DENARDI et al., 2013; VENTURINI et al., 2016; FELLI KUBIÇA et al., 2019; ROSSATO et al., 2019), demonstrando seu potencial também

para utilização em terapias combinadas com outros antifúngicos no contexto clínico. Diante de todo o exposto, essa molécula parece ser promissora como alternativa para o combate a infecções fúngicas, e maiores informações sobre seu potencial antifúngico frente a *Aspergillus* são necessários.

4. RESULTADOS

O trabalho realizado durante o período de doutoramento resultou em seis manuscritos como primeira autora, os quais serão apresentados nesta tese. Destes, três estão publicados em jornais revisados por pares de relevância na área da micologia. Além disso, um manuscrito de revisão está em processo de revisão também em um jornal revisado por pares de relevância na área da micologia, e mais dois manuscritos, os quais ainda não foram submetidos a nenhum jornal, estão formatados de acordo com a associação médica americana.

No período de doutoramento, foi realizada a identificação molecular de isolados clínicos de aves aquáticas, de aves de produção e de humanos, e investigamos a diversidade genética de *Aspergillus* baseado na técnica de microssatélites. Além disso, foi feito *screening* de todos os isolados para três antifúngicos azóis, e os isolados que apresentaram algum crescimento no teste foram selecionados para maiores investigações quanto a resistência antifúngica, com a realização de microdiluição seriada para determinação das concentrações inibitórias mínimas dos antifúngicos itraconazol, voriconazol e posaconazol frente a estes isolados; e investigação de mecanismos moleculares relacionados a resistência por PCR em tempo real para detecção das principais mutações de resistência no gene *cyp51A,* bem como por sequenciamento do *cyp51A* e do seu promotor.

Os resultados obtidos com o emprego dessas técnicas podem ser vistos nesta tese na forma dos manuscritos/artigos apresentados a seguir. Além disso, serão apresentados em forma de apêndices, dois artigos nos quais a candidata participa como co-autora, e teve a oportunidade de colaborar durante seu período de doutoramento sanduíche no Instituto Nacional de Saúde Doutor Ricardo Jorge, em Lisboa, Portugal.

O primeiro manuscrito a ser apresentado é um manuscrito de revisão, intitulado “Aspergillosis, avian species and the *One Health* perspective: the possible importance of birds in azole resistance”, o qual apresenta uma extensa revisão bibliográfica, que embasa nossa hipótese sobre a importância das aves na abordagem *One Health* de

Aspergillus e da aspergilose, bem como na emergência da resistência a azóis. Essa revisão reflete todo o embasamento teórico que foi utilizado para propor os trabalhos resultantes nesta tese.

O manuscrito II, intitulado “Genetic diversity of Aspergillus fumigatus from different hosts in southern Brazil: the usefulness of microsatellite data” analisa a diversidade genética de *Aspergillus fumigatus* sensu stricto provenientes de isolados de humanos e aves, especialmente pinguins. Neste artigo, demonstramos a versatilidade da técnica de microssatélites para aplicação no contexto clínico, principalmente em casos complexos, como a aspergilose pulmonar crônica, bem como no contexto epidemiológico, como na identificação de surtos de aspergilose em ambiente de reabilitação de pinguins, e a comprovação de estirpes geneticamente idênticas isoladas em diferentes hospedeiros, com implicações em futuros estudos no contexto *One Health*.

O manuscrito III, intitulado “Aspergillosis in Magellanic penguins: molecular identification and susceptibility patterns of clinical isolates” traz pela primeira vez a identificação molecular a nível de espécie de isolados clínicos de *Aspergillus* provenientes de pinguins-de-Magalhães com aspergilose comprovada. Reportamos que *Aspergillus fumigatus* sensu stricto é o principal agente etiológico da aspergilose em pinguins, além de reportar, pela primeira vez, aspergilose causada pela espécie críptica *A. sydowwi* neste hospedeiro. Também apresentamos resultados de susceptibilidade a antifúngicos azóis dos mesmos isolados, reportando um isolado resistente a posaconazol, e identificação de mutações no gene *cyp51A* deste isolado.

O artigo I, intitulado “Diphenyl diselenide and its interaction with antifungals against Aspergillus spp.”, e acessível pelo DOI: (doi.org/10.1093/mmy/myaa072) por sua vez, avalia a atividade do disseleneto de difenila $(PhSe)_2$ sozinho e em combinação com itraconazol, voriconazol e caspofungina foi avaliada frente a três seções de *Aspergillus* consideradas mais patogênicas: *Fumigati*, *Flavi* e *Nigri*. Os resultados destacam a atividade promissora do $(PhSe)_2$ frente as espécies de *Aspergillus*, com destaque para sua atividade em combinação com antifúngicos clássicos, como a caspofungina.

O artigo II, intitulado “Aspergillosis in albatrosses” , acessível pelo DOI (doi.org/10.1093/mmy/myz122) foi publicado no formato de brief report, e descreveu a ocorrência de aspergilose em albatrozes (*Thalassarche melanophris)* durante a

reabilitação e identificou os agentes etiológicos envolvidos na infecção. Os resultados obtidos neste trabalho sugerem que a aspergilose pode agir como um fator limitante na reabilitação de albatrozes.

Por fim, o artigo III, intitulado “Aspergillosis in free-ranging aquatic Birds”, acessível pelo DOI (doi.org/10.1016/j.mmcr.2020.04.005) relatou três casos de *Aspergillus fumigatus* causando doença fúngica em aves aquáticas de vida livre, com a identificação molecular do agente causal em nível de espécie. Até onde sabemos, este é o primeiro relato de aspergilose nas espécies de aves *Procellaria aequinoctialis, Chroicocephalus maculipennis e Nannopterum brasilianus*. Os dados obtidos sugerem que a aspergilose em aves selvagens pode ser mais prevalente do que se pensava anteriormente.

4.1. MANUSCRITO I. Aspergillosis, avian species and the One Health perspective: the possible importance of birds in azole resistance

Manuscrito submetido ao periódico Microorganisms.

O manuscrito é apresentado conforme as regras de formatação da revista para a qual foi submetido.

MDPI

microorganisms

Review

Aspergillosis, avian species and the *One Health* perspective: the possible importance of birds in azole resistance

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Abstract: The *One Health* context considers health based on three pillars: humans, animals, and environment. This approach is a strong ally in the surveillance of infectious diseases and in the development of prevention strategies. *Aspergillus* spp. are fungi that fit substantially in this context, in view of their ubiquity, as well as their importance as plant pathogens, and potentially fatal pathogens for, particularly, humans and avian species. In addition, the emergence of azole resistance, mainly in *Aspergillus fumigatus* sensu stricto, and the proven role of fungicides widely used on crops, reinforces the need for a multidisciplinary approach to this problem. Avian species are involved in short and long distance travel between different types of landscapes, such as agricultural fields, natural environments and urban environments. Thus, birds can play an important role in the dispersion of *Aspergillus*, and of special concern, azole-resistant strains. In addition, some bird species are particularly susceptible to aspergillosis. Therefore, avian aspergillosis could be considered as an environmental health indicator. In this review, aspergillosis in humans and birds will be discussed, with focus on the presence of *Aspergillus* in the environment. We will relate these issues with the emergence of azole resistance on *Aspergillus*. These topics will be therefore considered and reviewed from the "*One Health*" perspective.

Keywords: *Aspergillus*; azole resistance; avian aspergillosis; invasive aspergillosis; One Health context.

1. Introduction

Aspergillosis is a fungal disease caused by *Aspergillus*, which can affect humans, dogs, cats, horses, marine mammals, wild and domestic birds and even invertebrates, such as bees and corals [1]. The common route of infection for vertebrates is inhalation of conidia present in the environment and the respiratory tract is the most common anatomical site for initial site of infection [1,2]. To date, aspergillosis is considered a non-contagious disease, however recent studies have raised the possibility of transmission of this fungus among hospitalized patients [3,4]. In this sense, due to its clinical manifestation in avian species [5,6], the hypothesis of fungal transmission among captive birds, as in the case of aviaries, wildlife rehabilitation centers and zoological institutions must be raised. This transmission may occur especially through environmental (particularly air) contamination by sick birds.

Azoles such as itraconazole (ITC), voriconazole (VRC), posaconazole (POS) and isavuconazole (ISA) are the drugs of choice for prophylaxis and treatment of aspergillosis both in humans and animals [7–12]. Because of the difficulty on designing antifungal drugs that lack side effects in humans, effective therapeutic options to treat mycoses presently are limited [13]. Concurrently, triazoles are the main pesticides used in agriculture [14,15]. Thus, a concern about worldwide azole resistance arises from the large use of agricultural fungicides in the environment, leading to emerging resistant *Aspergillus* strains, and hence contributing to an increase of treatment failures' rate in humans.

In this review, we will discuss relevant microbiologic aspects of these fungi, and of its infection, aspergillosis, in humans and birds. Aspects concerning *Aspergillus* in the environment, and the emergence of antifungal resistance of this pathogen will also be approached. We highlight these topics from a *One Health* perspective, where humans, animals and environment are all connected.

2. Aspergillosis in humans

According to the Global Action Fund for Fungal Infections (GAFFI), it is estimated that the health of more than 15 million people is affected by aspergillosis, causing more than 1 million deaths per year [16]. As fungal infections increase in clinical medicine [17–19], the number of cases of aspergillosis is likewise increasing through the past decades. An epidemiological study performed in the United States suggests an increase from 3 cases of invasive aspergillosis (IA) per 10,000 people admitted for hospital care (data obtained in 1996) to 10 cases per 10,000 (data obtained between 2009 and 2013) [20].

The clinical manifestations of aspergillosis in humans are classified according to the extent of mycelial colonization or tissue invasion and are influenced by the host's immune response capacity. The range of consequences includes allergic reactions and invasive infections. Patients with co-morbidities such as chronic granulomatous disease, bone marrow or solid organ transplantation, prolonged neutropenia, AIDS, or with prolonged treatment with steroids or other immunosuppressive drugs are risk groups for IA [8,19], and mortality rates due to acute IA can reach 80% in the first year after diagnosis [21,22]. Recent studies emphasize the importance of IA in intensive care units, especially in patients with influenza, in patients receiving newer cancer chemotherapy modalities, and in patients diagnosed with COVID-19 [23–28].

Aspergilli in the section *Fumigati* are the most common etiological agents in all the aspergillosis presentations described above. The species *A. fumigatus* sensu stricto is the most frequently isolated agent of aspergillosis in humans. Since the beginning of the 2000's, however, *Aspergillus* sections have been studied more intensively at the molecular level, and cryptic species of the *Fumigati* section, such as *A. lentulus*, *A. thermomutatus*, and species of the *viridinutans* complex such as *A. pseudofischeri* and *A. felis*, among others, have also been described in aspergillosis cases [29–33].

Aspergillosis cases have been classified according to the guidelines of the European Organization for Research and Treatment of Cancer / Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC / MSG), [34]. However, with the increasing knowledge of new risk groups and with different characteristics, aspergillosis case definitions

may change, according to the analyzed group as well as the clinical manifestations. Case definitions specifically for chronic pulmonary aspergillosis [35] and for influenza-associated pulmonary aspergillosis in intensive care unit patients [36] have been described. Also, given some differences already found in cases of Covid-associated pulmonary aspergillosis (CAPA), epidemiological studies are being performed to support the case definition of CAPA [28,36,37].

3. Aspergillosis in avian species

Birds are especially susceptible to aspergillosis because of their anatomical and physiological characteristics, including the presence of air sacs with poor vascularization and limited mucociliary function, providing an ideal environment for fungal growth. Additionally, birds have heterophils instead of neutrophils, which could be less effective against hyphal invasion [6,38,39]. Aspergillosis is therefore a major cause of morbidity and mortality in birds, causing economic and ecological damages. Furthermore, this disease can be responsible for impacting avian production in zoological institutions and in wild animals during rehabilitation [38,40–42].

Aspergillus section *Fumigati* is responsible for up to 90% of deaths in birds with aspergillosis [12,39,40]. *A. fumigatus* sensu stricto is the only etiologic agent of this section identified in birds so far [42–44]. This may be related with the virulence traits of this species: the ability to grow at higher temperatures, such as the body temperature of birds (38-42 ° C); the reduced size of the conidia in comparison with other sections of the genus, facilitating penetration into the lower respiratory tract; and the enhanced production of gliotoxin [40,45,46].

The clinical signs of aspergillosis in birds are nonspecific and often not evident until the final stage of the disease. For certain avian species, the management of large groups is more common than individual management, and therefore an earlier diagnosis can be an even bigger challenge. In this context, early diagnosis is essential for determining treatments, and serological assays seems to be the most promising so far [47–53]. However, since an efficient and financially accessible method for making an early diagnosis is not available, the diagnosis of aspergillosis in birds is commonly made only on *post-mortem* examination. Macroscopic findings during necropsy are described as white-yellow granulomas in pulmonary parenchyma and/or air sac membranes, and in some cases the dissemination of the disease to other organs, such as heart, liver, kidneys and spleen. In addition, the growth of fungal colonies, with moderate to large production of conidia in the lower respiratory tract can occur, and it has been reported in different avian species [5,6,38,54,55] (Figure 1). Confirmation of the diagnosis is made by isolation of the fungus in culture associated with evidence of tissue invasion, and observation of hyphae in histopathology [6,40].

Aspergillosis is reported in poultry production and in different types of avian livestock, such as chickens, turkeys, geese, ducks, pigeons, emus and ostriches. Young birds are the most affected [51,56–58]. It is common to observe outbreaks of aspergillosis in poultry, with mortality rates varying between 4.5 and 90% [38]. The economic losses in this group can reach US$ 11 million per year in turkey production [54]. The losses are related to the condemnation of carcasses in slaughterhouses owing to air sacculitis and fungal pneumonia, reduction of the growth rate of the birds, and increase of the mortality rate [1,38,57]. The risk factors for poultry include high environmental humidity, lack of well-ventilated housing, accumulation of organic matter and use of bedding litter made rich with organic matter [38,59,60]. These environmental conditions are optimal for *Aspergillus* growth. High humidity levels and richness of organic matter are factors that contribute to fungal multiplication and more airborne conidia, consequently enhancing the chance of inhaling a larger inoculum, one of the risk factors for the development of avian aspergillosis [12,38,39,41,61].

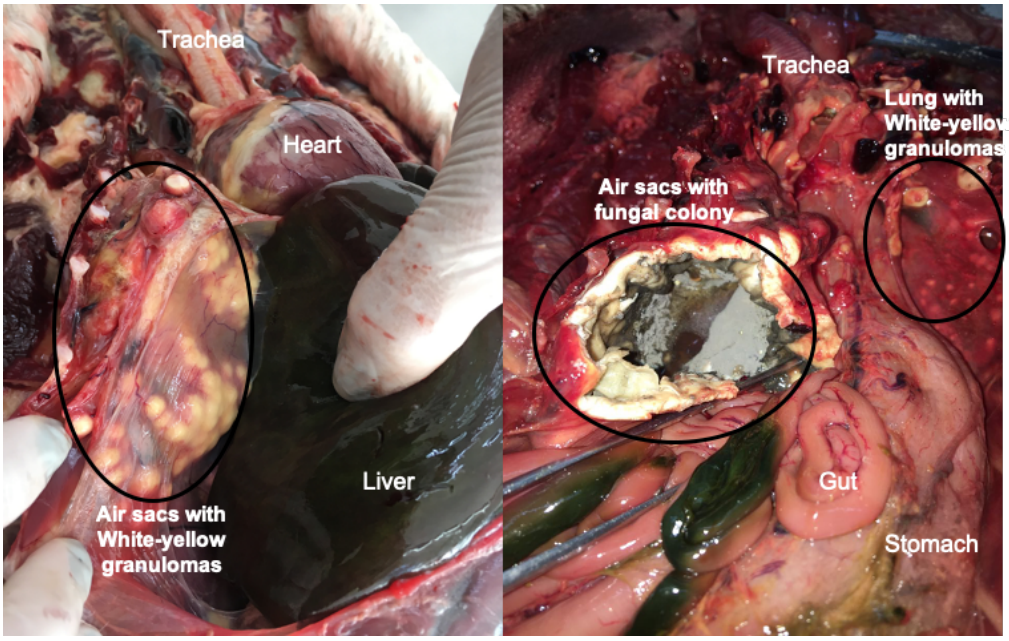

Figure 1: Common lesions of pulmonary aspergillosis found in avian species A: White-yellow granulomas on air sac membranes from a Magellanic penguin that died during rehabilitation at the Center of Rehabilitation of Marine Animals, Brazil, B: Growth of fungal colonies of Aspergillus fumigatus on air sac membranes with evidence of substantial sporulation, from a free-ranging Magellanic penguin, found dead in Cassino beach, Rio Grande, Brazil

Aspergillosis is also a concern given the high mortality of wild birds kept in captivity, such as zoological institutions, avian wildlife rehabilitation centers, and captive birds of prey used for falconry [41–43,62,63]. In wildlife rehabilitation centers, aspergillosis is considered an important fungal disease for penguins in rehabilitation [41], with a mortality rate of ~50% in Magellanic penguins (*Spheniscus magellanicus*) in captivity [12]. In zoological institutions, in addition to the occurrence of *Aspergillus* outbreaks, deaths caused by aspergillosis are common [64–66]. There are many knowledge gaps regarding free-ranging wild birds, although some reports have been published regarding the frequency of aspergillosis, in comparison to what is known regarding aspergillosis in captive birds [67–69].

Wild birds brought into captivity are commonly administered azoles, for both prophylaxis and treatment, given the high incidence of fungal diseases in rehabilitation centers and the potential for increased exposure [9,11,12,42,70]. Captive populations of birds in zoological institutions are also prophylactically treated and this can be related to species susceptibility and environmental challenges. Regarding poultry farms, the use of azoles for prophylaxis and treatment of aspergillosis is unusual, owing to concerns regarding the existence of drug residues in meat and the expense. However, unusual circumstances could lead to antifungal prophylaxis on farms [71]. On the other hand, the use of azoles for decontaminating bedding and environmental disinfection is common [38,58,59,71–74].

4. *Aspergillus*

Aspergillus is a ubiquitous saprophytic genus, with worldwide distribution. It is frequently found in decomposing plants, playing an important role in this process and contributing to carbon and nitrogen recycling [61]. Fungi of this genus are widely used in industry owing to their high capability to produce a diversity of enzymes, such as amylases; in generating chemical additives, such as citric acid; the production of soy sauces; and in bioremediation processes, among others [75].

The taxonomy of *Aspergillus* is in flux, and 4 to 8 subgenera, and from 16 to 25 sections, has been proposed by different authors, and more than 350 species. Most species are found in the environment, without reported clinical relevance thus far [76–79]. Sections *Flavi*, *Nigri*, *Nidulantes, Terrei* and *Fumigati* are of interest in clinical practice.

Species belonging to section *Fumigati* are the main etiologic agents of aspergillosis. *Aspergillus fumigatus* sensu stricto is the cause of the majority of those infections. However, it is estimated that between 3 to 6% of those infections are caused by cryptic species. *A. lentulus, A. udagawae, A. viridinutans, A. thermomutatus, A. novofumigatus* and *A. hiratsukae* are the most common cryptic species reported in medical practice to date. When compared to *A. fumigatus* sensu stricto, these cryptic species seem to have limited pathogenicity, since they have limited thermotolerance and have slower rates of production of different mycotoxins. On the other hand, most of these species have intrinsic resistance to azoles, which makes them refractory to the treatment of choice for aspergillosis [32,45,80,81].

Some characteristics present in *Aspergillus fumigatus* sensu stricto can partially explain why this species is the leading human and animal pathogen, such as rapid growth, small size of the conidia (1-4 μm), thermotolerance (growth between 15ºC and 55ºC, able to tolerate up to 70º C), tolerance to high pH, low nutritional requirements, and production of secondary metabolites such as gliotoxin. The latter represents an important virulence factor [32,45,61,82] helping in tissues' invasion. High thermotolerance is a factor that could benefit *A. fumigatus* in the environment, in comparison to other fungal species: its optimal growth temperature is 37ºC [83], which allows this species to be an important human pathogen. Given the current conditions of global warming, this feature could favor *A. fumigatus* growth and its dispersion in the environment [84].

5. *Aspergillus* azole resistance

Intrinsic antifungal resistance, already known in cryptic species of *Aspergillus* section *Fumigati*, has been also described for other species. High minimal inhibitory concentrations to antifungals were also found by different *Aspergillus* sections such as *Flavi*, *Nigri*, *Nidulantes* and *Circumdati* isolated mainly from environmental sources, such as hospital environments. This highlights the importance of establishing surveillance programs for *Aspergillus* antifungal susceptibility from different sources, as well as the need for monitoring the epidemiology of *Aspergillus* species other than *Fumigati* and avoiding underestimating these microorganisms as potential pathogens [85].

The increase in azole resistance described for *A. fumigatus* sensu stricto is a global concern [31,86,87]. The first report of azole resistance in clinical isolates was published in 1997 [88]. Since then, many reports of clinical and environmental azole resistant isolates from different regions of the world have been reported [86,88–95]. A retrospective/prospective study in a laboratory of the United Kingdom has shown that the resistance rate of *A. fumigatus* has increased from 0.43 (1998-2011) to 2.2% (2015-2017) [96], and another study from Denmark has shown an increase from 1.4 to 6% in azole resistance from human clinical isolates [97].

The emergence of azole resistance in *A. fumigatus* sensu stricto deserves special attention, since this is the most common species of *Aspergillus* related to human and animal aspergillosis, as stated. In addition, given the limited availability of antifungal drugs to treat fungal infections, this increase in resistance rates found in clinical practice poses a substantial problem and could result in treatment failure and a consequent increase in mortality rates [98]. Current local azole resistance rates vary between 0 and 26%, variation occurring according to geographic region and patient population [85,90,96,99–105]. An investigation, involving 13 countries in four continents, found an azole resistance rate of 6% among 2,026 *Aspergillus* isolates evaluated [106].

Regarding avian species, despite the importance of aspergillosis in these animals, there are few studies evaluating resistance or cryptic species. The majority of those reports are in poultry, and the rate of

resistance is considered low thus far [58,71,107,108]. On the other hand, a few reports on *Aspergillus* susceptibility studies isolated from captive wild birds are described [109,110]. Studies on aspergillosis in free-ranging wild birds are scarce, and consequently, knowledge about epidemiological trends [67–69] is scarce, and susceptibility assays on *Aspergillus* isolated from this group of birds have not been published so far.

6. Main mechanisms of azole resistance

Azole drugs act directly by inhibiting ergosterol biosynthesis, binding to the enzyme 14α-lanosterol demethylase (CYP51), preventing the conversion of lanosterol to ergosterol [87,90]. The *cyp51A* gene is directly related to the emergence of *A. fumigatus* sensu stricto azole resistance [86,90,111].

Long-term treatments, such as those carried out in cystic fibrosis patients, are an important route to azole resistance, and single nucleotide polymorphisms (SNP) which result in amino acids changes such as G54, G138, M220, and G448, among others, are more commonly found in these patients [112]. Point mutations as G54 and M220 can change the protein structure, which affects the docking of certain azole compounds for the whole protein [113–115]. On the other hand, mutations such as TR_{34}/L98H, which confers pan-azole resistance [99,100], and TR_{46}/Y121F/T289A, apparently more related to a high level of voriconazole resistance and variable susceptibility to itraconazole [113,116,117], are reported in environmental strains [100,118], in patients with long-term treatment as well as in patients with IA [89,105,119,120].

Overexpression of *cyp51* is another mechanism associated with resistance in *A. fumigatus* [87,112]. Despite the great importance of *cyp51A* mutations conferring azole resistance, a survey with Manchester isolates showed that 43% of resistant isolates were not *cyp51A* mutants [121]. Thus other mechanisms than *cyp51A* mutation may have an important role in azole resistance, such as point mutation in the subunit HapE, of the CCAAT-biding complex (CBC) [122,123], in *cox10* gene [124], and in *hmg1* gene [125]. In this sense, genome-wide sequencing is a good tool to identify possible mutations conferring azole resistance in *cyp51*-unrelated resistant strains [124,125].

The overexpression of efflux pumps has already been proven in resistant strains without changes in the *cyp51* gene [126,127], such as overexpression of genes that encodes proteins from the ATP-binding cassette of the ABC transporters class [127–131], as well as from the major facilitator transporter (MFS transporter) [132,133].

The production of an extracellular matrix (ECM) by *A. fumigatus*, in biofilm formation, plays a significant role in antifungal resistance, shielding the fungus from the drugs and reducing antifungal susceptibility [134,135]. In addition, the overexpression of efflux pumps in association with biofilm formation has been related to azole resistance [136]. More studies aiming to understand the real importance of biofilm formation and its contribution to the emergence of azole resistance are necessary.

7. The role of pesticides in emerging *Aspergillus* azole resistance

Treatment failures in IA patients have raised the discussion of the origin of resistance, since many of those patients had never been under previous azole therapy. Environmental exposure is the most reasonable possibility as the source of resistant strains [137]. In this context, investigations regarding possible sources of resistance for environmental strains were initiated and the large use of azole fungicides in agriculture was identified as a probable factor in the emergence of resistant strains in the environment [138].

This hypothesis was later supported by numerous other findings, such as the predominance of two mutations (TR_{34}/L98H and TR_{46}/Y121F/T298A) in the majority of clinical isolates from different care centers, especially in azole-naïve patients [86,120,139,140]. The presence of the same mutation in environmental isolates [91,100,141], the genetic similarity between clinical and environmental isolates [117,137,138,142], the global spread of these mutations [91,93,95,100,143,144] and most recently, studies showing the lower

diversity present in resistant strains with these mutations in relation to wild type strains [106,145] support this hypothesis. Since then, several studies have shown the role of azole pesticides used in agriculture in the selection of resistant strains of *A. fumigatus* [91,116,141,146,147].

Practices to control infestations in crops are essential, given that plant pathogens, particularly fungi, can cause huge economic losses in many agricultural endeavors, such as the culture of grapes [148], rice (an average of US$ 69.34 million lost annually in US [149]), soybeans [150], corn [151], among many others. It is estimated that such global losses in the five more important crops occur on a scale that, if mitigated, would be enough to feed 8.5% of 7 billion people in 2011[152]. Among the pest control mechanisms, azole pesticides are currently widely used on crops, such as bromucazole, cyproconazole, diphenoconazole, epoxiconazole, fluquiconazole, flutriafol, imibenconazole, ipconazole, metconazole, myclobutanil, propiconazole, tebuconazole, tetraxonazole and triticonazole [153,154]. The control of *Aspergillus* in cereal crops is important mainly because of the mycotoxin production by some species. Aflatoxins, which are produced especially by *A. flavus*, represent a high risk to human and animal health, with serious implications such as hepatotoxicity, teratogenicity and immunotoxicity [155]. Ochratoxin A, first described in *A. ochraceus*, and produced by several species of *Aspergillus*, with nephrotoxic and genotoxic effects, is a probable cause of cancer for humans [156]. *A. fumigatus* is not the main target of fungicides used in crops. However, since *Aspergillus* fungi are saprophytic and ubiquitous, the presence of its spores in these areas is common. The use of azole fungicides culminates in the selection of resistant strains, which then increase in the environment [145].

Many of these crop fungicides have molecular similarity with azoles used in clinical settings, sharing as the target, ergosterol biosynthesis [141]. Because of the same action target and the structural similarity of the molecules, the azole crop fungicides can lead to cross resistance with azole drugs available to treat aspergillosis in clinical cases [137]. Thus the search for alternative options to the use of fungicides for pest control in crops is urgently needed and, concurrently, the search for more effective drug options to treat fungal diseases [157].

8. Contamination of the environment with pesticides

The fungicide-driven route to the emergence of azole resistance in *A. fumigatus* is already a well-described fact. However, analyzing this issue in a broader ecological context, this problem spreads beyond the limits of the lands where those chemicals are used. Azoles fungicides are considered pesticides with high chemical and photochemical stability, low biodegradability and are easily transported into the environment, making them persistent in soil and water [158]. Fruits, vegetables and flowers cultured in farms that regularly use fungicides enter in thousands of homes daily. In this route, they can carry traces of those chemicals with them inside our homes [154]. In addition, there is contamination of drinking and irrigation water, and adjacent soils with these chemicals, mainly drained by rainfall [159]. It is true that the rains also end up diluting these fungicides; however, if they are in sub-inhibitory doses, this can also contribute to the emergence of resistance, such as by stimulating the overexpression of efflux pump genes [160].

Unfortunately, contamination of different environments by crop fungicides occurs worldwide. In this context, some countries have laws to determine the maximum acceptable concentrations of these products in both water and food, but in other countries, mainly those in development, there is no regulation of these limits yet. In Cameroon, where there is no government regulation, fungicides such as metalaxyl, carbendazim, tetraconazole and penconazole are found in between 22 and 100% of sampled surface waters from the Méfou watershed [159]. In Spain, a study regarding the concentration of new-generation fungicides released from crops in throughfall (the rainfall that is not arrested in the plant canopy) were determined during rainfall episodes, and concluded that these concentrations far exceeded the maximum permissible levels for drinking water established by the European Union (EU) regulations. Although this

throughfall water is not used directly for drinking, it contaminates soil, runoff and water courses, and concentrations of those fungicides could exceed the limits established by the EU [161]. In areas of broad use of fungicides in the USA, at least two fungicides were detected in 55% of bed sediments and 83% of suspended solid, sampled from three different geographic areas, showing that these chemicals can persist in the environment in variable concentrations [162]. Again, in a USA study, during a heavy fungicide application period, azole fungicides such as propiconazole and metconazole, in addition to other classes of fungicides, were found in different types of wetlands located in and near those fields [163]. In an important region for rice growing in Brazil, high levels of tebuconazole (up to 460 ng L^{-1}) were found in surface and drinking waters [164].

9. Use of crop areas and adjacent areas by birds

The presence of resident and migratory birds in plantation areas and their surroundings is very common. These areas serve both as feeding and resting grounds for many avian species, in large part due to the loss of their natural habitats, such as natural wetlands, on which waterbird species depend throughout their life cycle [165]. Most commonly, the presence of birds in cultivation areas results in conflict with an economic losses in agriculture, such as the case of wine grapes in single vineyards [166]. However, in some cases, this relationship can be beneficial for both sides, as for sustainable management in some rice fields in the Mediterranean area, where migratory waterbirds forage in rice fields, and farmers benefit from the nutrient enrichment of the soils due to the defecation of these birds [167].

In Europe, a total of 121 avian species were observed, at least occasionally, in rice fields; many of them using these fields to forage and/or breed [168]. In the Americas, 169 waterbird species and 166 landbird species were recorded in rice paddies [169]. In Asia, 135 different bird species were noted using Japanese and Korean rice paddies [170]; in India, at least 351 species [171] were counted in those areas. In West Africa, the density of shorebirds in shallow water is 4-6/hectare and of the waterbirds is 11/hectare in wet coastal rice fields [172]. In wine grape vineyards, the presence of birds in crop areas is also well recorded, such as in two farms of Canada, where 8 species and more than 1.000 individuals were recorded, many with foraging behavior [166]. Another study in the USA reported the presence of 29 fruit-eating avian species in different fruit crops, such as grapes, apples, blueberries and sweet cherries, some of them in large numbers [173]. Hence, a high diversity of birds (carnivorous, herbivorous, insectivorous and omnivorous) tend to be found in these areas. Many migratory birds use these areas as stop-over sites as well, such as the use of cover crops in the US Midwest Corn Belt region during spring migration [174]. In addition, flower crops, such as tulips, are important areas used by some birds, mainly passerines (perching birds), during some phases of their life-cycle [175,176]

The movements of avian species between these areas can vary according to season, migration, and rainfall patterns; and may or may not coincide with seasons of pesticide use. However, the direct interaction of birds with seasons of pesticide use is not a crucial factor for our analyses, since the main subject of our interest is the interaction of birds with azole-resistant *A. fumigatus* isolates selected due to the use of agricultural fungicides. These chemicals remain in the environment for a long time. In this sense, any birds that have contact with these lands and adjacent areas at any time may be exposed and colonized or infected by azole-resistant fungal strains. Estuaries and adjacent waterlands are natural habitats for a high diversity of endemic avian species such as passerines, waterbirds and shorebirds, as well as migratory birds [177]. As previously discussed, these areas are directly impacted by fungicide contamination, which can remain in the environment for extended periods. This is a potential route of exposure to azole resistant strains. The contamination of these environments can also occur through an alternative route: the migratory routes of birds is a potentially important route for the dispersion of resistant strains [165,178], as we will discuss next.

10. Bird migration and their role in pathogen dispersion

The importance of migratory birds in the spread of pathogens such as viruses, bacteria and fungi that can affect not only other animals, but also humans, is already known [179]. Pathogenic bacteria were isolated from fecal and blood samples of migratory birds captured along the Mediterranean and Black Sea, and some of these microorganisms were shown to be multi-drug resistant [178]. Perhaps the most well-known role of avian species in microorganism dispersion is with viruses [180–182], and genomic diversity studies have proven the spread of viruses through bird migration, as well as the potential risk of transmission from wild birds to poultry and *vice versa* [183].

In addition, a genomic study demonstrated that gulls are a reservoir of *Candida glabrata,* including fluconazole-resistant strains, and they can facilitate the spread of this microorganism and promote the indirect transmission to humans via environmental contamination [184]. Recently, the role of birds in the dispersion of the highly pathogenic multi-drug resistant yeast, *Candida auris,* was also proposed [185].

During migration, birds are exposed to several stressors, such as weight loss, fatigue, food deprivation, among others [186], which can be predisposing factors to infectious diseases, such as aspergillosis. They can become infected by the inhalation of conidia during foraging, or even during rest time. Since avian species can be considered as a health indicator of a certain ecosystem [187], the surveillance of aspergillosis in wild birds could be an important tool to better understand environmental azole resistance, particularly its geographic distribution. We may anticipate threats to human health defined in such studies, such as the introduction of *Aspergillus* isolates with new resistance profiles, the emergence of new resistance mechanisms and an increase in the rate of resistance in various regions.

Moreover, direct and/or indirect transmission of *Aspergillus* strains from wild birds to poultry and humans should not be disregarded, particularly since reproductive structures of the fungus have been found on air sac membranes of birds with aspergillosis [5,6,67]. However, given a bird's lack of a diaphragm and a negative pressure system in their thoracic cavity, it is unlikely that the birds can release conidia during respiration. Therefore, the more likely route of infection would be through their feces or contaminated carcasses that then contaminate the environment. Birds and humans share common geographic areas in rural areas (where many aviaries are found), but also urban and suburban areas, since most of the human population is located near water sources (rivers, estuaries, lagoons and the sea), essential environments throughout the life cycle of birds [188].

11. Why should we consider the role of birds in azole resistance in the One Health approach?

The *One Health* context considers health in several dimensions, addressing the health of humans, animals, and finally, the health of the ecosystem [189]. In the *One Health* approach, the role of environmental changes in the emergence of chronic and infectious diseases is highlighted. The concept is supported in the development of disease prevention strategies by several international agencies, such as the Food and Agriculture Organization of the United Nations, the World Organization for Animal Health and the World Health Organization [190].

This approach had been essential to better understand the role of crop fungicides in the emergence of azole resistance in *A. fumigatus*, with consequent treatment failure and mortality especially in humans with IA [98,117]. Thus, multidisciplinary studies, involving the three pillars of the *One Health* concept, should be considered in an attempt to circumvent this emerging problem, bringing to light new solutions for the control of agricultural pests, for the control of environment in, e.g., poultry production, and for the prophylaxis and antifungal therapy in human and veterinary medicine [191,192].

A recent study showed the low diversity of azole resistant strains harboring the TR_{34}/L98H and TR_{46}/Y121F/T289A mutations, when compared with wild strains. The global distribution of those clonal *A. fumigatus* isolates, harboring these mutations, demonstrates the ability of these strains to spread throughout the world [145]. Another study showed the gene flow among regional and global populations, with the

same genotypes found in countries up to 7,500 km apart, suggesting intermediate to short-distance dispersals and long distance dispersals as credible patterns of spread of *A. fumigatus* [106].

Environmental dispersion by winds or dispersion by human population movements can be sources of dissemination. However, given the movements that birds routinely perform during their life cycle, with short to medium distance displacements for food and rest, and long distance displacements for seasonal migrations, the role of birds as contributors to dispersion of *Aspergillus* strains (such as those carrying specific mutations that confer azole resistance) should be considered. Some avian species are highly susceptible to aspergillosis, presenting large fungal colonies on their air sac membranes. By analogy, these birds resemble *in vivo* cultures of *Aspergillus*, with high fungal growth and high levels of sporulation of the fungus. The mortality among these birds is very high. Contamination of other birds and of the environment with resistant *Aspergillus* strains occurs through the feces of sick birds or carcasses and dispersion of strains by healthy birds (in their feathers, for example) (Figure 2). In this sense, the surveillance of mortality in wild birds and the identification of this disease in these animals could be an important indicator of environmental health, and a tool to monitor the emergence of new mechanisms of azole resistance that could have implications for human health.

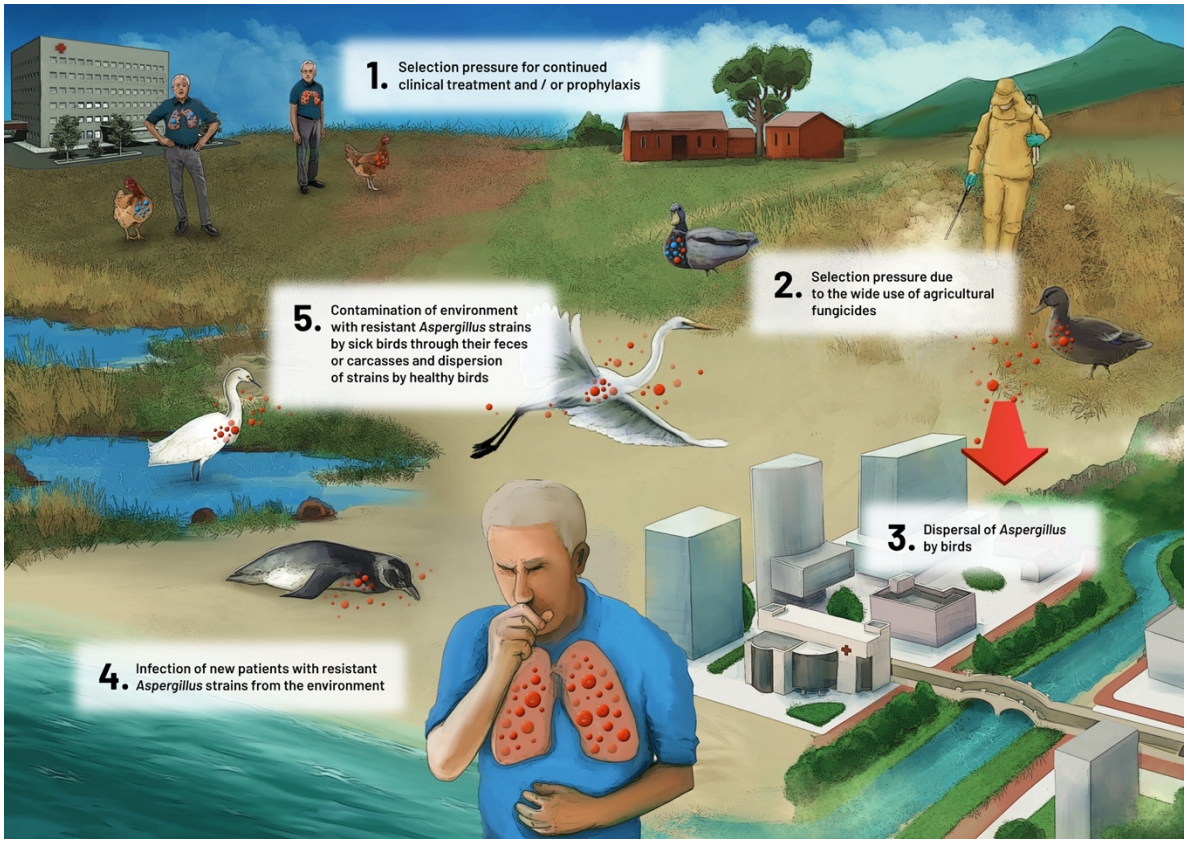

Figure 2: Proposed role of avian species in the dispersion of azole-resistant *Aspergillus fumigatus*

Birds can carry pathogens without being sick themselves. The transportation of pathogens from one crop field to another or to nature can occurs through their digestive tract (beak, gut microbiota and feces), legs, and feathers [179,184,193,194]. This dispersion can occur over short distances, such as from a foraging field to where the birds rest, or over large distances, as in the case of migratory birds [165,169,178,179]. For example, some birds such as *Calidris alba* (sanderling) migrate every year from the Artic region (where they breed in northern summer) to the southern hemisphere (where they feed during the northern winter),

traversing from 3,000 to 10,000 km twice a year. During this travel, they require some stop-overs to feed and rest [195]. This is an important example to show how far birds could transport strains of *Aspergillus* and the significant impact of the dispersion.

Finally, another point still scarcely investigated so far, is the consequence for the emergence of azole resistance given the routine use of these drugs as prophylactic therapy in rehabilitation of wild birds, and the use of azole-based chemicals for disinfecting poultry houses [11,12,38,71,196,197]. In the case of azole prophylaxis in avian species, or the use of azole-based chemicals in poultry farms, these exposures could contribute to azole resistance development.

12. Concluding remarks and future perspectives

In summary (Figure 3), there is much evidence suggesting the potential role of avian species in azole resistant *Aspergillus*. Important points, that act as overlapping circles regarding animals, humans and environment, to be highlighted are:

- *Aspergillus* species are ubiquitous fungi, present in many environments, and it is a potential pathogen of importance in animals (we emphasize birds) and humans;
- The emergence of azole resistance in *Aspergillus* species such as *A. fumigatus* sensu stricto is a major concern, limiting treatment success;
- The wide use of crop fungicides has an important role in the emergence of azole resistance;
- Avian species are highly susceptible to aspergillosis. Many species have regular migratory movements, moving between different environments, including those where large amounts of pesticides are used, and natural environments, such as estuaries, lagoons and beaches;
- In their movements between different types of environments, such as agricultural fields, natural environments and urban environments, birds may have an important role in the dispersion of *Aspergillus* isolates, especially resistant strains;
- Considering the characteristics of *Aspergillus* fungi, the importance of aspergillosis in birds and humans, and the emergence of azole resistance, it is essential to promote more investigations in a *One Health* approach.
- Avian aspergillosis can be used as an indicator of environmental health, including surveillance of the introduction of new resistant strains, changes in resistance rates, and emergence of new mechanisms of azole resistance.

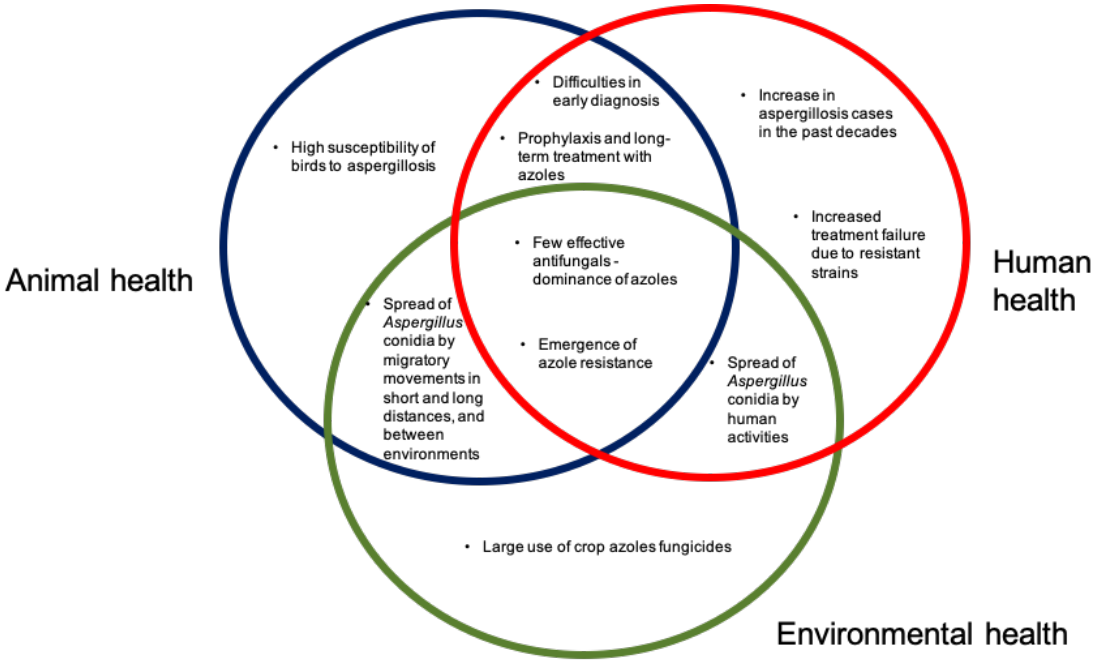

Figure 3: The One Health scheme, demonstrating the implications for *Aspergillus* and aspergillosis, in the environment, in animal health (with emphasis on birds), and in human health.

After discussing these topics, we suggest some measures that deserve attention in global efforts to overcome the serious and emerging problem of resistance to azoles by *A. fumigatus*, and its consequences to human and animal health, among them:

- Regular cleaning of where birds are kept, with non-azole products, aiming at environmental control of the amount of fungal inoculum;
- Avoid contact of people, in groups at risk for aspergillosis, with poultry farms, zoological institutions, avian wildlife rehabilitation centers;
- Search for more efficient early diagnosis techniques for both humans and birds;
- Implementation of antifungal stewardship programs for both humans and birds;
- Search for new antifungal molecules different from those presently used on crops, with different mechanisms of action, and dissimilar to those used in human therapy;
- Surveillance of antifungal susceptibility in *Aspergillus* strains in environment, birds, and humans;
- Measures to control the dispersion of *Aspergillus* strains in agricultural products transported by humans from farms to urban areas;
- Surveillance of the main routes of bird migration and correlation with spread of azole resistant isolates;
- Search for new fungal control options for fungal control for crops, such as biological rather than chemical control.

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4.2. MANUSCRITO II - Genetic diversity of *Aspergillus fumigatus* from different hosts in southern Brazil: the usefulness of microsatellite data

O manuscrito é apresentado conforme as regras de formatação da American Medical Association.

Genetic diversity of *Aspergillus fumigatus* from different hosts in southern Brazil: the usefulness of microsatellite data

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Abstract

Aspergillosis is a fungal infection of global importance in humans and animals, especially avian species. Among *Aspergillus'* clinical manifestations in humans, chronic pulmonary aspergillosis (CPA) presents many challenges as its difficult diagnosis, and invasive aspergillosis (IA) is difficult to manage, especially in immunocompromised patients. In addition, aspergillosis is also one of the most important infectious diseases regarding avian species such as in captive penguins. In this study we aimed to demonstrate the versatility of microsatellites as molecular markers to be used in the clinical practice, to detect outbreaks, as well as to be used in the epidemiological context, comparing genetic diversity of clinical *A. fumigatus* strains. Using this technique, we found out that a patient with a complex case of CPA was colonized/infected by the same *Aspergillus* strain for at least 5 years. We were also able to identify a possible aspergillosis outbreaks in a Center of Recovery of Marine Animals. In addition, we found an identical *A. fumigatus* genotype in two different hosts: a penguin and a human. These findings highlight the usefulness of this typing method in clinical practice, in the detection of outbreaks, as well as in epidemiological studies, with a great potential to be used in the *One Health* context.

Key words: microsatellite, chronic pulmonary aspergillosis, avian aspergillosis, genotyping,

Introduction

Aspergillosis is a fungal infection of global importance in humans and animals, especially avian species [1,2]. It is caused by *Aspergillus* and section *Fumigati* is involved in more than 90% of the invasive infections caused by this genus. High rates of morbidity and mortality are described in different hosts [3–6]. The identification of *Aspergillus* at species level is performed by molecular methods, and *Aspergillus fumigatus* sensu stricto is the main species associated with animal and human aspergillosis.[7–9]

Chronic pulmonary aspergillosis (CPA) is a medical condition often misdiagnosed as tuberculosis (TB) [1] due to similar symptoms in both conditions, culminating in a delay in start the antifungal therapy [10]. Despite the availability of diagnosis tests nowadays, and the established criteria for case definition [11], diagnosing CPA is still a challenge, especially in complex cases, in which several pre-existing conditions are involved [12]. In this sense, some specific molecular tools

could be useful to provide additional information to support clinical decision and results interpretation.

Aspergillosis is also one of the most important infectious diseases in avian species, and can cause several economic and ecological losses [13–15]. It is considered a limiting factor to birds rehabilitation, namely of penguins, and a previous epidemiological study with captive penguins raised the hypothesis that some penguins could be colonized/infected by *Aspergillus* in nature before being take to rehabilitation [5]. Although there are some studies describing aspergillosis in free-ranging birds [16–19], there is just one report on aspergillosis in Magellanic penguins (*Spheniscus magellanicus*) in nature [20]. Another study with different avian species showed that the recovery center is a potential source of infection of *Aspergillus* [21]. In the case of penguins' rehabilitation, the main infection source of *Aspergillus* is not proven so far.

A. fumigatus is a fungus with high genetic diversity, and the study of this diversity is used for detection of outbreaks, identification of patients chronically colonized/infected and monitoring antifungal therapy efficacy in patients with aspergillosis [22–24], as well as for molecular epidemiological studies[21,25]. Distinct molecular techniques can be applied for *Aspergillus* typing [23]. A good typing tool has high discriminatory power, applicability, and inter laboratorial reproducibility. In this sense, microsatellite markers are suitable for typing *A. fumigatus* [22,26,27].

A recent study using microsatellite genotyping data has shown that *Aspergillus*-resistant isolates with identical genotypes can be found in clinical as well as in environmental isolates, in geographically distant areas from each other [28], reinforcing the global importance of *Aspergillus* as a public-health pathogen. Considering that *Aspergillus* is a *One Health* pathogen [29], and in this context, environment, humans and animals are important, the use of molecular markers to study genetic diversity is useful to a better understand of whether the same genotypes could also be found in different hosts.

Given the importance of *Aspergillus* in both humans and animals (especially in avian species) as a potential fatal pathogen, the aim of this work was demonstrate the versatility of the use of molecular markers such as microsatellites in the clinical context, as well as in the epidemiological context, detecting outbreaks and also comparing genetic diversity of clinical *A. fumigatus* strains isolated from humans and avian species from southern Brazil.

Material and Methods

Aspergillus isolates

A total of 87 *A. fumigatus* isolates were included in this study, 44 of which were obtained from human patients (28 of which with cystic fibrosis), 34 from penguins with proven invasive aspergillosis, and 9 from poultry. The strains were isolated from 2008 to 2019 and were maintained frozen in fungal collection of the Mycology Lab.

Confirmation of the species identification was done by partial sequencing of calmodulin gene (*calM*) [30], and/or partial sequencing of β-tubulin gene (*benA*) [31]. Sequence analysis were performed using MEGA software version 10.0.5, and obtained sequences were compared with those deposited in the GenBank database (Bethesda, MD, USA) in order to achieve the identification to species level. All of them were identified as *A. fumigatus* sensu stricto.

Analysis of genetic diversity of A. fumigatus sensu stricto

Isolates typing was performed based on the microsatellites markers method using the multiplex PCR M3 combination, which amplifies three trinucleotide loci, with a calculated discriminatory power of 0.9968 [26]. The three forward primers (STRAf 3A, STRAf 3B and STRAf 3C) were labeled at the 5'end with carboxyfluorescein (FAM), hexachloro carboxyfluorescein (HEX), or dichloro carboxyfluorescein (NED), respectively.

PCR reactions were carried out in a 25 µl volume containing 1× PCR buffer (Applied Biosystems Inc., Foster City, CA, USA), 1 µM of each primer [STR*Af* 3A (Forward FAM-GCTTCGTAGAGCGGAATCAC and reverse GTACCGCTGCAAAGGACAGT), STR*Af* 3B (Forward HEX-CAACTTGGTGTCAGCGAAGA and reverse GAGGTACCACAACACAGCACA), and STR*Af* 3C (Forward NED-GGTTACATGGCTTGGAGCAT and reverse GTACACAAAGGGTGGGATGG)], 0.2 mM deoxynucleoside triphosphates, 3.0 mM MgCl2, 1 U of Taq DNA polymerase (Applied Biosystems Inc., Foster City, CA, USA), and 1 ng of genomic DNA. PCR conditions were carried out in a thermal cycler as follow: after an initial denaturation step of 95°C for 10 min, 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. A 15µL of ultrapure formamide mixture was added in 1µL of PCR product, and then a denaturation at 95ºC for 3 min was performed at thermo cycler. After this step, fragments analysis was performed by capillary electrophoresis in a 3500 Genetic Analyzer (Applied Biosystems) instrument, using the

molecular weight marker GeneScan 500 ROX Size Standard (Applied Biosystems), and the GeneMapper 6.0 software (Applied Biosystems) was used to data analysis. Simpson diversity index was calculated using untb package, and the neighbor joining tree was constructed using the ape package, both in Rstudio® software.

Results

Microsatellite data analysis showed high genetic diversity, with 69 different genotypes (Simpson's index of diversity = 0.98), with 34 alleles for STRAf3A *loci* (Simpson's index of diversity = 0.97), 20 alleles for STRAf3B *loci* (Simpson's index of diversity = 0.89), and 27 alleles for STRAf3C *loci* (Simpson's index of diversity = 0.94) (Table 1).

Table 1: Alleles of *Aspergillus fumigatus* sensu stricto from different hosts, obtained from three different loci (*STRAf3A, STRAf3B and STRAf3C*). Data for each allele are presented in fragment size (molecular weight).

Host	*STRAf3A*			*STRAf3B*			*STRAf3C*		
	Number of alleles	Minimum Fragment size	Maximum Fragment size	Number of alleles	Minimum Fragment size	Maximum Fragment size	Number of alleles	Minimum Fragment size	Maximum Fragment size
Human	24	129	333	15	162	245	18	76	180
Penguin	16	129	294	12	147	225	18	72	171
Poultry	8	141	228	5	165	213	5	81	180
All hosts	34	129	333	20	147	245	27	72	180

A phylogenetic tree using the neighbor-joining tree estimation of Saitou and Nei (1987), was built to evaluate the genetic relationship among the isolates. Isolates in the same branch of the tree correspond to identical genetic strains based on the analyzed molecular markers (Figure1).

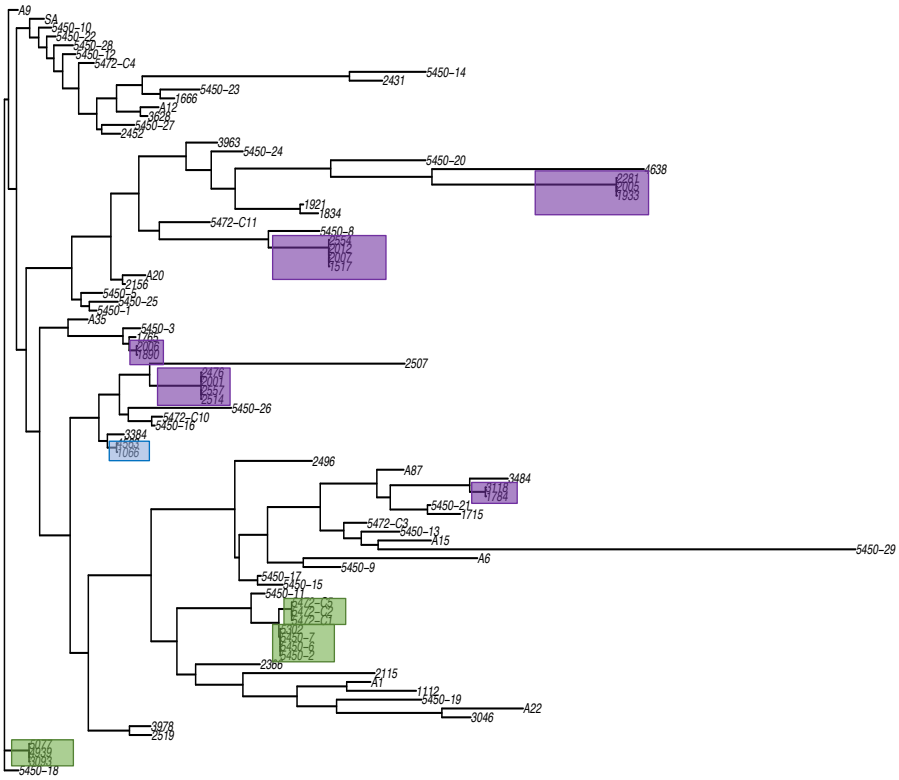

Figure 1 – Genetic clustering of human and avian clinical isolates of Aspergillus fumigatus sensu stricto genotyped with 3 microsatellite markers. The relationship between isolates was determined using the neighbor-joining tree estimation of Saitou and and Nei (1987). Identical genotypes were found in different isolates from the same human patient in three cases (in green). Another genotype was shared by a human and a penguin' isolate (in blue). Also, identical genotypes were found in two to four cases of strains isolated from different penguins (in purple).

Application of the previously mentioned microsatellite genotyping method in the study of selected cases

- *In a human clinical context*

We selected one of the three cases in which we had identical microsatellite genotypes obtained from different isolates of the same patient, to illustrate the applicability of this genotyping method in the clinical practice context.

Case report

Man, 73 years-old, with HIV diagnosis in 2000 (LTCD4= 354 viral charge (VC)= 20,000 copies) in virological suppression since 2010 (LTDC4 = 396 and undetectable VC). Ex-drinker and ex-user of intravenous drugs and tobacco (abstainer since 2008). Thymoma diagnosis in 2002, with

resection and radiotherapy, resulting in lung parenchyma damage as a sequel (fibroatelectasis in the middle field of the right hemithorax and pleural thickening in the right apex, and deviation of the ipsilateral mediastinum). Hepatitis C, genotype 2, treated with viral bleaching in 2017. Diabetes mellitus type 2 under drug control since 2015. Diagnosed with non-tuberculous mycobacteria infection by *Mycobacterium avium* in 2006 and again in 2009. Chronic obstructive pulmonary disease (COPD) under treatment with inhaled corticosteroid and b2agonist since 2010.

Presenting recurrent respiratory infections and progressive deterioration of the lung parenchyma on imaging studies since 2010. Given its previous clinical history, several (more than 10) investigations to mycobacteria infection were performed, all of them negative after the treatment conclusion (2009). *Aspergillus fumigatus* was isolated at the first time in a sputum sample in 2013, and a second time in a bronchoalveolar lavage (BAL) sample in 2015. However, in both cases it was interpretated as contamination/colonization, and no further investigations were performed, neither any antifungal treatment. After a new respiratory manifestation, *A. fumigatus* was isolated once more from a sputum sample in January, 2019. In this occasion, serological investigations for aspergillosis were performed.

Detection of antibody anti-*Aspergillus* (IgG) using Bio-Rad PlateliaTM *Aspergillus* IgG EIA (Marnesla-Coquette, France), and detection of antigen (LFA *Aspergillus* GM, IMMY®) were performed according to manufacturer's instructions, being positive in both of them (OD=3.5 and OD=1, respectively).

Based on the clinical and on laboratorial evidences, diagnosis of aspergillosis (CPA) was confirmed, and patient was hospitalized for treatment with amphotericin B. However, the patient did not tolerate this therapy, and then itraconazole (ITC) was prescribed, but treatment was not correctly followed by the patient. In May 2019, *A. fumigatus* was isolated once again from a sputum sample. In December 2019, patient worsened his clinical condition, and then azole therapy (ITC, 200 mg; 12/12h) was again prescribed. After 6 months of treatment, he had clinical improvement and stabilization of the radiological images (Figure 2). Also, a new anti-IgG test was performed, and the result was negative. The maintenance of the ITC therapy for more 6 months was indicated. Case report approved by the health ethics committee of the federal university of Rio Grande FURG-CEPAS number 229/2018.

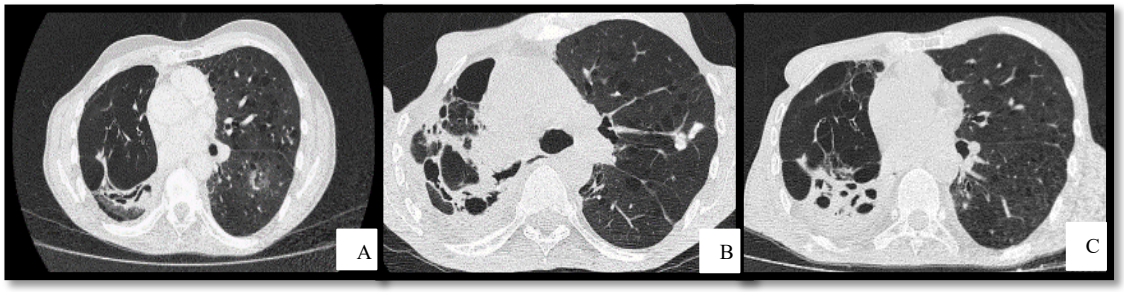

Figure 2- Imaging exams of the patient: A) 2016-Subtotal atelectasis of the upper, middle and lower lobes and extensive bronchiectasis, B) 2018- Complete atelectasis LSD and LID, bronchiectasis, bullous areas and cavitation with mucoid secretion, C) 2020- Sequelae in LSD, segmental atelectasis and bronchiectasis.

The microsatellite genotyping of three isolates (one from 2015 and two from 2019) from this patient showed the same multilocus genotype (186/165/105), which suggests that the patient was at least colonized by the same strain since 2015.

- *In a veterinary context*

In *Aspergillus* isolates collected from penguins (in rehabilitation centers and free-living), we observed that the same multilocus genotype was found in different animals that shared the same facilities at a rehabilitation center in the same period (Table 1), suggesting the occurrence of aspergillosis outbreaks in the rehabilitation environment. In addition, we were able to identify identical strains infecting penguins among different years, suggesting the maintenance of certain strains for a long period at the environment (Table 2).

Table 2: Identical genotypes of *Aspergillus fumigatus* sensu stricto isolated from Magellanic penguins that died from aspergillosis during rehabilitation.

Genotype	Penguin ID	Year of isolation
29 (195/159/76)	2001	2008
	2514	2011
	2476	2011
	2557	2011
35 (201/182/102)	1890	2008
	2006	2008
41	1784	2008

(207/222/102)	3118	2015
50 (216/193/139)	1517	2008
	2007	2008
	2012	2008
	2554	2011
59 (295/165/168)	2005	2008
	2281	2008
	1933	2008

- *In a One Health context*

Finally, we also found the same genotype (180/168/92) in an *A. fumigatus* isolate collected from a human source and from a free-ranging penguin, which can have implications in molecular epidemiology of *Aspergillus* from a *One Health* context.

Discussion

In this work, we characterized the genetic diversity of *A. fumigatus* from clinical isolates of different hosts from southern Brazil, and demonstrated the versatility of microsatellite markers to be used in human medicine, veterinary medicine and epidemiological contexts, as follows: (1) promoting deeper clinical information on the colonization/infection of a patient, demonstrating the persistence of the same genotype in a patient over a period of at least 4 years (2) identifying possible aspergillosis outbreaks during the rehabilitation of penguins and (3) identifying an identical genotype in isolates from a human and a penguin aspergillosis cases.

Use of data in human clinical context

In the *A. fumigatus* isolates collected from the same (human) patients, we observed that all isolates obtained in different moments (sometimes different years) showed identical microsatellite genotypes. One genotype was found in three different patients (131/170/111). As previously demonstrated [23], a patient can be colonized with several *A. fumigatus* strains, but probably one of them overlaps the others for tissue invasion. However, our findings suggest that the patients included in this study were colonized or infected by one single strain.

In what concerns to the case reported in this study, the difficulty of the CPA diagnosis was highlighted and was due to the several health condition of the patient. It is estimated that CPA

affect 3 million people worldwide, among them, 1.2 million cases are classified as sequel of pulmonary tuberculosis (TB) [32], with 373,000 new cases per year within 12 months of completion TB treatment [10]. Beyond TB, non-tuberculous mycobacterial infection (NTM), allergic bronchopulmonary aspergillosis (ABPA), chronic obstructive pulmonary condition (COPD), emphysema, pneumothorax and prior treated lung cancer are some underlying conditions in CPA [33]. In this case, patient had previous diagnosis of NTM, twice, and COPD.

The diagnosis of CPA requires a combination of evidences, such as: one or more cavities with or without a fungal ball or nodules on thoracic imaging for ≥3 months, evidence of *Aspergillus* infection (mycological and/or histopathological exams) or serological tests, and the exclusion of other alternative pathologies. However, this last point make diagnosis more difficult since it is very common the occurrence of co-infections in CPA cases [12]. These criteria were applied from the third isolation of *Aspergillus*, when serological tests were performed, with positive results. The confirmation of the involvement of the same *Aspergillus* strain in the CPA manifestation of this patient could acted as an auxiliary tool to support the clinical decision if it was done at time.

Interpreting the isolation of *A. fumigatus* from a respiratory sample is a challenge, since the isolation may be contamination, colonization or, in fact, an active infection. Our case illustrates this context, in which this diagnosis was considered only after several fungal isolations. The fact that the same fungal strain was isolated in different years, suggests the association of this agent with the progressive deterioration of the lung parenchyma; or even a previous colonization that culminated in progression to active disease after tissue damage as result of other etiologies and / or use of corticosteroids. In both cases, it is worth to emphasize the importance of clinical and laboratory deep investigation of a patient with chronic pulmonary involvement whose respiratory samples' cultures result in the isolation of *A. fumigatus*. In these cases, molecular typing of *Aspergillus* isolates may provide valuable information which can help clinical decision regarding treatment.

Use of data in epidemiological context

Since this methodology has high discriminatory power [26], its application in this study proved to be very useful, determining a high genotypic diversity among the *A. fumigatus* isolates collected from penguins and humans. The occurrence of clonal strains among penguins' isolates reinforces that the rehabilitation environment can be considered a potential source of infection [21].

The majority of clonal *A. fumigatus* were isolated from penguins that shared the same facilities at the same period. These findings reinforce the utility of genetic typing of *Aspergillus* to identify an outbreak and to adopt more stringent containment measures in the moment of the occurrence of an outbreak, mainly based on environmental disinfection measures and constant monitoring of birds in rehabilitation [14,34]. Thus, based on these findings and on the easy reproduction of this methodology, we encourage the application of this technique in the rehabilitation' routine, since it can contribute to the early identification of an aspergillosis outbreaks, increasing the chances of adopting effective control measures. Based on our findings in the rehabilitation context, we can also reinforce the utility of this molecular technique to monitor the occurrence of *Aspergillus* outbreaks in human hospitals as well, as already demonstrated in other studies [25,35], but scarce applied so far.

Also, we detected the probable clonal maintenance of some strains in this environment for a long time, since we have found two genotypes (195/159/76 and 216/193/139) occurring in 2008 and again in 2011, and another one (207/222/102) occurring in 2008 and again in 2015. The environmental long periods-permanency of *A. fumigatus*, was already shown in other studies [24,25]. In fact, even with the adoption of measures to control the fungal load in the environment [14,34], it is impossible to achieve a total of success in particular in these places where several animals are kept at the same time, and with different management areas.

We also have found a clonal genotype shared by a human and a penguin' isolate, each of one found with about 7 years of difference. The clonal expansion of *A. fumigatus* TR mutant strains worldwide was shown recently by Sewell and collaborators [28]. Authors described *Aspergillus* clones in both clinical and environmental isolates and also, in very large geographic distances. Since this phenomenon can occur with mutant strains, it is most likely that the same can occur in wild-type strains too, according to our results. This finding has also an interesting implication in the *One Health* context, considering the role of birds in the dispersion of *Aspergillus* strains, since a clone is able to infect different hosts, such as a penguin and a human. This evidence reinforces our recent hypothesis that birds develop an important role in the dispersion of *Aspergillus* strains worldwide as well as they may represent an environmental health indicator, mainly in case of sick birds. To better understand this important and scarce explored subject so far, broader analyzes related to genetic diversity of *Aspergillus* in different hosts, as well as analyzes focused on bird migration routes are suggested for future work.

In this work, we could achieve several conclusions regarding *Aspergillus* and aspergillosis by interpretation of the data obtained from molecular typing of *A. fumigatus* sensu stricto, the most important *Aspergillus* species involved in human and avian infections. Here, we demonstrated that the same technique was able to provide valuable information, opening future perspectives regarding clinical as well as epidemiological context, such as the improvement of information obtained from a routine diagnosis of aspergillosis, early identification of outbreaks, and better understand of *Aspergillus* local and global dispersion as well as the main dispersion routes.

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4.3. MANUSCRITO III - Aspergillosis in Magellanic penguins: molecular identification and susceptibility patterns of clinical isolates

O manuscrito é apresentado conforme as regras de formatação da American Medical Association.

Aspergillosis in Magellanic penguins: molecular identification and susceptibility patterns of clinical isolates

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Abstract

Aspergillosis is considered the most important infection disease in captive penguins, and *Aspergillus* section *Fumigati* is reported in up to 99% of aspergillosis' cases in penguins. The correct identification of *Aspergillus* at species level is made by molecular methods, and it is important since different species present different virulence and antifungal susceptibility patterns. Despite the antifungal resistance presented by cryptic species, the emergence of acquired resistance in *A. fumigatus* sensu stricto is a global concern. No data regarding molecular identification and

azole resistance are available to Magellanic penguins so far. Then, the aim of this work was to perform the molecular identification of *Aspergillus* at species level and evaluate the susceptibility patterns of *A. fumigatus* sensu stricto with characterization of *cyp51A* gene in clinical *A. fumigatus* strains isolated from Magellanic penguins with proven aspergillosis. *A. fumigatus* sensu stricto was the only species identified in section *Fumigati*, reinforcing the importance of this species in aspergillosis in penguins. The cryptic species *A. sydowii* from *Nidulantes* section was identified for the first time in this host. One *A. fumigatus* sensu stricto was resistant to posaconazol, but the mutations found in *cyp51A* gene of this isolate are not related with phenotypic resistance, suggesting that other mechanisms of resistance could be involved in the resistance of this isolate.

Introduction

Aspergillosis is considered the most important infection disease in captive penguins, and it has been considered a limiting factor for penguins' rehabilitation, in which the disease presents a proportionate mortality of 48.5%, and incidence density of 7.3% per 100 penguins/month [1,2]. As also observed in other hosts [3,4], the inhalation of fungal conidia is the main route of infection, and the respiratory system is the most affected in penguins [5].

Aspergillus section *Fumigati* is reported in up to 99% of aspergillosis' cases in penguins[1]. *Aspergillus* identification at section level is made by macro- and microscopic characteristics, and its identification at species level is currently possible only by molecular techniques [6]. Clinical implications in identify cryptic species (species from the same section that are difficult to distinguish phenotypically from each other) are associated with intrinsic azole resistance [7,8], and their differences concerning gliotoxin production, a mycotoxin capable to induce immunosuppression, which birds are particularly sensible [9,10].

Despite the intrinsic azole resistance that cryptic *Aspergillus* species can present, the emergence of azole-resistance in *A. fumigatus* has becoming a global concern, since the availability of effective antifungal drugs molecules is limited, resulting in increase of morbidity and mortality rates in aspergillosis disease, due to treatment failure [11–13]. It is also important to understand the molecular mechanisms involved in phenotypic resistance to an epidemiological perspective, since different mechanisms can suggest the origin of the resistance. Given that the main fungal target of azoles is the biosynthesis of ergosterol, mutations in *cyp*51 gene are reported as the most common molecular mechanisms involved in phenotypic azole resistance so far [14,15]. Most of these mutations

can be related to agriculture procedures, being most likely associated with cross resistance by the use of crop fungicides [16,17], or to long-term azole therapy patients [18,19].

The surveillance of *Aspergillus* azole-resistance has been recommended worldwide [20–25], however it is not a routine in veterinary medicine practice, even with the routine use of antifungals as prophylaxis and/or treatment option[1,26]. Specifically regarding the susceptibility patterns of *Aspergillus* isolated from avian species, few works have been published, most of them, with isolates from poultry [27–30]. In addition, no data concerning molecular identification at species level of *Aspergillus* isolates from Magellanic penguins are available so far. Thus, the aim of this work was to perform the molecular identification of *Aspergillus* at species level and to evaluate the susceptibility pattern of *A. fumigatus* sensu stricto with detection of mutations in *cyp51A* gene in clinical *A. fumigatus* strains isolated from Magellanic penguins with proven aspergillosis.

Material and Methods

Aspergillus isolates

A total of 37 *Aspergillus* strains, recovered from penguins clinical specimens between 2008 and 2019 (33 from captive penguins and four from free-ranging penguins)[1] were included in the study. Data regarding the origin of the animals (rehabilitation or free-living) and administrations of antifungal prophylaxis before death were also collected and recorded. The collected *Aspergillus* strains were stored at the fungal collection of the Mycology Laboratory of Universitary Hospital of Federal University of Rio Grande (HU-FURG), Rio Grande do Sul, Brazil.

Identification of Aspergillus at species level

Aspergillus strains were identified at section level by macro- and microscopic characteristics, and at species level by molecular techniques. The identification at section level classified the isolates as: 33 belonging to section *Fumigati,* 1 to section *Flavi*, 1 to section *Terrei* and 1 to section *Nidulantes* - complex *Versicolores* (previously known as section *Versicolores*).

Genomic DNA used in all molecular techniques described below was extracted with the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

The identification of *Aspergillus* at species level was performed by partial sequencing of calmodulin gene (*calM*) [31], using the primers cmd5 (5' CCG AGT ACA AGG AGG CCT TC 3') and cmd6 (5' CCG ATA GAG GTC ATA ACG TGG 3'), and/or partial sequencing of β-tubulin gene (*benA*) [32], using the primers β-tub1 (5' -AATTGGTGCCGCTTTCTGG-3'), and β-tub2 (5' -AGTTGTCGGGACGGAATAG-3'). PCR for both amplifications was carried out in a 25 µl volume containing 1× PCR buffer (Applied Biosystems Inc., Foster City, CA, USA); 1 mM $MgCl_2$ (Applied Biosystems); 0.1 mM each of dATP, dGTP, dCTP, and dTTP (Applied Biosystems); 0.2 µM of each primer; 1 U of Taq DNA polymerase (Applied Biosystems); and 20–50 ng of *Aspergillus* genomic DNA.

For partial *calM* amplification, PCR was carried out in a thermal cycler as follow: after an initial denaturation step of 95°C for 10 min, 38 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 7 min. For partial *benA* amplification, PCR was carried out as follow: after an initial denaturation step of 94°C for 2 min, 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. PCR products were confirmed by 2% agarose gel electrophoresis and the purification of the PCR product was made using the ExoSAP-IT enzyme system (USB Corporation, Cleveland, OH), according to the manufacturer's instructions.

Sequencing was performed using the forward primers the respective PCR products (cmd5 or β-tub1), with the BigDye terminator v 1.1 Cycle sequencing kit (Applied Biosystems) in the thermal cycler with the following conditions: an initial denaturation at 96°C for 5 s, 30 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min, and a final extension at 72°C for 5 min. Sequence analysis was performed using MEGA software version 10.0.5, and obtained sequences were compared with those deposited in the GenBank database (Bethesda, MD, USA) in order to achieve the identification to species level.

Detection of azole-resistant strains

The azole-resistance screening method was performed in all *A. fumigatus* sensu stricto strains based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) procedure [33,34]. Three different Sabouraud dextrose agar (SDA) plates were prepared containing 4 mg/L itraconazole (ICZ), 2 mg/L voriconazole (VCZ), and 0.5 mg/L posaconazole (PCZ) each one. A 0.5 McFarland turbidity inoculum suspension prepared from fresh conidia from a 5 -7-day-old

culture were inoculated by dipping a sterile swab into the inoculum suspension and swabbing the agar surface. Plates were incubated at 35ºC, and observation of fungal growth were performed in 48h. A pan-azole resistant strain with known TR_{34}/L98A mutation was used as resistance control, and the *A. fumigatus* strain ATCC 204305 was used as susceptible control. Also, each isolate suspension tested was inoculated in a plate containing Sabouraud dextrose agar without chloramphenicol and without any antifungal drug as growth control. Those isolates that were not able to growth in the plates with antifungal drugs were considered as "susceptible", and those that showed any growth in antifungal media, were selected for further investigations.

Broth microdilution antifungal susceptibility testing

The isolates that grew in screening agar plates were submitted to broth microdilution test according to M38 protocol of Clinical Laboratory Standards Institute (CLSI 3rd Edition) [35]. Tested antifungal concentrations ranged from 0.0156 to 8µg/ml for all drugs tested (ICZ, VCZ, PCZ). *A. flavus* strain ATCC 204304 was used as control of drugs' potency. Standard conidia inoculum was prepared from a conidia suspension with optical density ranging from 0.09 and 0.11 (80 to 82% transmittance) subsequently diluted in 1:50 in standard media (RPMI-1640). The inoculum control was performed by plating 100 µL of 10^{-2} dilution of the final suspension of standard conidia inoculum and incubated at 30ºC. Colony forming unit (CFU) has been verified after 48h incubation at 25ºC.

Tests were performed in a 96 well sterile microplate, in which 100 µL of the standard conidia inoculum was inoculated in each well containing 100 µL of the previous distributed drugs concentrations. In each microplate, sterile media control and the inoculum growth control was performed. Visual reading of the results was performed after 48h incubation at 35ºC determining the minimum inhibitory concentration (MIC) as the lowest concentration able to inhibit 100% of fungal growth. Tests were performed at least in duplicate, and in case of conflicting results, a triplicate was performed. Given that just epidemiological cutoff values (ECVs) were proposed for ICZ (1µg/ml), VCZ (1µg/ml), PCZ (0.25µg/ml) based on CLSI method [36], the determination of azole resistant strains followed the MIC breakpoints for ICZ (>2µg/ml), VCZ (>2µg/ml), and PCZ (>0.5µg/ml) proposed based on the EUCAST protocol [37].

Investigation of the mechanisms of resistance

Isolates that grew in screening agar plates were submitted to a multiplex real-time PCR assay for identification of azole resistance markers TR_{34}/L98H and TR_{46}/Y121F/T289A (AsperGenius®, PathoNostics, Maastricht, Netherlands), performed on the RotorGene Q instrument (Qiagen, Hilden, Germany), following the manufacturer's instructions.

In addition, the sequencing of *cyp51A* gene and its promoter was performed from an amplification reaction in a final volume of 25 µL consisting of 2,5X PCR buffer (Applied Biosystems Inc., Foster City, CA, USA); 2 mM $MgCl_2$ (Applied Biosystems); 0.2 mM each of dATP, dGTP, dCTP, and dTTP (Applied Biosystems); 0.4 µM of each primer [CYP51A-AF1 (5-ATGGTGCCGATGCTATGG-3) and CYP51AR2 (5'-AGTGAATAGAGGAGTGAATCC-3')]; 1 U of Taq DNA polymerase (Applied Biosystems); and 20–50 ng of *Aspergillus* genomic DNA. PCR reaction was performed by an initial denaturation step of 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, and a final extension at 72ºC for 7 min [38]. Purification was performed as described above. For each PCR product, 4 sequencing reactions was performed, using two forward primers [CYP51AF1 and CYP51AF2 (5'-GACATCTCTGCGGCAATGG-3')] and two reverse primers [CYP51AR2 and CYP51AR3(5'-CCATTGCCGCAGAGATGTC-3')] following the sequencing conditions described previously.

For sequencing of *cyp51A* promoter, the primers PA5 (5'TCTCTGCACGCAAAGAAGAAC3') and PA7 (5'TCATATGTTGCTCAGCGG3') [39], were used, following the same volume reactions as described to amplification of *cyp51*A. PCR conditions were an initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 30 s, 66°C for 45 s and 72°C for 2 min, and a final extension at 72°C for 7 min. PCR products confirmation, purification and sequencing were performed as described above, using both forward and reserve primers. The edition of nucleotide sequences, assembly of the consensus sequences and alignment were performed using MEGA software version 10.0.5.

Results

Molecular Identification of Aspergillus isolated from Magellanic penguins

In what concerns to the four clinical isolates of *Aspergillus* from free- ranging penguins, three were identified as *A. fumigatus* sensu stricto and one as *A. terreus* sensu stricto. Regarding

the 33 isolates from captive penguins, 31 were identified as *A. fumigatus* sensu stricto, one as *A. flavus* sensu stricto and one as a cryptic species from section *Nidulantes, A. sydowii*.

Susceptibility patterns and mechanisms of resistance

Thirty-four *A. fumigatus* sensu stricto isolates were screened for azole resistance in azole-supplemented agar media. From those, three showed some level of growth in screening plates, being selected for further characterization. In the microdilution broth test, one isolate showed a MIC=1 to PCZ, being considered as resistant (; the other two were considered susceptible for all the tested azoles.

No mutations were detected in the multiplex real-time PCR assay for azole resistance markers in any of the three selected isolates. Analyzing the nucleotide sequencing of *cyp51A* and its promoter, no mutations were found in two isolates and only the PCZ resistant strain showed several nucleotide mutations, resulting in four amino acids substitutions F46Y/N248T/D255E/M172V.

Antifungal prophylaxis (with itraconazole at 20mg/k for 15 days) was prescribed to 36% (n=12) of the captive penguins; 55% (n=18) had not received antifungal prophylaxis, and in 9% (n=3) no information regarding antifungal prophylaxis was available. The three isolates selected for further investigation regarding their possible resistance to azoles were all from captive penguins. Two of them were collected from penguins that had received antifungal prophylaxis, and one (which presented in vitro resistance to PCZ) was collected from a penguin that did not received any antifungal prophylaxis.

Discussion

In this work we performed the molecular identification of *Aspergillus*, and evaluated the susceptibility profile to ICZ, VCZ, and PCZ, with characterization of the molecular sequence of the *cyp51A* gene of *A. fumigatus* clinical isolates collected from Magellanic penguins. To the best of our knowledge, this is the first work regarding molecular identification of *Aspergillus* from Magellanic penguins with proven aspergillosis. This data is important for a better understanding the molecular epidemiology of this disease in these animals but also in the *One Health Context.*

Our study proved that the majority of aspergillosis cases diagnosed in penguins (91%) were caused by *A. fumigatus* sensu stricto, reinforcing the importance of this species as the main fungal pathogen for penguins, as already described for other birds and humans [40,41]. No cryptic species belonging to section *Fumigati* were identified in this study. Several factors may favor the infection of the animals by this species, including the higher thermotolerance of *A. fumigatus* sensu stricto [42], . In fact, the body temperature of these birds normally varies between 39 and 41 degrees, which favors the growth of *A. fumigatus* sensu stricto. Also, the higher level of gliotoxin production, is also attributed to its higher virulence [9,43]. Gliotoxin is a mycotoxin that presents immunosuppressive effects in hosts, which avian species are particularly sensible to.

Also, to the best of our knowledge, this is the first report of *A. sydowii* causing infection in a penguin. *A sydowii* is a cryptic species of the complex *versicolores*, included into the section *Nidulantes* according to the most recent taxonomic data [44]. Despite less common, this species had been identified in clinical samples from humans [45,46]. Also, this is the main etiological agent of *Aspergillus* diseases identified in sea fans from Caribbean sea [47–49]. This data reinforces the capacity of this agent be found in the sea, an essential environment of penguins life-cycle. Interestingly, the penguin that was diagnosed with aspergillosis by this *Aspergillus* species died in the first 24 hours after their admission in a Rehabilitation Center, suggesting that he was recovered already infected from nature.

The 3118 *A. fumigatus* sensu stricto isolate was resistant to PCZ and showed amino acids substitutions in *cyp51A.* Although already reported in a pan azole resistant isolate [50], the combination of these substitutions seems to not be related with azole-resistance, since they were also found several times in different susceptible strains [50–52]. Thus, probably any other mechanism such as in efflux pumps [53], or mutations in other genes [54–56] could be associated with its lower susceptibility to PCZ. None of the isolates showed phenotypic pan-azole resistance, and in accordance with these findings, no TR mutation was detected in real time PCR or in *cyp51A* sequencing.

Despite almost half of penguins had received antifungal therapy as prophylaxis, no phenotypic resistance was observed in the majority of the isolated *A. fumigatus* sensu stricto strains. In Magellanic penguins, guidelines for rehabilitation includes prophylaxis to aspergillosis, with itraconazole at 20mg/kg/day during 15 days from the beginning of the rehabilitation process [1]. In this context, the use of azoles as prophylaxis in these animals, as well as the treatment of

aspergillosis in captivity penguins, are very common [57,58]. The data obtained in this study suggest that the prophylaxis applied to these animals may not have been acting as a relevant factor to contribute to the emergence of resistance, probably because, unlike treatment, prophylaxis is not performed for a long time.

In this work we found that *A. fumigatus* sensu stricto is the most important etiologic agent of aspergillosis in penguins, and the identification of a cryptic species of section *Nidulantes* demonstrate the importance of the use of molecular techniques to the correct identification of *Aspergillus* at species level. These data are unprecedented in the scientific literature and important bases for a better understanding of the molecular epidemiology of aspergillosis in penguins. Also, we can suggest that prophylaxis performed in these animals during rehabilitation may not have implications on the emergence of resistance, and should be continued with caution and preferably together with the surveillance of the emergence of the resistance, since its use can be beneficial for better outcomes in the rehabilitation of penguins.

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4.4. ARTIGO I. Diphenyl diselenide and its interaction with antifungals against *Aspergillus* spp.

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Diphenyl diselenide and its interaction with antifungals against *Aspergillus* spp.

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Abstract

Given the few antifungal classes available to treat aspergillosis, this study aimed to evaluate the *in vitro* antifungal activity of diphenyl diselenide $(PhSe)_2$ alone and in combination with classical antifungals against *Aspergillus* spp., and its *in vivo* activity in a systemic experimental aspergillosis model. We performed *in vitro* broth microdilution assay of $(PhSe)_2$ against 32 *Aspergillus* isolates; and a checkboard assay to test the interaction of this compound with itraconazole (ITC), voriconazole (VRC), amphotericin B (AMB), and caspofungin (CAS), against 9 *Aspergillus* isolates. An experimental model of invasive aspergillosis in mice was studied, and survival curves were compared between an untreated group and groups treated with 100 mg/kg ITC, or $(PhSe)_2$ in different dosages (10 mg/kg, 50 mg/kg and 100 mg/kg). All *Aspergillus* non-*fumigatus* and 50% of *A. fumigatus* were inhibited by $(PhSe)_2$ in concentrations ≤64 μg/mL, with significant differences in MICs between the sections. Synergism or additive effect in the *in vitro* $(PhSe)_2$ interaction with VRC and CAS was observed against the majority of isolates, and with ITC against the non-*fumigatus* strains. In addition to the inhibitory interaction, $(PhSe)_2$ was able to add a fungicidal effect to CAS. Survival curves from the systemic experimental aspergillosis model demonstrated that the inoculum caused an acute and lethal infection in mice, and no treatment applied significantly prolonged survival over that of the control group. The results highlight the promising activity of $(PhSe)_2$ against *Aspergillus* species, but more *in vivo* studies are needed to determine its potential applicability in aspergillosis treatment.

Key words: selenium, *Aspergillus*, aspergillosis, synergism, checkerboard, animal model

Lay summary

The activity of diphenyl diselenide $(PhSe)_2$ alone and in combination with itraconazole, voriconazole, and caspofungin, is described against three of the most pathogenic *Aspergillus* sections. $(PhSe)_2$ may prove useful in therapy of infection in future; further study is required.

Introduction

Aspergillosis is a major opportunistic fungal disease, and invasive aspergillosis is the most severe common mycosis, reaching mortality rates of 90% in high-risk groups.[1] The availability of drugs to treat fungal diseases is limited. Azoles or amphotericin B, which are the first choices for antifungals in the treatment of aspergillosis, are potentially toxic to animals and humans. Echinocandins, which act on the fungal cell wall and have less toxicity to mammalian hosts, are not fungicidal and require parenteral administration.[1]

Moreover, susceptibility to classical antifungals differs between sections and species of *Aspergillus*. For example, *A. lentulus*, a cryptic species of *Aspergillus* section *Fumigati*, has intrinsic resistance to azoles and amphotericin B;[2] *A. felis* shows high MIC values to voriconazole;[3] *A. terreus* commonly has intrinsic resistance against amphotericin B;[4] and some cryptic species of *Aspergillus* section *Nigri* have high itraconazole MIC values.[5]

Beyond the intrinsic resistance observed in some species of *Aspergillus*, there is a global concern about the emergence of acquired resistance, mainly in *Aspergillus fumigatus sensu stricto,* with an incidence rate ranging from 0.6 to 4.2% and a prevalence rate of 3.2% of resistance to triazoles, according to a multicenter international surveillance network.[6] In some countries and patient groups, such as in hematopoietic stem cell transplant patients in Germany, *Aspergillus* resistance to triazoles can even reach an incidence of 30%.[7]

Thus, searching for new antifungal drugs is necessary. Organoselenium compounds have shown promising antimicrobial activity.[8,9] Among these compounds, diphenyl diselenide $(PhSe)_2$

is a simple and chemically stable molecule with proven low toxicity to animals.[10,11] The distribution of $(PhSe)_2$ tends to accumulate in adipose tissue, liver, kidneys, lungs and brain, in rats and mice.[12] The acute lethal dose (LD_{50}) of $(PhSe)_2$ in rats by the intraperitoneal route is 1200 μmol/kg,[13] and for a single dose orally administrated in mice is estimated to be >312 mg/kg.[14] Although not fully defined so far, it is supposed that $(PhSe)_2$ interacts with sulfhydryl groups and has as its main mechanisms of action a pro-oxidative activity for microorganisms owing to glutathione depletion.[15] Against fungal species, $(PhSe)_2$ had demonstrated *in vitro* activity against *Sporothrix brasiliensis*, *Fusarium* spp., *Candida* spp., *Cryptococcus* spp.,.[9,16–18]

Given the need for new antifungal drugs to treat aspergillosis, and the good *in vitro* activity of $(PhSe)_2$ as an antifungal drug against other species of fungi, the aim of this study was to evaluate the *in vitro* antifungal activity of diphenyl diselenide alone and in combination with classical antifungals against *Aspergillus* spp., and its *in vivo* activity in a systemic experimental aspergillosis model.

Methods

1. In vitro assays

1.1.Compounds tested

The synthesis of $(PhSe)_2$ was performed according to the Paulmier method,[19] in the Chemistry Department of the Federal University of Santa Maria (UFSM), Brazil. Spectral analyses were performed for ^{1}H NMR and ^{13}C NMR to evaluate whether the synthesized compound presented analytical and spectroscopic data according to the structure expected for $(PhSe)_2$, and 99.9% chemical purity was determined by GC/HPLC.

Itraconazole (ITC; Janssen Pharmaceutica N.V., Beerse, Belgium), voriconazole (VRC; Pfizer Inc., NY, USA), caspofungin (CAS; Merck & Co. Inc, Whitehouse Station, NJ, USA), and

amphotericin B deoxycholate (AMB; Bristol-Myers Squibb Co., Princeton, NJ, USA) were obtained as powders. Stock solutions were prepared in 100% dimethyl sulfoxide (DMSO) for azoles or AMB, or in sterile de-ionized water for CAS. Stock solutions of each drug were kept at −70° C.

1.2 Isolates tested

Thirty-two clinical isolates of *Aspergillus* spp. from the fungal collection of the Mycology Laboratory of the Medicine Faculty of Federal University of Rio Grande, Rio Grande do Sul, Brazil, were included in the *in vitro* study. Among the isolates, 20 were *Aspergillus fumigatus sensu stricto* (confirmed by sequencing of partial *benA* and partial *calM* genes),[20] 7 were *Aspergillus* section *Nigri* and 5 were *Aspergillus* section *Flavi* (both sections confirmed by macro- and microscopic characteristics).

1.3 Microdilution assay

Microdilution broth assay was performed according to the M38-A2 protocol of Clinical and Laboratory Standards Institute (CLSI).[21] The final inoculum concentration of 0.8 x 10^4 to 10^5 Colony Forming Units (CFU) per ml in RPMI 1640 with MOPS was standardized with conidia from young *Aspergillus* spp. colonies by spectrophotometer measurement (530 nanometres) and confirmed by pour plate method. Seven serial dilutions of $(PhSe)_2$ in DMSO (final concentration 1%) were tested, ranging from 1 to 64 µg/ml. Microplates were filled with 100 µl of inoculum and 100 µl of concentrations of the compounds and incubated for 48 hours at 35ºC. Minimum Inhibitory Concentration (MIC) was determined by visual reading as the lowest concentration able to inhibit 100% of the fungal growth (clear tube). All isolates were tested four times, in a duplicate row in each microplate and in a duplicate experiment.

1.4 Checkerboard assay

Nine of the 32 isolates tested were randomly chosen to perform the checkerboard assay,[22] including 5 *Aspergillus fumigatus sensu stricto*, 2 *Aspergillus* section *Nigri* and 2 *Aspergillus* section *Flavi*. The inoculum was standardized as previously described in section 1.3. Drug concentrations ranged from 1 to 64 µg/ml for $(PhSe)_2$; from 0.125 to 8 for ITC and VRC; from 0.015 to 8 for CAS; and from 0.03 to 16 for AMB. DMSO was used for the $(PhSe)_2$, VRC and ITC dilutions (final DMSO concentration 1%), and sterile de-ionized water for CAS and AMB dilutions. *A. fumigatus* 10AF strain and *Candida kefyr* strain SA were used as quality control.[23]

In brief, 50 µl of drug A (diphenyl diselenide) at various concentrations were distributed in the rows and 50 µl of drug B (antifungal) in the columns for the interaction. Then, 100 µl of standardized inoculum were added to the wells. Microplates were incubated for 48 hours at 35ºC for reading of MICs. Fractional Inhibitory Concentration index (FICi) was determined by the equation: FICi = (MIC_A in combination/MIC_A tested alone) + (MIC_B in combination/MIC_B tested alone). All assays were done in duplicate.

To determine the Minimum Fungicidal Concentration (MFC), 50 µl of each well without visual growth was plated on Sabouraud agar and incubated at 35ºC for five days. MFC was considered the minimal concentration of the drug resulting in killing ≥99% of the inoculum. The Fractional Fungicidal Concentration index (FFCi) was calculated and interpreted in the same way as described for FICi.

The interactions between the compound and the drugs was classified as strong synergism when FICi or FFCi <0.5; weak synergism when 0.5 ≤ FICi or FFCi <1; additive when 1 ≤ FICi or FFCi < 2; indifferent when FICI or FFCi =2; and antagonistic when FICi or FFCi >2[24].

1.5 Statistics

Descriptive analyses were used to calculate MIC_{50} (lowest concentration capable of inhibiting 50% of fungi), MIC_{90} (lowest concentration capable of inhibiting 90% of fungi) and the

geometric mean of $(PhSe)_2$ MIC for each *Aspergillus* section tested. Values of MICs from isolates with MIC higher than the maximum concentration tested were considered for analyses to be one dilution higher. The Kruskal-Wallis test was performed to compare the *in vitro* inhibitory activity of $(PhSe)_2$ with respect to the section of the isolates (*A.* section *Fumigati*, *A.* section *Flavi* and *A*. section *Nigri*). Statistical program SPSS 20.0® (IBM Corp., Armonk, NY) was used, and p-values <0.05 were considered statistically significant.

2. In vivo experiment

2.1. Animals

An animal model of systemic aspergillosis was studied as described previously[25–28]. Forty female CD-1 mice, six weeks old (Charles River Laboratories, Hollister, CA, USA), weighing approximately 25 grams each, were arranged in 4 groups of 10 animals (five in each cage). Food and water were provided *ad libitum.* Animal experimentation was done with the approval of the Institutional Animal Care and Use Committee of the California Institute for Medical Research.

2.2. Strain and Infection

Young colonies of *A. fumigatus* 10AF strain, sub-cultured in Sabouraud agar for 7 days at 35ºC, were used for inoculum standardization.[25] Briefly, conidia from the surface of the colonies were gently harvested, by adding saline with Tween 80 (0.05%) and transferred to a 15 ml tube. The solution was adjusted to a concentration of 4×10^7 conidia/ml in a hematocytometer chamber, and confirmed by CFU counting on Sabouraud dextrose agar. Mice were infected by intravenous injection of 250 µl of the inoculum, resulting in a concentration of 10^7 conidia/mouse (Day 0).

2.3. Treatments

All mice received oral treatment, by gavage (250 µl once a day) for 10 days, starting at day 1 (24 hours post-infection). Canola oil had been used as the diluent to adjust the distinct

concentrations of $(PhSe)_2$. Treatment experimental groups were organized as follow: **G1**: canola oil (untreated control group); **G2**: 100 mg/kg ITC; **G3**: 10mg/kg $(PhSe)_2$; **G4**: 50 mg/kg $(PhSe)_2$; **G5**: 100 mg/kg $(PhSe)_2$.

Mice were evaluated daily for eleven days to determine the survival curves in each treatment group. Evaluation of survival was done by using a log-rank test and survival curves compared by the non-parametric Wilcoxon test using the statistical program, Prism® (version 7.00 for Windows, GraphPad Software, La Jolla California USA).

Results

1. Microdilution and Checkerboard assays

All *Aspergillus* section *Nigri* and section *Flavi* isolates tested (n=12) were inhibited by $(PhSe)_2$ in the concentration ranges tested. However, 50% of the *A. fumigatus* isolates (10/20) were able to grow even in the highest concentration of the compound (MIC >64 µg/mL). MIC_{50} and MIC_{90} against all *Aspergillus* spp. isolates ranged from 16 to 64 µg/mL and from 16 to >64 µg/mL, respectively. *A. fumigatus* showed the highest MIC geometric mean value (84.4 µg/mL) with MIC values ranging from 16 to >64 µg/mL, and *A.* section *Nigri* showed the lowest MIC geometric mean value (16 µg/mL) with MIC values ranging from 8 to 32 µg/ml; ($p<0.001$) (Table 1).

Synergism or additive inhibition effect in the *in vitro* $(PhSe)_2$ interaction with VRC were found against all *A. fumigatus,* and with ITC were found against non-*fumigatus* isolates (3/4). $(PhSe)_2$ interaction with CAS was also positive, resulting in an additive effect in 67% (6/9) of the isolates tested. On the other hand, $(PhSe)_2$ was indifferent (IND) or even worsened AMB antifungal activity against all *Aspergillus* sections, and also the ITC activity against *A. fumigatus* isolates (Table 2). In addition, both synergic and antagonistic effects were sometimes found in different wells of the checkerboard matrix for an isolate (asymmetric result; these were characterized as

indifferent in the interpretation). Regarding fungicidal activity, a beneficial effect was seen in the $(PhSe)_2$ interaction with CAS, showing synergism in more than half of the isolates (6/9) (Table 3).

3. In vivo assay

None of the treatments applied in this study was able to significantly prolong survival over that of the untreated controls, or to protect mice from deaths in this severe systemic aspergillosis study. Deaths started to occur day 3 post-infection, and one day before the end of the experiment all mice had already died (Figure 1). Although the lowest dosage of $(PhSe)_2$ (10mg/kg/day; G3 group) produced a better survival curve than that for mice from the untreated group (G1), this difference was at the limit of statistical significance ($p=0.051$). On the other hand, survival curves of groups that had received high dosages of $(PhSe)_2$ (50 mg/kg/day and 100 mg/kg/day) were similar to those found in the untreated group ($p>0.05$), but significantly worse than those found in the ITC group ($p=0.04$ and 0.03, respectively), and in the $(PhSe)_2$ at 10 mg/kg/day ($p=0.008$ and 0.007, respectively).

Discussion

Our study shows for the first time the *in vitro* organoselenium compound $(PhSe)_2$ activity against three pathogenic *Aspergillus* sections and its interaction with azoles (VRC and ITC), CAS, and AMB, as well as study in a murine model of systemic aspergillosis. Inorganic selenium is a trace element with an important role in human and animal nutrition due to its biological activity, however it is more toxic to mammals than selenium in its organic forms.[29,30] In this context, several organoselenium compounds have been studied, showing a diversity of beneficial biological effects and pharmacologic potential for mammalian hosts, such as hepatoprotection, antinociceptive, anti-inflammatory and antioxidant effects.[31,32]

Its antimicrobial activity has also been proposed, with *in vitro* studies suggesting potential to be used against bacteria, such as *Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli, Staphylococcus aureus*.[33–35] Our MIC results corroborate the potential of $(PhSe)_2$ to be used as an antifungal drug, as already shown with other fungi, including yeasts *Candida glabrata* and *Cryptococcus* spp.,[17,36] dimorphic fungi such as *Sporothrix brasiliensis,*[9] and moulds such as *Fusarium* spp..[8] Moreover, our results are similar to some described in other *in vitro* studies of organoselenium compounds against a few isolates of *Aspergillus* species.[16,35,37,38] Given that different species can present different antifungal susceptibility patterns,[2,5] the lack of identification at species level of isolates from sections *Flavi* and *Nigri* was a limitation of our study. We utilized literature information regarding the main species of these sections (*A. flavus* and *A.niger*) for our discussion.

Since the probable mechanism of action of $(PhSe)_2$ is via glutathione depletion, culminating in reactive oxygen species (ROS) accumulation and consequently leading the fungi cells to apoptosis,[33,39,40] the variation in the concentration of melanin in the fungal cell wall could explain the major resistance of *A. fumigatus* to $(PhSe)_2$ found in our study in comparison to the other *Aspergillus* sections. Some studies had reported higher concentrations of melanin in *A. fumigatus* than in *A. flavus* and *A. niger*.[41,42] It is well documented that melanized cells have higher antioxidant properties, which can improve the antifungal resistance, than non-melanized cells.[43,44] In addition, *A. fumigatus* is efficient in upregulating the production of important enzymes involved in a detoxification process, and its conidia are capable of tolerating hydrogen peroxide in concentrations up to 15 mM.[45,46]

On the other hand, *A. niger*, which produces many metabolites, such as citric acid,[47] also produces more oxalic acid than *A. fumigatus* and *A. flavus.*[48] The production of these metabolites by *A. niger* is related to the formation of hydrogen peroxide.[49] In this sense, the significantly lower

MICs of $(PhSe)_2$ against *A. niger* in comparison with other *Aspergillus* sections could be attributed to the excessive generation of ROS by this section, associated with detoxication inability due to the depletion of antioxidant enzymes as glutathione.

Positive interaction of $(PhSe)_2$ with VRC against *A. fumigatus* isolates, and with ITC against *non-fumigatus* sections deserves more investigation since VRC is a commonly used drug for treating invasive aspergillosis. Among diseases caused by *A. niger* and *A. flavus,* non-invasive and chronic aspergillosis, is more commom than invasive disease for which ITC is a therapy of choice for treatment.[50] On the other hand, an *in vitro* antagonism of the compound with AMB was observed against all isolates tested. Besides the well-known action of AMB on preformed ergosterol, it is also known that production of ROS in fungi cells is an additional mechanism of action of this drug.[51] Thus, a possible opposing competition between the two drugs for the same target could have occurred.

Interestingly, a lesser inhibition was clearly observed in some fungal isolates in the wells with AMB or ITC interacting with low concentrations of $(PhSe)_2$ in comparison to the same concentration of the antifungals alone. Similar behavior was observed with sub-inhibitory concentrations of biogenic selenium nanoparticles against *A. niger*.[52] It is possible that in this case, the fungal cells could immobilize the selenium present in the medium and use it to their own benefits, contributing to its better growth in the presence of an otherwise inhibitory action of the classical antifungal.[53]

Our study showed another important interaction occurring between $(PhSe)_2$ and CAS, an antifungal drug that targets the fungi cell wall, more specifically 1,3 beta-D-glucan synthesis.[54] The combination of these two drugs not only decreased the CAS MIC, but also promoted greater inhibition, even in the CAS combination with the lowest concentrations of $(PhSe)_2$, in all isolates tested. In addition, fungicidal synergism found in the interaction of the drugs is promising, since

CAS alone does not have fungicidal activity against *Aspergillus*. A positive interaction between CAS and AMB against *Aspergillus* spp. was previously reported,[55] possibly supporting the hypothesis that $(PhSe)_2$ and AMB have similar mechanisms of action in the production of ROS. Thus, although $(PhSe)_2$ can be antagonistic to AMB, in combination with CAS or azoles (VRC or ITC) $(PhSe)_2$ presents a positive interaction, presumably because they have different mechanisms of action that can be complementary to each other in antifungal therapy.[56]

The survival curve of our *in vivo* experiment shows that the inoculum caused an acute, severe and lethal infection in mice. In this aggressive model of systemic aspergillosis, $(PhSe)_2$ was barely able to significantly alter the mortality. Since ITC, a sometimes effective drug against aspergillosis,[57] also had negligible effect on the survival rate, other *in vivo* studies with a chronic or less acutely lethal model of infection are necessary to investigate the possible beneficial activities of $(PhSe)_2$ alone. Such models would be relevant to more chronic forms of clinical aspergillosis, including allergic forms. Now that we have defined the nature of $(PhSe)_2$ drug interactions *in vitro*, studies *in vivo* of combination with classical antifungals should also proceed.[58]

Although low toxicity of $(PhSe)_2$ had been reported, even in comparison with other organoselenium compounds,[10,11,59] the toxicity of $(PhSe)_2$ to mice is greater than to rats;[30,59] therefore, the acute toxicity in mice may be due to the faster metabolism of the compound in the liver.[30] The LD_{50} of $(PhSe)_2$ orally administrated in mice is estimated to be >312 mg/kg.[14] However, the toxicity is dose-dependent and toxicity tests in high dosages had not been performed in association with study of fungal infections. It is known that *Aspergillus* produces toxins, many of them with cytotoxic effects on mammals, such as gliotoxin, trypacidin, fumiquinazolins, among many others.[60] In this context, the significatively higher mortality rate observed in the groups receiving the highest $(PhSe)_2$ dosages (50mg/kg and 100mg/kg), corresponding to 80% at day 3

post-infection, in contrast to the lower $(PhSe)_2$ dose, may be a result of the toxicity of $(PhSe)_2$ for mice associated with the cytotoxic effects of fungal infection.

Given the lowest $(PhSe)_2$ MICs found against *A.* section *Nigri*, additional tests regarding studies using an experimental model of infection with this *Aspergillus* section are indicated. In addition, given the better results of the CAS in the *in vitro* interaction assay, and promising preliminary results suggesting decreased mortality in the *in vivo* assay combining ITC and $(PhSe)_2$, that requires confirmation [data not shown], indicate additional studies regarding the *in vivo* interaction of these drugs against aspergillosis should be performed. Presently, combination of drugs in antifungal therapy is indicated in some cases of invasive fungal infections,[56] therefore, $(PhSe)_2$ should be investigated in this context, since, suggested by some *in vitro* results, it might add options to failing antifungal therapy.

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Tables and Figure Legends

Table 1: Results of microdilution assay for $(PhSe)_2$ against 32 *Aspergillus* spp. clinical isolates.

Table 2: Results of inhibition interactions between classical antifungal drugs and $(PhSe)_2$ against *Aspergillus* spp. isolates (n=9) in the checkerboard assay.

Table 3: Fungicidal results, interaction between classical antifungal drugs and $(PhSe)_2$ against *Aspergillus* spp. isolates (n=9).

Figure 1: Survival rate of an invasive aspergillosis murine model (10 mice per group challenged intravenously with a high-inoculum of 10AF *Aspergillus fumigatus* strain) according to treatment group.

Table 1: Results of microdilution assay for $(PhSe)_2$ against 32 *Aspergillus* spp. clinical isolates.

***Aspergillus* section**	**MIC GM (range)** µg/mL	**MIC_{50}** µg/mL	**MIC_{90}** µg/mL
Fumigati (n=20)	84.4 (16- >64)	64	>64
Flavi (n=5)	36.7 (16- 64)	32	64
Nigri (n=7)	16 (8- 32)	16	16
All isolates (n=32)	47.2 (8- >64)	64	>64

MIC: Minimal Inhibitory Concentration; GM: geometric mean; MIC_{50}: MIC value inhibiting 50% of the isolates tested; MIC_{90}: MIC value inhibiting 90% of the isolates tested.

Table 2: Results of inhibition interactions between classical antifungal drugs and $(PhSe)_2$ against *Aspergillus* spp. isolates (n=9) in the checkerboard assay*.

Aspergillus Section	ISOLATE ID	$(PhSe)_2$		VRC				$(PhSe)_2$		ITC				$(PhSe)_2$		AMB				$(PhSe)_2$		CAS			
		MIC alone	MIC comb	MIC alone	MIC comb	FICi	IN	MIC alone	MIC comb	MIC alone	MIC comb	FICi	IN	MIC alone	MIC comb	MIC alone	MIC comb	FICi	IN	MIC alone	MIC comb	MIC alone	MIC comb	FICi	IN
Fumigati	10 AF	32	8	0.5	0.125	0.5	WS	32	32	1	2	2.06	AN	64	64	1	2	2.01	AN	64	32	0.5	0.5	1.5	AD
	1066	16	8	0.5	0.25	1	AD	16 16	8 2	0.5 0.5	0.125 1	0.75 2.12	IND**	64	32	1	2	2.5	AN	64	16	0.5	0.5	1.25	AD
	1517	≥64	8	1	0.5	0.56	WS	64	64	2	2	2	IND	64	4	1	2	2.06	AN	64	64	0.5	0.5	2	IND
	2007	64	8	2	0.125	0.185	SS	64 64	1 >64	1 1	0.5 0.25	0.51 ≥2.25	IND**	64	16	0.5	1	2.25	AN	64	8	0.5	0.5	1.125	AD
	2507	32	8	1	0.5	0.75	WS	32	32	1	1	2	IND	64	16	1	2	2.25	AN	64	32	0.5	0.5	1.5	AD
Flavi	3844	32	16	1	2	2.5	AN	32	1	1	0.5	0.53	WS	32 32	16 1	2 2	0.038	0.51 4.03	IND**	32	16	0.25	0.125	1	AD
	5230	32	32	2	2	2	IND	32	1	1	0.5	0.53	WS	32 32	16 1	2 2	0.034	0.51 2.03	IND**	32	32	0.25	0.25	2	IND
Nigri	3585	8	8	2	2	2	IND	8	8	1	1	2	IND	8	2	0.25	2	8.25	AN	8	8	0.25	0.25	2	IND
	5335	8	4	2	1	1	AD	16	8	2	0.125	0.56	WS	8 8	4 2	1 1	0.125 4	0.62 4.25	IND**	8	2	0.25	0.25	1.25	AD

$(PhSe)_2$: diphenyl diselenide; VRC: Voriconazole, ITC: Itraconazole, AMB: Amphotericin B, CAS: Caspofungin; MIC: Minimal Inhibitory Concentration; FICi: Fractional Inhibitory Concentration index; IN: Interpretation. SS: strong synergism; WS: weak synergism; AD: additive; IND: indifferent; AN: antagonism. *MIC expressed as µg/ml. ** a few wells showed a synergistic interaction, and others, antagonism, in a test of the same isolate.

Table 3: Fungicidal results*, interaction between classical antifungal drugs and $(PhSe)_2$ against *Aspergillus* spp. isolates (n=9).

Aspergillus Section	Isolate ID	$(PhSe)_2$		VRC				$(PhSe)_2$		ITC				$(PhSe)_2$		AMB				$(PhSe)_2$		CAS			
		MFC alone	MFC comb	MFC alone	MFC comb	FFCi	IN	MFC alone	MFC comb	MFC	MFC comb	FFCi	IN	MFC alone	MFC comb	MFC alone	MFC comb	FFCi	IN	MFC alone	MFC comb	MFC alone	MFC comb	FFCi	IN
Fumigati	10 AF	≥64	≥64	8	8	2	IND	≥64	≥64	8	8	2	IND	64	16	1	8	8.25	AN	64	32	>8	2	≤0.62	WS
	1066	≥64	≥64	8	8	2	IND	≥64	≥64	>8	>8	2	IND	64	1	4	8	2.01	AN	64	16	>8	1	≤0.37	SS
	1517	≥64	≥64	8	8	2	IND	≥64	2	4	8	2.01	AN	64	2	1	4	4.03	AN	≥64	64	>8	1	≤0.56	WS
	2007	≥64	≥64	8	8	2	IND	≥64	≥64	8	8	2	IND	64	1	0.5	1	2.01	AN	64	32	>8	0.5	≤0.56	WS
	2507	≥64	1	4	8	2.01	AN	≥64	≥64	>8	>8	2	IND	64	4	1	4	4.06	AN	64	32	>8	1	≤0.61	WS
Flavi	3844	32	1	1	4	4.01	AN	32	1	1	0.5	0.53	WS	32 32	16 1	4 4	0.03 8	0.5 2.01	IND**	64	16	>8	0.125	≤0.26	SS
	5230	32	32	2	2	2	IND	32	1	1	0.5	0.53	WS	32	16	4	0.03	0.5	WS	64	16	>8	1	≤0.37	SS
Nigri	3585	8	8	2	2	2	IND	8	8	2	2	2	IND	8	2	0.25	2	4.25	AN	8	8	>8	>8	2	IND
	5335	16	4	2	1	0.75	WS	16	4	2	8	4.25	AN	16	2	1	4	4.12	AN	16	2	>8	0.25	≤0.15	SS

$(PhSe)_2$: diphenyl diselenide; drugs: VRC: Voriconazole, ITC: Itraconazole, AMB: Amphotericin B, CAS: Caspofungin; MFC: Minimal Fungicidal Concentration; FFCi: Fractional Fungicidal Concentration index; IN: Interpretation; SS: strong synergism; WS: weak synergism; AD: additive; IND: indifferent; AN: antagonism. *MFC expressed as µg/ml. ** a few wells showed a synergistic interaction, and others, antagonism, in a test of the same isolate.

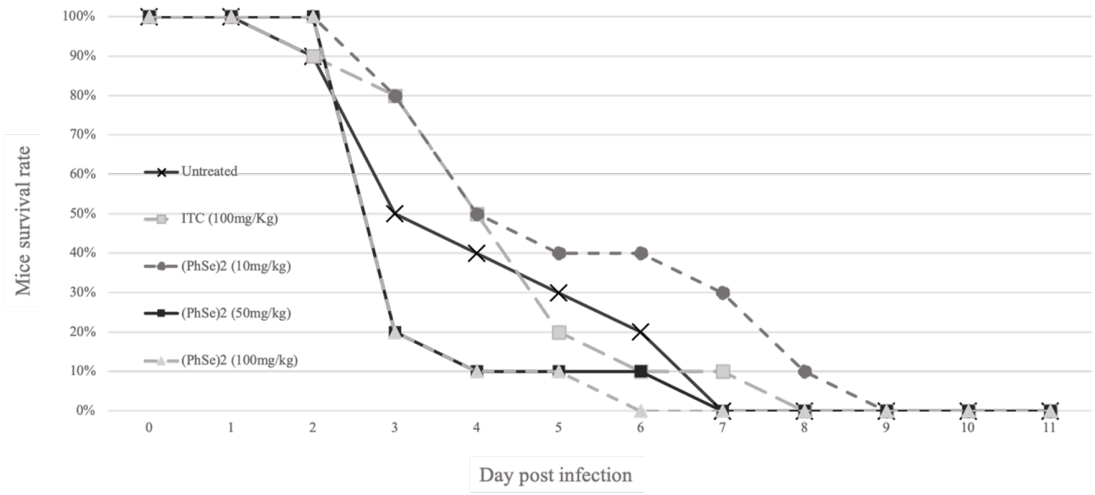

Figure 1: Survival rate of an invasive aspergillosis murine model (10 mice per group challenged intravenously with a high-inoculum of 10AF *Aspergillus fumigatus* strain) according to treatment group.

4.5. ARTIGO II. Aspergillosis in albatrosses

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Aspergillosis in albatrosses

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Abstract

Aspergillosis is a respiratory fungal disease of importance in captive marine birds. The aim of this study was to describe the occurrence of aspergillosis in *Thalassarche melanophris* during rehabilitation events, and to identify the etiological agent. All the albatrosses that were received for rehabilitation and died within a two year period were included in the study. The proportionate mortality rate caused by aspergillosis was 21.4% (3/14). One of the etiological agents was *Aspergillus flavus/oryzae* lineage and the other two were *A. fumigatus sensu stricto*. Our study suggests that aspergillosis can act as a limiting factor in the rehabilitation of albatrosses.

Keywords: *Aspergillus fumigatus sensu stricto*, procellariiformes, seabirds, molecular identification, *Aspergillus* section *Flavi*.

Aspergillosis is a respiratory fungal disease of great importance in captive marine birds.[1,2] However, there are few reports about *Aspergillus* infection in procellariiformes, probably because rehabilitation of a large group of albatrosses on a single occasion is not common.[3]

Albatrosses are marine birds with a long life (ranging between 30 and 80 years depending on the species), a high adult survival rate and delayed sexual maturity.[4] The waters of the southern region of Rio Grande do Sul, Brazil, are usually feeding areas for individuals of *Talassarche melanophris* and *Talassarche chlororhynchos* among other procellariforms, and interaction with fishing activities is currently one of the main threats to these birds.[5]

Currently, the definitive identification of *Aspergillus* species requires the addition of molecular methods.[6] The knowledge of the causative agent of aspergillosis is not only epidemiologically relevant, but also clinically important, since different species belonging to the same section show different characteristics of virulence, including gliotoxin production, and resistance to antifungal agents.[7,8]

The study of aspergillosis in marine birds in captivity is important to understand the magnitude of its impact on these animals, allowing the establishment of preventive protocols according to the needs and management of each species.[1] A rare event of the rehabilitation of a large group of albatrosses was seen as an opportunity to determine the occurrence and relevance of aspergillosis in these seabirds. Therefore, the aim of this study was to describe the occurrence of aspergillosis in *T. melanophris* during rehabilitation events, and to identify the etiological agent at the species level by molecular techniques.

In this context, all the albatrosses (*T. melanophris* and *T. chlororhynchos*) that were received for rehabilitation (n=32) and died within a two year period (2015–2017), at the Rehabilitation Center of Marine Animals of Federal University do Rio Grande (CRAM-FURG), in Rio Grande, Rio Grande do Sul, Brazil (32º01’34” S, 52º06’21” W), were included in this study (S1).

Necropsy was performed in all birds to evaluate macroscopic alterations. Samples of lesions, lungs, trachea and/or air sacs of all animals were collected for histopathological analyses and also submitted for mycological culture onto Sabouraud Dextrose Agar at 30ºC for 7 days, in triplicate. Albatrosses that showed gross lesions at necropsy, associated with the isolation of *Aspergillus* sp. in the mycological culture as well as visualization of hyaline, septate and 45º branched hyphae in the tissue fragments in the

histopathological analysis were classified as having aspergillosis.

In order to perform molecular identification, DNA extraction was performed according to Woods et al., (1993)[9], the amplification of partial *benA* was carried out according to Staab et al., (2009),[10] and where *benA* sequencing did not identify isolates to species level, partial *calM* sequencing was carried out according to Hong et al., (2005).[11] Sequencing of samples was performed using an automatic sequencer. The resulting data collection files were analyzed using MEGA software version 7.0 and the species homology was defined comparing the nucleotide sequences by BLAST on the website https://blast.ncbi.nlm.nih.gov/Blast.cgi.

To determine itraconazole susceptibility, the broth microdilution technique was performed according to the M38-A2 protocol of Clinical and Laboratory Standards Institute (CLSI, 2008),[12] in duplicate, and the standard strain Af 71 (kindly provided by Prof. Dr. David W. Denning, National Aspergillosis Centre, UK) was used as quality control to validate the test results.

In a two-year period, 14 albatrosses died during rehabilitation (12 *T. melanophris* and 2 *T. chlororhynchos*), which represents a 56% rehabilitation success. Among dead birds, three were identified as aspergillosis cases. The proportionate mortality rate of aspergillosis was 21%, and all animals diagnosed with aspergillosis belonged to the species *T. melanophris* (Table 1).

Table 1 Data from the aspergillosis cases in albatrosses

Avian Number	Sex	Age (Y)	Body weight (g)	Captive time (days)	Causative agent	Lesion place	Macroscopic lesions	Microscopy		
								Ce	N	P
3177	M	1	2224	18	*A. fumigatus stricto sensu*	D	G; FC	He Mo CE	3	H FB

3179	F	1	2448	14	*A. section Flavi*	R	G	He Mo	2	H FB
3380	F	1	2216	10	*A. fumigatus stricto sensu*	R	G; FC	Mo	2	H

Legend: Sex: Male (M), Female (F); Lesion place: Disseminated (D), Respiratory system (R); Macroscopic lesions: Granuloma (G), Fungal colony (FC); Microscopy: Cell (Ce) – Heterophils (He), Macrophages (Mo), Epithelioid cells (CE); Necrosis (N) 0- absent, 1- scarce, 2 –moderate, 3- exhuberant; Pattern: Hyphal (H), Fungal ball (FB)

Macroscopic lesions were predominantly located in the respiratory system, with white-yellow granulomatous nodules ranging between 0.1 and 0.5 cm diffusely distributed in the lungs and air sacs. Two birds showed fungal colonies in the bronchi, and one bird also had white-yellow granulomatous nodules in the kidneys (S2). Only one albatross had clinical signs before death, including dyspnea, loss of appetite and regurgitation.

In the histopathological analysis, tissue invasion by fungal hyphae were observed inside the lesions, which were visualized by Hematoxylin and Eosin (HE) supported by Periodic Acid-Schiff (PAS) and Grocott (S3) stains, in addition, foci of necrosis and presence of inflammatory infiltrates were observed (Table 1).

The other 11 birds did not show anatomo-pathological alterations, and the mycological examinations of their lung samples were negative. Thus, the *causa mortis* of these animals was not conclusive.

Aspergillus section *Flavi* was isolated in one bird, while *Aspergillus* section *Fumigati* was isolated in the two other birds. The molecular identification of the isolates

belonging to section *Fumigati* shows that both sequences showed 100% homology with *Aspergillus fumigatus sensu stricto* (reference sequence n° LC377774.1 of GenBank). Regarding the *Aspergillus* section *Flavi* isolate, in addition to partial *benA* sequencing, partial *calM* sequencing was performed. Even so, the identification of this isolate at species level was not conclusive. A phylogenetic relationship analyses showed that the isolate is more closely related with *A. flavus sensu stricto* [reference sequences of GenBank nº MG517619.1 (*benA)* and KJ175539.1 (*calM*)] and *A. oryzae* [reference sequences of GenBank nº MH 279883.1 (*benA)* and MH279853.1 (*calM*)] than with other species belonging to *A. flavus* clade (Figure 1). The obtained sequences were deposited in NCBI databases [GenBank Accession numbers MN244293, MN244294 (*Fumigati*) and MN244295 (*benA*) and MN422267 (*calM*) (*Flavi*)].

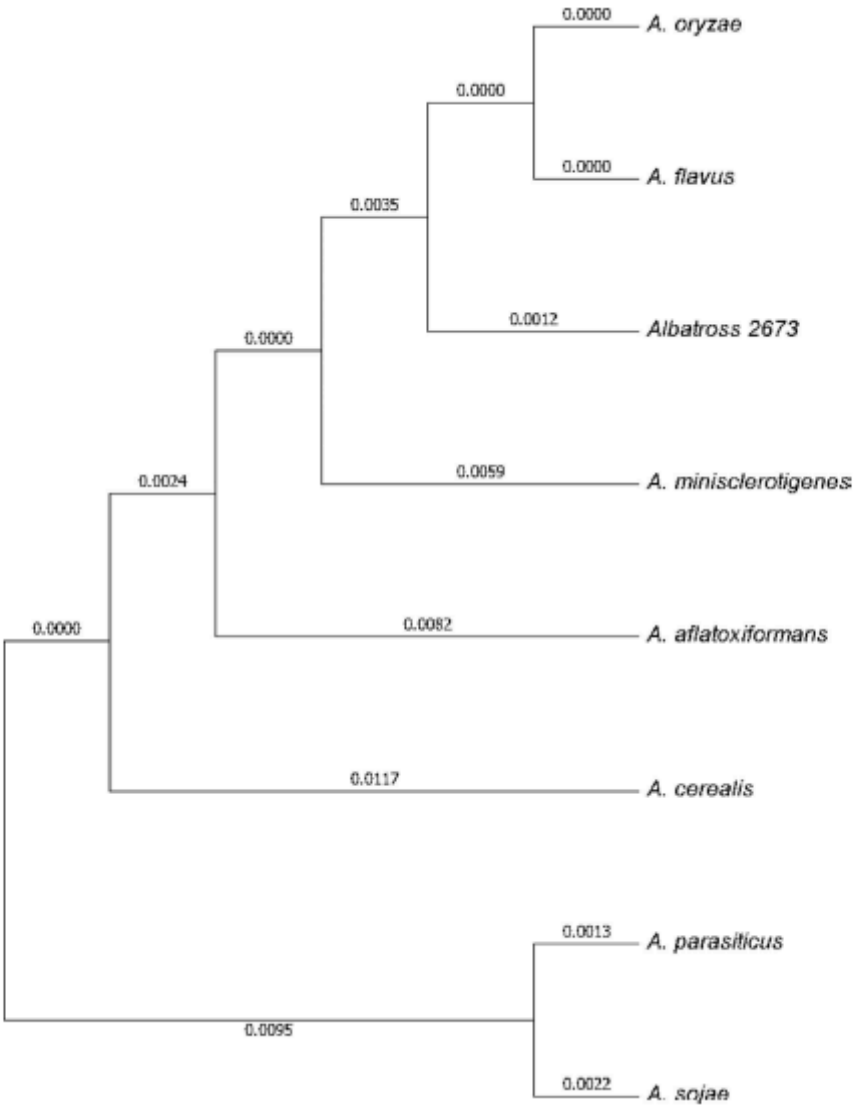

Figure 1 - Phylogenetic tree inferred from nucleotide data (partial *benA* and partial *calM*) using the Neighbor-Joining method, showing the relationship of the *Aspergillus* section *Flavi* isolate [Albatross 2673 (Accession numbers MN244295 and MN422267)] with other *Aspergillus* section *Flavi* species. Accession numbers of GenBank: *A. oryzae* MH279883.1, MH279853.1; *A. flavus* MG517619.1, KJ175539.1; *A. minisclerotigenes* EF203148, MG518009; *A. aflatoxiformans* MG517709, MG518079; *A. cerealis* MG517693, MG518063; *A. parasiticus* EF661481, EF661515; *A.sojae* EF203168, EF202041.

The three clinical isolates were susceptible to itraconazole, with a MIC of 0.25 µg/ml in both *Aspergillus fumigatus sensu stricto* isolates (ECV/CLSI ≤ 1 µg/ml) and 0.5 µg/ml in the *Aspergillus* section *Flavi* isolate (ECV/CLSI ≤ 1 µg/ml).[13]

To the best of our knowledge, this is the first study of aspergillosis in captive albatrosses with descriptions of the lesions and molecular identification of the causative agent. Studies with albatrosses are not easy to perform, since these seabirds are too fragile (since they have large and sensitive wings, sensitive legs; feathers must be in perfect condition for the birds to be able to fly and stay in contact with water for a long time; and, therefore they are easily stressed), and require the shortest possible period in captivity.[4,8]

The majority of the birds were admitted to the center after a mass stranding event, and were kept in large number in the facilities of the rehabilitation centre. In general, birds were weak, emaciated and dehydrated on admission. Whenever possible, birds were kept in the outer areas of the center, aiming to keep them in a better ventilated environment. Besides that, measures towards environmental control of *Aspergillus* conidia counts, such as disinfection with chlorhexidine 2% were applied during the albatrosses' rehabilitation, and no prophylactic treatment was performed.

Aspergillus fumigatus sensu stricto was the etiological agent found in both *A.* section *Fumigati* sequenced isolates. This species has several virulence factors, which could contribute to its higher prevalence in vertebrate hosts.[14] *A. fumigatus sensu stricto* is the main species reported in birds to date,[2, 15, 16] and this is the first report of this species in albatrosses.

On the other hand, the species identification of the *Aspergillus* isolate belonging to section *Flavi* was not conclusive. Although most species of *A.* section *Flavi* can be recognized using *benA* and *calM* sequences, there are some cases where the molecular identification is not possible, as the *A. flavus* / *A. oryzae* lineage, which our isolate is closely related; however phylogenetic analysis can be helpful as well as sequencing other regions, such as partial *RPB2* gene and partial actin gene. [17]

The study of aspergillosis during rehabilitation is important to enable understanding of the relevance of this disease in albatrosses during captivity, as the prevention of secondary diseases could support a greater rehabilitation success. Although there are few *Aspergillus* isolates analyzed, our results of a proportional mortality higher than 20% suggest that aspergillosis can act as a limiting factor in the rehabilitation of albatrosses. Further discussion of aspergillosis in birds can be found in the supplementary text.

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4.6. ARTIGO III. Aspergillosis in free-ranging aquatic birds

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Aspergillosis in free-ranging aquatic birds

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ABSTRACT

Background: Due to the difficulty in the access to free-ranging birds, data regarding Aspergillus infections in wild avian species is rare compared to captive wild and domestic birds. Objective: report three cases of Aspergillus section Fumigati causing fungal disease in free-ranging aquatic birds, with the identification of the causal agent to the species level. Case reports: The diagnosis of aspergillosis was performed by macroscopic lesions found during the necropsy and confirmed by culture and histopathology. Molecular identification by partial sequencing of the calM and benA genes allowed to confirm Aspergillus fumigatus sensu stricto as the etiological agent of aspergillosis in Procellaria aequinoctialis (White-chinned petrel) (n=1), Nannopterum brasilianus (Neotropical cormorant) (n=1) and Chroicocephalus maculipennis (Brown-hooded gull) (n=1). Conclusion: Larger studies regarding the importance of aspergillosis in free-ranging aquatic birds are necessary, as well as it potential role in the One Heath context.

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1. Introduction

Aspergillosis is a common fungal disease in birds, mainly due to their physiological and anatomical characteristics of the respiratory system (1). This mycosis is widely reported in captive (1–3), but less frequently in free-living birds (4).

Aquatic birds are species that live in marine or freshwater environments mainly for foraging activities. *Procellaria aequinoctialis* (White-chinned petrel), classified as vulnerable according to Red List of the International Union for Conservation of Nature (IUCN), has a circumpolar distribution, breeds in sub-Antarctic oceanic islands, and migrates to the southern Brazilian ocean to feed, mainly during non-breeding season, in the austral winter. *Chroicocephalus maculipennis* (Brown-hooded gull) is classified as a "least concern" resident species according to IUCN. In Brazil, it breeds in the interior of the country and migrates to the coast in the non-breeding period. *Nannopterum brasilianus* (Neotropical cormorant) is, according to IUCN, a "least concern" species, non-migrant, with distribution from South America to southern United States (5,6).

Due to the difficulty in the access to free-ranging birds, data about *Aspergillus* infections in wild avian species is scarce. There are some reports of epizootic deaths related to feeding of contaminated food as moldy corn and rotten silage (4), beyond this, reports of aspergillosis in aquatic birds are less common than those in terrestrial birds (4).

Given that migration is a natural behavior in some species, birds could be associated with the introduction of new and foreign fungal strains in distinct regions. Since fungal reproductive structures are commonly produced in air sacs of these animals, it is possible that they can exhale conidia during breathing (1). It is also possible that transmission could occur from decaying cadavers. Additional information about aspergillosis in free-ranging birds is an important tool for a better comprehension and monitoring of the disease in nature. Therefore, this study aimed to describe three cases of the *Aspergillus* infection in free-ranging aquatic birds, with the molecular identification of the causal agent to species level.

2. Cases

During the years 2017 and 2018, three fatal proven cases of aspergillosis in free-ranging aquatic birds from three distinct species were diagnosed in the Center of Recovery of Marine Animals (CRAM-FURG) in Rio Grande, Rio Grande do Sul, South Brazil. The three birds were severely

ill, were gathered in Cassino beach (32° 9'40.71"S e 52° 5'51.71"O), Rio Grande, Rio Grande do Sul, South Brazil and died during their transportation to the Center.

Infected avian individuals were a male *N. brasilianus* weighing 840 grams (reference weight 1200 – 1400g), a female *P. aequinoctialis* weighing 762 grams (reference weight 1020 – 1550g), and a male *C. maculipennis* weighing 308 grams (reference weight 290 - 360g). The three birds were adult and none of them showed evidence of traumatic lesions during the carcass examination.

White-yellow granulomas (ranging from 0.1cm to 0.5cm) were found in the air sacs and in the lungs of the three birds. The petrel and the cormorant showed thickening of air sacs and fungal colonies in the air sacs and in the bronchi. In addition, granulomas were also found in the kidneys of the petrel. (Fig. 1).

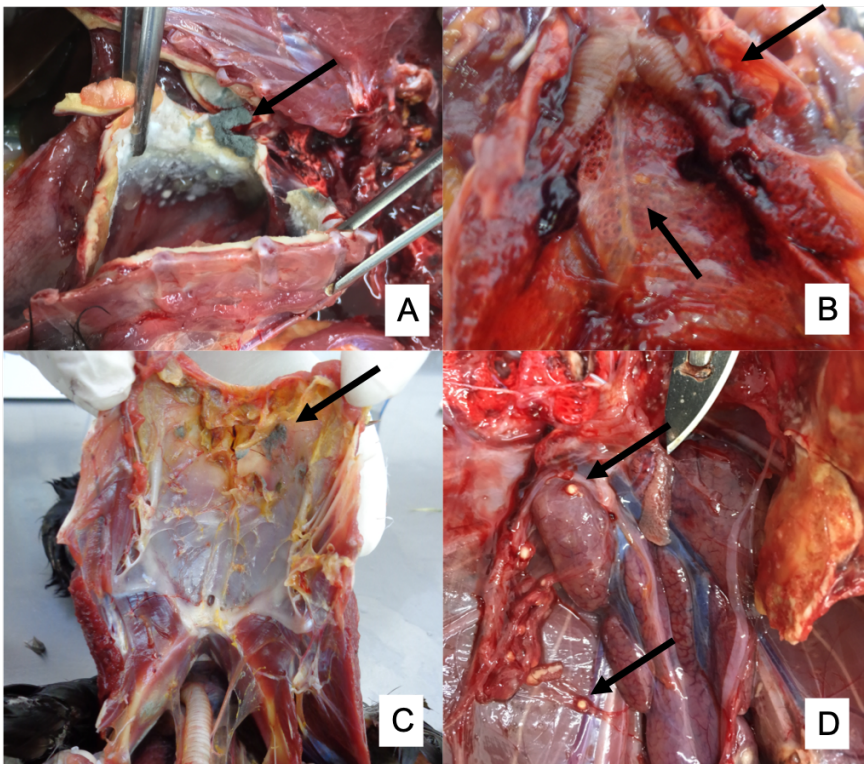

Figure 1: Macroscopic lesions of aspergillosis in free-birds: A) Fungal colonies in the air sacs of *Procellaria aequinoctialis*; B) White-yellow granulomas in the lungs and air sacs in *Chroicocephalus maculipennis*; C) Fungal colonies in air sacs in *Nannopterum brasilianus*; D) White-yellow granulomas in kidney and peritoneum of *Procellaria aequinoctialis*

Tissue samples from the three birds were cultured on Sabouraud Dextrose Agar and pure blue-green fungal colonies were obtained after 48h incubation at 30ºC. Isolates were identified

phenotypically (macro- and micromorphology) as *Aspergillus* section *Fumigati*. DNA extraction was performed and the identification of isolates at the species level was done by molecular sequencing of partial *calM* (7) and partial *benA* genes (8).

Sequencing of partial *benA* was performed by ACTGene Análises Moleculares Ltd. (Center for Biotechnology, UFRGS, Porto Alegre, RS, Brazil), and sequencing of partial *calM* was performed at National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal. Nucleotide sequences were aligned using the "Mega" software version 7.0 and species homology was defined by comparing the nucleotide sequences by BLAST on the website https://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequencing data showed that *Aspergillus fumigatus sensu stricto* was the etiological agent of aspergillosis in the three birds. Sequences obtained were deposited in GenBank data base [Accession numbers (*benA*: MN250367, MN250368 and MN250369) and (*calM*: MN746387, MN746388 and MN746389)].

3. Discussion

It is well established that aspergillosis is a highly prevalent disease in captive birds (2,9). However, there is no agreement in the scientific literature about the importance of this disease in free-living birds. While some authors consider aspergillosis as rare in free birds (10), others affirm that this is the most common disease among medical conditions not induced by trauma (11,12). Our studies, focused on three different aquatic avian species found in nature (in south Brazil) and infected by *Aspergillus* spp., indicate the need for more investigation about aspergillosis in free-ranging birds. Both the petrel and the cormorant were in poor physical condition at the time of the diagnosis, but we cannot assume that this condition was a predisposing factor to aspergillosis development, since it could also be a consequence of the disease progression (13). In contrast, the gull was in good general physical condition, and no evidence of a known predisposing factor was observed, suggesting that aspergillosis could have occurred as a primary disease in this bird (12).

Some species of aquatic birds travel for long periods during the migration, even crossing oceans, and are, therefore, difficult to access (14). The occurrence of neotropical cormorants is more common among the Patos lagoon estuary (32° 02' 6.00" S/ 52° 05' 55.00" W), but groups of these birds in the Cassino beach are frequently observed. It is important to highlight that the region of Patos lagoon estuary is highly populated, with animals and humans are sharing the same environment. Similarly, the brown-hooded gull occurs in the both environments cited previously

but in the open-air garbage dump too. On the other hand, the white-chinned petrel, a migrating oceanic bird, feeds in the Brazilian waters, and reproduces in sub Antarctic islands. The occurrence of these birds in the beaches may occurs as consequence of injury, debilitation, or diseases.

Aspergillus fumigatus sensu stricto, the etiologic agent of all the aspergillosis cases from our study, remains the main causal agent of the *Fumigati* section involved in avian aspergillosis (2,3,9), as well as in mammals affected by the disease (1). Besides the production of gliotoxin, an immunosuppressive mycotoxin (15), the thermotolerance of *A. fumigatus sensu stricto* could be a factor that promotes a better fungal growth at avian body temperatures (16).

Our data, showing the occurrence of aspergillosis in free-living birds acquired in a natural environment, stress that the dissemination of pathogens by migrating birds should also be considered (17). With the emergent concern regarding azole resistance in *Aspergillus fumigatus*, the environment plays an important role in this context. Since most environmental strains can infect and cause aspergillosis in susceptible hosts, without differences in virulence attributes of clinical *versus* environmental isolates (18), there is a need of thinking about the spread of potential pathogenic fungi as a global concern (19).

The human population in coastal areas is higher than inland (20), and humans share this environment with other animals, such as aquatic birds. In this context, and taking in consideration the previously mentioned concerns on *Aspergillus* resistance and the potential importance of dissemination of pathogens by migrating birds, free-ranging aquatic birds should be monitored for aspergillosis as in order to increase the knowledge of *Aspergillus* epidemiology in the context of *One Health*, in which humans, animals and environments are understood to be all connected (19).

To our knowledge, this is the first report of aspergillosis in the bird species *Procellaria aequinoctialis*, *Chroicocephalus maculipennis* and *Nannopterum brasilianus*. The obtained data suggests that aspergillosis in wild birds could be more prevalent than previously thought.

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5. Conflict of Interest

There are none.

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5. CONCLUSÕES GERAIS

- *Aspergillus fumigatus* sensu stricto é o principal agente etiológico da aspergilose em aves aquáticas. O estudo descreveu pela primeira vez, um caso de aspergilose em pinguim causado pela espécie críptica *A. sydowwi*, pertencente à seção *Nidulantes*.

- Alta diversidade genética entre os isolados de *A. fumigatus* sensu stricto foi encontrada em diferentes hospedeiros. Com relação a aspergilose humana, a técnica de microssatélites mostra-se útil principalmente no auxílio em compreender melhor a colonização/infecção por *Aspergillus* na prática clínica. No contexto de epidemiologia molecular, a técnica foi capaz de identificar possíveis surtos de aspergilose em pinguins em reabilitação. Por fim, o estudo demonstrou que estirpes geneticamente idênticas são capazes de infectar diferentes hospedeiros, o que abre perspectivas para investigações sobre a epidemiologia molecular de *Aspergillus* no contexto *One Health*, com enfoque no papel que as aves podem desempenhar tanto na dispersão de *Aspergillus*, bem como indicadores da saúde do ambiente.

- Resistência panzol não foi evidenciada no estudo, e, apenas uma estirpe de *A. fumigatus* sensu stricto apresentou resistência fenotípica ao posaconazol. A profilaxia com antifúngico aplicada em pinguins-de-Magalhaes durante a reabilitação não parece ter maiores implicações na emergência da resistência em *A. fumigatus* sensu stricto.

- A análise do gene *cyp51A* da estirpe resistente ao posaconazol demonstrou a presença de algumas mutações nucleotídicas com substituição de aminoácidos. Entretanto, as mutações encontradas não foram relacionadas até o momento com padrões de resistência, sugerindo que outro mecanismo possa estar envolvido nesse padrão desse isolado.

- Por fim, o composto disseleneto de difenila $(PhSe)_2$ demonstrou potencial antifúngico anti-*Aspergillus* in vitro, com destaque frente a estirpes da seção *Nigri*. Além disso, resultados promissores foram obtidos quando analisada a interação do composto com antifúngicos clássicos. Dessa forma é indicada a continuação das investigações quanto ao potencial antifúngico desta molécula, principalmente com modelos *in vivo*.

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APÊNDICES

Apêndice I – Artigo I. Diphenyl diselenide and its interaction with antifungals against *Aspergillus* spp.

Neste apêndice a primeira página pré print do artigo "*Diphenyl diselenide and its interaction with antifungals against Aspergillus spp."* publicada no periódico Medical Mycology é apresentada.

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Original Article

Original Article

Diphenyl diselenide and its interaction with antifungals against *Aspergillus* spp.

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Abstract

Given the few antifungal classes available to treat aspergillosis, this study aimed to evaluate the *in vitro* antifungal activity of diphenyl diselenide $(PhSe)_2$ alone and in combination with classical antifungals against *Aspergillus* spp., and its *in vivo* activity in a systemic experimental aspergillosis model. We performed *in vitro* broth microdilution assay of $(PhSe)_2$ against 32 *Aspergillus* isolates; and a checkboard assay to test the interaction of this compound with itraconazole (ITC), voriconazole (VRC), amphotericin B (AMB), and caspofungin (CAS), against nine *Aspergillus* isolates. An experimental model of invasive aspergillosis in mice was studied, and survival curves were compared between an untreated group and groups treated with 100 mg/kg ITC, or $(PhSe)_2$ in different dosages (10 mg/kg, 50 mg/kg and 100 mg/kg). All *Aspergillus* non-*fumigatus* and 50% of *A. fumigatus* were inhibited by $(PhSe)_2$ in concentrations $\leq$ 64 μg/ml, with significant differences in MICs between the sections. Synergism or additive effect in the *in vitro* $(PhSe)_2$ interaction with VRC and CAS was observed against the majority of isolates, and with ITC against the non-*fumigatus* strains. In addition to the inhibitory interaction, $(PhSe)_2$ was able to add a fungicidal effect to CAS. Survival curves from the systemic experimental aspergillosis model demonstrated that the inoculum caused an acute and lethal infection in mice, and no treatment applied significantly prolonged survival over that of the control group. The results highlight the promising activity of $(PhSe)_2$ against *Aspergillus* species, but more *in vivo* studies are needed to determine its potential applicability in aspergillosis treatment.

Lay Summary

The activity of diphenyl diselenide $(PhSe)_2$ alone and in combination with itraconazole, voriconazole, and caspofungin, is described against three of the most pathogenic *Aspergillus* sections. $(PhSe)_2$ may prove useful in therapy of infection in future; further study is required.

Key words: selenium, *Aspergillus*, aspergillosis, synergism, checkerboard, animal model.

Apêndice II – Artigo II. Aspergillosis in albatrosses

Neste apêndice a versão pré print da primeira página do artigo "*Aspergillosis in albatrosses"* publicada no periódico Medical Mycology é apresentada.

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Brief Report

Brief Report

Aspergillosis in albatrosses

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Abstract

Aspergillosis is a respiratory fungal disease of importance in captive marine birds. The aim of this study was to describe the occurrence of aspergillosis in *Thalassarche melanophris* during rehabilitation events and to identify the etiological agent. All the albatrosses that were received for rehabilitation and died within a 2-year period were included in the study. The proportionate mortality rate caused by aspergillosis was 21.4% (3/14). One of the etiological agents was *Aspergillus flavus/oryzae* lineage, and the other was *A. fumigatus sensu stricto*. Our study suggests that aspergillosis can act as a limiting factor in the rehabilitation of albatrosses.

Key words: *Aspergillus fumigatus sensu stricto*, procellariiformes, seabirds, molecular identification, *Aspergillus* section *Flavi*.

Aspergillosis is a respiratory fungal disease of great importance in captive marine birds.[1,2] However, there are few reports about *Aspergillus* infection in procellariiformes, probably because rehabilitation of a large group of albatrosses on a single occasion is not common.[3]

Albatrosses are marine birds with a long life (ranging between 30 and 80 years depending on the species), a high adult survival rate, and delayed sexual maturity.[4] The waters of the southern region of Rio Grande do Sul, Brazil, are usually feeding areas for individuals of *Talassarche melanophris* and *Talassarche chlororhynchos* among other procellariforms, and interaction with fishing activities is currently one of the main threats to these birds.[5]

Currently, the definitive identification of *Aspergillus* species requires the addition of molecular methods.[6] The knowledge of the causative agent of aspergillosis is not only epidemiologically relevant but also clinically important, since different species belonging to the same section show different characteristics of virulence, including gliotoxin production, and resistance to antifungal agents.[7,8]

The study of aspergillosis in marine birds in captivity is important to understand the magnitude of its impact on these animals, allowing the establishment of preventive protocols according to the needs and management of each species.[1] A rare event of the rehabilitation of a large group of albatrosses was seen as an opportunity to determine the occurrence and relevance of aspergillosis in these seabirds. Therefore, the aim of this study was to describe the occurrence of aspergillosis in *T. melanophris* during rehabilitation events, and to identify the etiological agent at the species level by molecular techniques.

Apêndice III – Artigo III. Aspergillosis in free-ranging aquatic birds

Neste apêndice a primeira página do artigo "*Aspergillosis in free-ranging aquatic birds"* publicada no periódico Medical Mycology Case Reports é apresentada.

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Aspergillosis in free-ranging aquatic birds

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ABSTRACT

Background: Due to the difficulty in the access to free-ranging birds, data regarding *Aspergillus* infections in wild avian species is rare compared to captive wild and domestic birds. Objective: report three cases of *Aspergillus* section *Fumigati* causing fungal disease in free-ranging aquatic birds, with the identification of the causal agent to the species level. Case reports: The diagnosis of aspergillosis was performed by macroscopic lesions found during the necropsy and confirmed by culture. Molecular identification by partial sequencing of the *calM* and *benA* genes allowed to confirm *Aspergillus fumigatus sensu stricto* as the etiological agent of aspergillosis in *Procellaria aequinoctialis* (White-chinned petrel) (n = 1), *Nannopterum brasilianus* (Neotropical cormorant) (n = 1) and *Chroicocephalus maculipennis* (Brown-hooded gull) (n = 1). Conclusion: Larger studies regarding the importance of aspergillosis in free-ranging aquatic birds are necessary, as well as it potential role in the One Heath context.

1. Introduction

Aspergillosis is a common fungal disease in birds, mainly due to their physiological and anatomical characteristics of the respiratory system [1]. This mycosis is widely reported in captive [1–3], but less frequently in free-living birds [4].

Aquatic birds are species that live in marine or freshwater environments mainly for foraging activities. *Procellaria aequinoctialis* (White-chinned petrel), classified as vulnerable according to Red List of the International Union for Conservation of Nature (IUCN), has a circumpolar distribution, breeds in sub-Antarctic oceanic islands, and migrates to the southern Brazilian ocean to feed, mainly during non-breeding season, in the austral winter. *Chroicocephalus maculipennis* (Brown-hooded gull) is classified as a "least concern" resident species according to IUCN. In Brazil, it breeds in the interior of the country and migrates to the coast in the non-breeding period. *Nannopterum brasilianus* (Neotropical cormorant) is, according to IUCN, a "least concern" species, non-migrant, with distribution from South America to southern United States [5,6].

Due to the difficulty in the access to free-ranging birds, data about *Aspergillus* infections in wild avian species is scarce. There are some reports of epizootic deaths related to feeding of contaminated food as moldy corn and rotten silage [4], beyond this, reports of aspergillosis in aquatic birds are less common than those in terrestrial birds [4].

Given that migration is a natural behavior in some species, birds could be associated with the introduction of new and foreign fungal strains in distinct regions. Since fungal reproductive structures are commonly produced in air sacs of these animals, it is possible that they can exhale conidia during breathing [1]. It is also possible that transmission could occur from decaying cadavers. Additional information about aspergillosis in free-ranging birds is an important tool for a better comprehension and monitoring of the disease in nature. Therefore, this study aimed to describe three cases of the *Aspergillus* infection in free-ranging aquatic birds, with the molecular identification of the causal agent to species level.

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Apêndice IV - MANUSCRITO IV. Trends on *Aspergillus* epidemiology – perspectives from a National Reference Laboratory surveillance program

O apêndice a seguir corresponde a um manuscrito submetido ao periódico Journal of Fungi. A colaboração que resultou na co-autoria deste trabalho foi realizada durante o período de doutoramento sanduíche desta autora pelo programa de internacionalização PDSE da CAPES, no Departamento de Doenças Infeciosas/ Unidade de Referência de Infeções Parasitárias e Fúngicas do Instituto Nacional de Saúde Dr. Ricardo Jorge, em Lisboa, Portugal.

Neste trabalho, realizei a identificação molecular, o screening para azóis e a microdiluição seriada de parte das estirpes de *Aspergillus*. Além disso, participei da revisão da redação do manuscrito.

Article

Trends on *Aspergillus* epidemiology – perspectives from a National Reference Laboratory surveillance program

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Abstract: The correct identification of *Aspergillus* species is important given that sibling species may show variable susceptibilities to multiple antifungal drugs and also because a sharper definition of species may facilitate epidemiological studies. Two retrospective laboratory studies were conducted on *Aspergillus* surveillance at the Portuguese National Mycology Reference Laboratory. The first, covering the period 2017-2018, aimed to study the molecular epidemiology of *Aspergillus* isolates obtained from patients with respiratory, subcutaneous or systemic infections and from environmental samples. The second, using our entire collection of clinical and environmental isolates, collected between 2012 and 2019, aimed to determine the frequency of azole-resistant *A. fumigatus* isolates. *A. fumigatus* sensu stricto was the most frequent species in both clinical and environmental samples. A high frequency of cryptic species was identified, based on beta-tubulin or calmodulin sequencing (37% in clinical and 51% in environmental isolates). Our findings underline the demand for correct identification and susceptibility testing of *Aspergillus* isolates. To determine the frequency of azole resistance of *A. fumigatus*, isolates were screened for azole resistance using azole-agars, and 53 possible resistant isolates were tested by the CLSI microdilution reference method. Nine *A. fumigatus* sensu stricto and six *Fumigati* cryptic isolates showed high minimal inhibitory concentrations to itraconazole, voriconazole and/or posaconazole. Real-time PCR to detect *cyp51A* mutations and sequencing of *cyp*51A gene and its promotor were performed. The overall frequency of resistance to azoles in *A. fumigatus* sensu stricto was 3.0%. With this retrospective analysis, we were able to detect an azole-resistant G54R mutant *A. fumigatus*

environmental isolate, collected in 2015. The TR_{34}/L98H mutation, linked to environmental transmission route of azole resistance, was the most frequently detected mutation (1.4%).

Keywords: *Aspergillus*; Surveillance; Molecular Epidemiology; Cryptic species; Azole resistance mutations

1. Introduction

According to the LIFE organization, more than 30 million patients are at risk of invasive aspergillosis (IA) and about 300,000 patients develop the disease annually [1]. The genus *Aspergillus* is composed of several hundred species, some of which are considered to be potentially pathogenic, causing serious infections with a fatality rate that can reach 50% if treated and more than 99% if not treated [1]. The main pathologies associated with infections by *Aspergillus* affect the lungs, including allergic bronchopulmonary aspergillosis (ABPA), and chronic pulmonary aspergillosis (CPA), but the infection can also spread to other organs leading to the development of IA [2]. Patients at high risk of acquiring IA generally suffer from severe granulocytopenia (leukemia, bone marrow or solid organ transplant patients). In addition, prolonged use of corticosteroids, diabetes, severe burns and major surgery are considered predisposing risk factors for IA [3].

Molecular studies [4, 5] have shown the limitation of conventional identification through morphological characteristics to distinguish *Aspergillus* species. In fact, an isolate identified using morphological methodologies can only be included in one of the "species sections", such as *Fumigati*, *Flavi*, *Nidulantes*, *Usti* or *Terrei*. Species that are morphologically identical and only distinguishable from each other using molecular methodologies are called cryptic species. Molecular differences also reflect a difference in susceptibility to antifungals, with cryptic species being in general less susceptible [4].

The *Aspergillus* species most frequently isolated in the clinical context are *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*. The *Fumigati* section, composed of *A. fumigatus* sensu stricto and its cryptic species, is the most frequently isolated from clinical products and is also frequently isolated from environmental sources. Moreover, the prevalence of clinical isolates of the *Fumigati* section with azole resistance has been increasing [6]. The proposed reasons for this include the development of resistance by prolonged antifungal prophylaxis or therapy. However, the inhalation of environmental strains resistant to antifungals is currently one of the biggest and most recent concerns of the scientific community [6-8].

It has been shown that azole-resistant *A. fumigatus* isolates have, for the most part, a resistance mechanism mediated by the *cyp51A* gene. Depending on the specific mutation, they may show resistance to an azole or to any azole class [6]. TR_{34}/L98H and TR_{46}/Y121F/T289A are the most common mutations associated with environmental pan-azole resistance. However, other mutations in the promotor of the *cyp51A* gene, as TR_{53}, $TR_{46}{}^{3}$, and $TR_{46}{}^{4}$, have also been described as from environmental origin [9]. Other mutations at various positions of the *cyp51A* gene such as G54, M220, P216, G138, and G448 have also been associated with resistance, of which the G54 and M220 mutations are the most common [10, 11].

Azoles are the first line of prophylaxis and treatment of infections by *A. fumigatus* and, therefore, there is a high concern inherent to treatment failure. Resistance to triazoles can severely limit treatment options and be associated with worse patient prognosis [12].

The European Center for Disease Prevention and Control (ECDC) published in 2013 [13] a recommendation for epidemiological surveillance to be carried out, to collect information at the local level, both in clinical and environmental context. In Portugal, few reports on *Aspergillus* prevalence and resistance have been published [14, 15]. As such, we hypothesized that the reported prevalence of azole-resistant *A. fumigatus* may not represent the true prevalence of azole resistance and, therefore, surveillance studies are warranted.

Here, we report the results from the laboratory-based national surveillance programme for *Aspergillus* that is established in our country since 2012, coordinated by the National Reference Laboratory for Mycology at the National Institute of Health Dr. Ricardo Jorge in Lisbon. Our laboratory provides reference diagnostic services to several hospitals from the National Health Service and for with a wide range of microbiological and clinical specialties from different regions of Portugal. In the context of this program, our laboratory receives clinical and environmental specimens or isolates for identification and antifungal susceptibility profiling of *Aspergillus*. Thus, with the two retrospective laboratory studies here presented, we are able to evaluate the frequency and diversity of *Aspergillus* species and the resistance profile to azoles of *A. fumigatus* at national level and, in doing so, to contribute to informed decisions and policies on the control of aspergillosis and on the use of azole antifungals, both in medicine and in the environment.

2. Materials and Methods

2.1 *Aspergillus identification and diversity*

During 2017 and 2018, *Aspergillus* isolates were obtained from clinical (respiratory, subcutaneous or systemic) and environmental (soil, air and surfaces from different sources, including hospitals, dwellings and greenhouses) samples, analyzed at the Mycology National Reference Laboratory or received from collaborating institutions in the context of the *Aspergillus* surveillance program.

All isolates included in the surveillance program were assigned with a unique and sequential designation (VA1, VA2,...). All isolates were plated for growth as single colonies on malt extract agar with chloramphenicol (MEA). The morphological identification of the *Aspergillus* section was carried out based on macro and microscopic characteristics and using identification atlases [16, 17].

Molecular identification of the species was carried out by sequencing the genes encoding calmodulin or beta-tubulin. Genomic DNA was prepared from each isolate using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Amplifications of the partial beta-tubulin gene were performed using primers Bt2a and Bt2b, as described previously [18], with the following modifications: PCR was carried out in a 25 µl volume reaction of Illustra PuReTaq Read-to-Go PCR beads (GE Healthcare, Buckinghamshire, UK), 0.4 µM of each primer Bt2a and Bt2b, and 20 to 50 ng of *Aspergillus* genomic DNA. PCR conditions were as follows: an initial denaturation at 94ºC for 5 min, followed by 30 cycles of 94ºC for 30 sec, 55ºC for 45 sec, and a final extension step of 72ºC for 2 min.

Calmodulin gene amplification was performed using the set of primers cmd5 and cmd6 [19]. Amplifications were performed in a 25 µl volume reaction of Illustra PuReTaq Read-to-Go PCR beads (GE Healthcare, Buckinghamshire, UK), containing 0.6 µM of each primer and 20 to 50 ng of *Aspergillus* genomic DNA. Amplifications were carried out with an initial denaturation at 95ºC for 10 min, followed by 38 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1 min, and a last final extension step of 72ºC for 7 min.

PCR products were analyzed by electrophoresis through 2% agarose gels and the resultant PCR amplicons were purified using the ExoSAP-IT enzyme system (USB Corporation, Cleveland, OH, USA), according to the manufacturer's instructions. Sequencing was performed with the BigDye terminator v 1.1 Cycle sequencing kit (Applied Biosystems). For calmodulin, the same set of primers as those used in the PCR (cmd5 and cmd6) were used. The conditions were: an initial denaturation at 96ºC for 5 sec, followed by 30 cycles of 96ºC for 10 sec, 50ºC for 5 sec and 60ºC for 4 min, followed by one cycle of 72ºC for 5 min. For beta-tubulin sequencing, another set of primers (Btub1 and Btub4) was used [20] and the amplification was done under the following conditions: an initial denaturation at 94ºC for 3 min, followed by 25 cycles of 96ºC for 10 sec, 50ºC for 5 sec and 52ºC for 4 min, followed by one cycle of 60ºC for 5 min. The resultant nucleotide sequences were edited using the program Chromas Lite v 2.01 and aligned with the program CLUSTALX v 2.1. Edited sequences were then compared with sequences deposited in the GenBank (Bethesda, MD, USA) and WI-KNAW

Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands) databases, in order to achieve the identification to species level, accepted when the obtained homology was ≥98%.

For both groups of clinical and environmental samples, species richness (total number of species), their abundance and diversity were evaluated through the Simpson diversity index (D = 1-{[Σn(n-1)]/[ΣN(N-1)]}, where n is the total number of organisms of a particular species and N is the total number of organisms of all species; the index ranges from 0 to 1, the greater the value, the greater the diversity in the group), and the Shannon diversity index $\boldsymbol{H' = \Sigma pi \ln pi}$, where pi is the proportion of individuals of species; the more unequal the abundance of species, the smaller the index; if abundance is primarily concentrated into one species, the index will be close to zero) [21].

2.2 *Azole susceptibility profiling*

For the study of azole-resistance patterns in *A. fumigatus* in Portugal, our entire collection 2012-2019 of isolates identified as belonging to *Fumigati* section was analyzed, which included both clinical and environmental isolates from different regions of the country.

Clinical isolates were obtained from biological specimens collected from different sites (mainly from the respiratory tract), from patients from 23 independent clinical institutions. Environmental isolates were collected from soil, air and surfaces from previous referred sources (hospitals, dwellings and greenhouses) but also from thermal spas, animal production farms and a waste sorting plant.

The pattern of susceptibility of the isolates to antifungal agents was carried out using an initial screening media for azole resistance. Plates of Sabouraud dextrose agar (SDA) (Oxoid, Hampshire, United Kingdom) supplemented with concentrations of 4 mg/mL of itraconazole (ICZ), 1 mg/mL (until 2017) [8] and 2 mg/mL (2018 to present) of voriconazole (VCZ) or 0.5 mg/mL of posaconazole (PCZ) were used [22-24]. Fresh conidia from a 7-day-old culture grown on MEA were suspended in saline solution at a turbidity equivalent to a 0.5 McFarland standard. Plates were inoculated by swabbing and incubated at 37ºC for 48 hours. To control strain viability, an SDA plate without chloramphenicol was used. The reference strain *Aspergillus fumigatus* ATCC 204305 was used as negative control and a pan-azole resistant strain TR_{34}/L98H (kindly provided by Professor Jacques Meis, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands) was used as positive control. *Aspergillus* growth was observed visually and classified as "Negative (-)" (no growth, susceptible), "Relevant growth (+)" (growth similar to positive control) and "Residual growth (±)" (growth of only one or few small colonies). Strains that were not able to grow in the azole-supplemented plates were considered as susceptible to the respective azoles, according to EUCAST guidelines [24]. In case of doubt or to confirm the screening results (for residual or relevant growths, respectively), the antifungal susceptibility profiles were determined by broth microdilution reference method for susceptibility testing of molds. The M38-A2 protocol from the Clinical and Laboratory Standards Institute was applied for determining the Minimal Inhibitory Concentrations (MIC) for ICZ, VCZ, PCZ. The final concentrations of drugs in the wells ranged from 0.0156 to 8 μg/mL for all. An internal control strain (*Aspergillus flavus* ATCC 204304) with known susceptibility was included in each run as a positive control of the antifungals' potency. SDA plates were inoculated with the final inoculum to check the number of colony-forming units in the inoculum. Plates were incubated at 35ºC and examined after 48h of incubation. Absence of visual growth defined the MIC. Isolates with high MICs were tested in triplicate to confirm the obtained results. Breakpoints for mold testing have not been established by the CLSI except for voriconazole and *A. fumigatus*. The CLSI epidemiological cut-off values (ECVs) used were 1 mg/L for ICZ, and 0.5 mg/L for PCZ and, for VCZ, 1 mg/L as breakpoint [25-27].

Working breakpoints were assigned according to Moin et al., [28] as follows: itraconazole: susceptible (MIC≤1 mg/L), intermediate (MIC=2 mg/L) and resistant (MIC≥4 mg/L); voriconazole: susceptible (MIC≤1 mg/L), and resistant (MIC≥2 mg/L); posaconazole: susceptible (MIC ≤0.5 mg/L), and resistant (MIC≥1 mg/L).

All isolates with a MIC for voriconazole, posaconazole, and/or itraconazole above these ECVs were retested and then screened for tandem repeats and point mutations. This was performed using the

AsperGenius® Resistance multiplex real-time polymerase chain reaction (PCR) assay to detect TR_{34}/L98H, and TR46/T289A/Y121F mutations and the AsperGenius® G54/M220 RUO PCR assay to detect G54/M220 mutations (PathoNostics, Maastricht, The Netherlands) on the Qiagen RotorGene Q instrument (Qiagen, Hilden, Germany) following the manufacturer's instructions. To confirm the real-time PCR results, the *cyp51A* gene and its promotor were sequenced according to Prigitano et al. and Mellado et al. [29, 30].

The resultant nucleotide sequences were edited using the program GeneStudio ™ Professional Edition version 2.2.0.0 and aligned with the program MEGA version 10.0.5. The edited sequences were compared with sequences deposited on the GenBank (Bethesda, MD, USA).

3. Results

3.1 Molecular epidemiology

From the 256 isolates collected during the study period (2017-2018), 156 were from clinical (human) sources, and 99 from different environmental sources.

One hundred forty four clinical isolates (92.3%) were from respiratory specimens, including bronchial / bronchoalveolar lavage (N=109), bronchial secretions (N=21), sputum (N=12), and bronchial aspirate (N=2). Other products included chest drain pus (N=1), tissue sample (N=1) and clinical products from unknown body source (N=10). These clinical products were collected from 129 patients (58 males, 66 females, 5 not known), with ages ranging from 17 to 88 years old at 14 hospital centers distributed throughout the country. The clinical information associated with the biological product sent to the laboratory was scarce, but the majority of the patients presented bronchiectasis in their image exams (Table 1).

Table 1 Clinical information available for the patients from whom *Aspergillus* was isolated

Clinical information	No. Patients
Bronchiectasis	32
Cavitary lung lesions/nodules/abscess/ infiltrates	11
Respiratory infection / Pneumonia	9
HIV+/ previous or active tuberculosis	9
Asthma	2
Invasive aspergillosis	2
Neoplasms	3
Cystic fibrosis	1
Chronic obstructive pulmonary disease	1
Diabetes	1
Admitted at the ICU, surgery or infectiology units	4
Not referred	54
Total	129

A. fumigatus sensu stricto was the most frequent species in both clinical and environmental sources. Among the 156 clinical isolates, eight different *Aspergillus* sections were identified (*Fumigati*, *Flavi*, *Nigri*, *Terrei, Circumdati, Clavati, Aspergilli* and *Nidulantes*, the latter now including previous section *Versicolores*) [31]. A total of 25 different species were identified (Table 2).

231 **Table 2.** Clinical and environmental *Aspergillus* species collected at the National Reference
232 Laboratory in the scope of the *Aspergillus* surveillance program (2017-2018)

	Clinical Isolates			Environmental Isolates		
Section	**Species**	**N**	**Total**	**Species (N)**	**N**	**Total**
Fumigati	*A. fumigatus* sensu stricto	61 (1*)	70	*A. fumigatus* sensu stricto		39
	A. lentulus	4 (2*)				
	A. felis/*A. pseudofelis*	2				
	A. udagawae	2 (1*)				
	Not identified to species level	1				
Flavi	*A. flavus* sensu stricto	8 (1*; 1#)	11			2
	A. sergii/*transmontaneensis*	1				
	Not identified to species level	2				
				A. nomius		
Nidulantes	*A. nidulans* sensu stricto	2	13			12
	A. delacroixii	1				
	A. minutus /*insuetus*	1 (1*)				
	A. teneensis	1		*A. teneensis*		
	A. jensenii	3 (1*)				
	A. fructus	1		*A. fructus*		
	A. tabacinus	1		*A. tabacinus*		
	A. creber / *A. paulaauensis*	1				
	A. versicolor sensu stricto / *A. tabacinus*	1				
	Not identified to species level	1				
				A. sydowii		
				A. protuberus		
Nigri	*A. niger* sensu stricto	5	40	*A. niger* sensu stricto		21
	A. welwitschiae	22 (2*)		*A. welwitschiae*		
	A. tubigensis	4 (1*)		*A. tubigensis*		
	A. brasiliensis	1				
	Not identified to species level	8				
Clavati	*A. giganteus*	1	1			
				A. clavatus sensu stricto		1
Terrei	*A. terreus* sensu stricto	12 (2*)	13			
	A. alabamensis	1				
Circumdati	*A. ochraceopetaliformis* / *A. flocculosus* (N=1)	1	2			3
	A. westerdijkiae (N=1)	1		*A. westerdijkiae*		
				A. melleus		
Aspergilli	*A. chevalieri*i (N=1)		1			
Flavipedes				*A. iizukae* (2*)		2
Usti				*A. ustus* sensu stricto		19
				A. insuetus		
				A. minutus		
				A. germanicus		

Not identified to section level	*Aspergillus* sp.	5	
TOTAL		**156**	**99**

Legend:

(*) Number of isolates not identified to section level by morphological methods (all from respiratory products)

(#) Number of isolates misidentified by morphological methods (as belonging to other section)

Twenty clinical isolates could not be identified to section level by morphological methods, all of them collected from respiratory products (bronchial/ bronchoalveolar lavage and bronchial secretions) (Table 2).

From those, 15 were identified by calmodulin and/or beta-tubulin sequencing, 11 of which were identified as cryptic species, some of them described as less susceptible to antifungals (as *A. lentulus*). In five isolates no PCR product was obtained. Molecular identification to species level was obtained in 139 isolates. In total, cryptic species represented 36.7% (51/139) of the total clinical isolates.

More than one *Aspergillus* species were isolated from the clinical samples of 19 patients (Table 3). Three patients (#6, #9 and #17) were infected/colonized by *A. fumigatus* sensu stricto and its cryptic species *A. lentulus.* This cryptic species was also isolated from patient #13, together with *A. niger* and *A. terreus*. Seven patients (#6, #7, #8, #10, #11, #13 and #19) were simultaneously colonized/infected with *A. terreus* together with other species.

Table 3. Patients infected/colonized by more than one *Aspergillus* species

Patient #	Isolate	Product	Species
1	VA141	LBA	*A. fumigatus* sensu stricto
	VA142	LB	*A. welwitschiae*
2	VA151	LBA	*A. welwitschiae*
	VA152		*A. terreus* sensu stricto
3	VA153	LB	*A. fumigatus* sensu stricto
	VA154		*A. welwitschiae*
4	VA159	LBA	*A. welwitschiae*
	VA160	LB	*A. teneensis*
5	VA191	LBA	*A. fumigatus* sensu stricto
	VA192		*A. welwitschiae*
6	VA193	LBA	*A. terreus* sensu stricto
	VA194		*A. fumigatus* sensu stricto
	VA198	LB	*A. fumigatus* sensu stricto
	VA199		*A. lentulus*
7	VA228	LBA	*A. fumigatus* sensu stricto
	VA229		*A. terreus* sensu stricto
8	VA230	LB	*A. niger* (section)
	VA231		*A. terreus* sensu stricto
9	VA242	LBA	*A. lentulus*
	VA243		*A. fumigatus* sensu stricto
10	VA244	Respiratory Product	*A. fumigatus* sensu stricto
	VA245		*A. terreus* sensu stricto
11	VA251		*A. fumigatus* sensu stricto

	VA252	Respiratory Product	*A. terreus* sensu stricto
12	VA272	LB	*Aspergillus* sp.
	VA273		*A. fumigatus* sensu stricto
	VA274	LBA	*A. giganteus*
	VA275		*Aspergillus* sp.
13	VA276	LBA	*A. terreus* sensu stricto
	VA277	LBA	*A. lentulus*
	VA278		*A. niger* (section)
14	VA280	LB	*A. flavus* (section)
	VA289	LBA	*A. fumigatus* sensu stricto
15	VA281	LB	*A. flavus* sensu stricto
	VA285		*A. fumigatus* sensu stricto
16	VA296	Bronchial secretions	*A. fumigatus* sensu stricto
	VA297		*A. chevalierii*
17	VA298	Bronchial secretions	*A. fumigatus* sensu stricto
	VA299		*A. lentulus*
18	VA301	LBA	*A. niger* (section)
	VA304		*A. fumigatus* sensu stricto
19	VA302	LB	*A. terreus* sensu stricto
	VA303		*A. fumigatus* sensu stricto (TR_{34}/L98H mutant)

The 99 isolates were recovered from hospital environment (air, surfaces) (N=17), soil (N=1), air conditioning filters from different settings (N=22), dwellings (air, surfaces) (40) and air from agricultural environments (N=17). Among these environmental isolates, eight different *Aspergillus* sections were identified (*Fumigati*, *Flavi*, *Nigri*, *Usti*, *Circundati*, *Clavati*, *Flavipedes* and *Nidulantes*). Within these sections, 18 different species were identified (Table 2). Cryptic species represented 51.5% (50/99) of the total isolates.

Species diversity was compared between clinical and environmental isolates. Simpson index showed greater species diversity in environmental samples compared with clinical samples (D= 0.82 versus D=0.76, respectively). On the other hand, the abundance of a specific species is slightly greater in clinical samples when compared to environmental samples (Shannon index = -2.07 versus -2.12, respectively).

3.2 Characterization of the resistance pattern of isolates from Fumigati section

A total of 337 isolates belonging *Fumigati* section were collected in the period between 2012 and 2019 (Table 4).

Table 4. *Aspergillus* species from *Fumigati* section collected in the period 2012-2019

Species	Number of isolates	Source
Aspergillus fumigatus sensu stricto	319	Clinical and environmental
Aspergillus lentulus	8	Clinical (respiratory products)
Aspergillus felis/parafelis/pseudofelis	5	Clinical (respiratory products)
Aspergillus hiratsukae	1	Environmental (Hospital environment)
A. udagawae	3	Clinical (respiratory products)
A. oerlinghauensis	1	Clinical (ear exsudate)

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272 Among those, and through morphological identification, it was not possible to achieve the
273 identification (not even to section level) in five cases, but their molecular identification allowed
274 the identification to species level as *A. fumigatus* sensu stricto. The frequency of cryptic species
275 within this section was 5.3% (18/337). Seven of these clinical cryptic species were from the same
276 hospital (2 *A. felis*, 3 *A. lentulus*, 2 *A. udagawae*). Twenty-three *A. fumigatus* sensu stricto and one
277 *A. felis* isolates lost their viability and it was not possible to test them regarding their antifungal
278 susceptibility. From the remaining 296 *A. fumigatus* sensu stricto and 17 cryptic isolates, 250
279 sensu stricto and 10 cryptic did not grow in any of the screening media supplemented with
280 azoles. Relevant and residual growth in azole resistance screening media were obtained for 46
281 out of 296 sensu stricto isolates and for 7 out of 17 *Fumigati* cryptic species. Per published
282 epidemiological cut-off values established using the CLSI M38A2 broth microdilution method,
283 4.4% of *A. fumigatus* isolates would be considered non-wild type to itraconazole (MIC >1 mg/L),
284 2.0% to voriconazole (MIC >1 mg/L) and 2.0% to posaconazole (MIC >0.5 mg/L) (Table 5).
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291 **Table 5.** Characterization of the isolates presenting growth on azole screening media

											Azole Screening media			Minimal inhibitory concentration (mg/L)			*cyp51A* Mutation
Isolate	**Year isolation**	**Geographic Location NUTS II**	**Location Code**	**Source**	**Biological product**	**Age**	**Gender**	**Clinical informations**	***Aspergillus* section**	**Molecular identification**	**ICZ**	**VCZ**	**PCZ**	**ICZ**	**VCZ**	**PCZ**	
VA7	2013	Lisbon	1	Clinical	Sputum	22	M	Cystic fibrosis	*Fumigati*	*A. fumigatus* sensu stricto	±	-	±	1	0.5	0.5	N.P.
VA8	2013	Lisbon	1	Clinical	Bronchial secretions	39	M	Cystic fibrosis	*Fumigati*	*A. fumigatus* sensu stricto	+	-	±	0.5	0.25	0.25	N.P.
VA9	2013	Lisbon	1	Clinical	Sputum	63	M	Cystic fibrosis	*Fumigati*	*A. fumigatus* sensu stricto	+	-	-	0.5	0.5	0.25	N.P.
VA10	2013	Lisbon	1	Clinical	N.R.	73	F	Cystic fibrosis	*Fumigati*	*A. fumigatus* sensu stricto	+	-	-	0.25	0.5	0.125	N.P.
VA16	2013	Lisbon	1	Clinical	Sputum	16	F	Cystic fibrosis	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	0.125	0.25	0.0625	N.P.
VA21	2013	Lisbon	1	Clinical	Bronchoalveolar lavage	48	M	Liver transplant	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	1	0.5	0.5	N.P.
VA35	2014	North	2	Clinical	Bronchial aspirate	67	F	N.R	*Fumigati*	*A. fumigatus* sensu stricto	+	-	-	1	0.5	0.5	N.P.
VA37	2014	Lisbon	3	Environment	N.R.	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	0.5	0.5	0.5	N.P.
VA44	2014	North	2	Clinical	Bronchial aspirate	79	M	Solid Tumor	*Fumigati*	*A. fumigatus* sensu stricto	±	-	±	0.5	0.5	0.5	N.P.
VA46	2014	North	2	Clinical	Bronchial aspirate	88	F	N.R	*Fumigati*	*A. fumigatus* sensu stricto	±	-	±	0.5	0.25	0.5	N.P.
VA54	2015	Lisbon	4	Clinical	N.R.	N.R	N.R	N.R	*Fumigati*	*A. fumigatus* sensu stricto	±	-	±	1	0.5	0.5	N.P.
VA55	2015	Lisbon	4	Clinical	Bronchial secretions	58	F	Chronic Liver disease	*Fumigati*	*A. fumigatus* sensu stricto	±	-	±	1	0.5	0.5	N.P.
VA63	2015	Lisbon	4	Clinical	Sputum	64	M	Sarcoidosis	*Fumigati*	*A. fumigatus* sensu stricto	±	-	±	2	0.5	0.5	N.P.
VA65	2015	Lisbon	4	Clinical	Bronchial secretions	21	M	Polytraumatized	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	0.5	0.25	0.5	N.P.
VA67	2015	Lisbon	4	Clinical	Sputum	55	M	Tuberculosis	*Fumigati*	*A. fumigatus* sensu stricto	±	-	±	0.5	0.25	0.5	N.P.
VA70	2015	Lisbon	4	Clinical	Bronchial secretions	78	M	Chronic obstructive pulmonary disease	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	1	0.5	0.5	N.P.
VA71	2015	Lisbon	4	Clinical	N.R.	N.R	N.R	N.R	*Fumigati*	*A. fumigatus* sensu stricto	+	-	±	1	0.5	0.5	N.P.
VA73	2015	Lisbon	4	Clinical	Bronchoalveolar lavage	69	M	Diabetes	*Fumigati*	*A. fumigatus* sensu stricto	+	-	±	1	1	0.5	N.P.
VA74	2015	Lisbon	4	Clinical	Bronchial secretions	81	F	Sepsis	*Fumigati*	*A. fumigatus* sensu stricto	±	-	±	1	0.5	0.5	N.P.
VA76	2015	Lisbon	4	Clinical	Broncoalveolar lavage	41	F	Pulmonary emphysema	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	1	0.25	0.5	N.P.

VA77	2015	Lisbon	4	Clinical	Pulmonary tissue	57	M	HIV+	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	1	0.25	0.125	N.P.
VA78	2015	Lisbon	4	Clinical	Bronchial secretions	57	M	HIV+	*Fumigati*	*A. fumigatus* sensu stricto	-	-	±	1	0.5	0.5	N.P.
VA83	2015	Lisbon	4	Clinical	Peritoneal fluid	82	M	Liver cirrhosis	*Fumigati*	*A. fumigatus* sensu stricto	+	-	-	1	0.25	0.5	N.P.
VA85	2015	North	5	Clinical	Bronchial lavage	40	M	Tuberculosis and Aspergilloma	*Fumigati*	*A. fumigatus* sensu stricto	+	±	+	2	2	1	No amplification
VA86	2015	Centre	6	Environment	Swine air	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	+	-	+	>8	0.5	2	G54R
VA95	2015	Lisbon	7	Environment	Air	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	+	±	+	2	2	1	No mutation detected
VA137	2016	Lisbon	8	Clinical	Bronchial secretions	87	M	N.R.	*Fumigati*	*A. fumigatus* sensu stricto	±	-	±	1	0.25	0.125	N.P.
VA116	2016	North	2	Clinical	Bronchial secretions	70	F	N.R.	*Fumigati*	*A. lentulus*	+	±	+	2	8	1	N.P.
VA118	2016	North	3	Clinical	Sputum	80	M	Bronchiectasis	*Fumigati*	*A. lentulus*	+	+	+	2	>8	1	N.P.
HSMA67	2017	Lisbon	1	Environment	Hospital surface	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	+	-	-	1	0.25	0.5	N.P.
VA146	2017	Leiria	9	Clinical	Bronchoalveolar lavage	45	M	HIV+	*Fumigati*	*A. fumigatus* sensu stricto	+	-	-	2	0.5	0.5	N.P.
VA149	2017	North	5	Clinical	Bronchoalveolar lavage	59	F	Bronchiectasis	*Fumigati*	*A. fumigatus* sensu stricto	+	-	-	1	0.5	0.5	N.P.
VA176	2017	North	6	Clinical	Bronchial lavage	67	M	Pulmonary infection	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	0.25	0.03	0.03	N.P.
VA144	2017	North	4	Clinical	Bronchial aspirate	57	M	N.R.	*Fumigati*	*A. felis*	+	+	+	4	>8	1	N.P.
VA182	2017	Lisbon	4	Clinical	N.R.	N.R.	F	HIV+, pulmonary aspergillosis, previous tuberculosis	*Fumigati*	*A.felis*	+	+	-	2	1	0.25	N.P.
VA199	2017	North	6	Clinical	Bronchial lavage	62	M	Cavitated pneumonia	*Aspergillus* sp.	*A. lentulus*	+	+	+	2	2	1	N.P.
VA244	2018	Lisbon	8	Clinical	Bronchial secretions	75	F	N.R.	*Fumigati*	*A. fumigatus* sensu stricto	±	±	-	1	1	0.5	N.P.
VA285	2018	North	6	Clinical	Bronchial lavage	83	F	Pulmonary abscess	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	1	0.25	0.5	N.P.
VA303	2018	North	6	Clinical	Bronchial lavage	65	M	Bronchiectasis	*Fumigati*	*A. fumigatus* sensu stricto	+	+	+	>8	8	2	TR_{34}/L98H
VA242	2018	North	7	Clinical	Bronchoalveolar lavage	48	M	Bronchiectasis	*Aspergillus* sp.	*A. lentulus*	±	±	-		LV		N.P.
VA254	2018	Lisbon	7	Clinical	Ear exudate	14	F	N.R.	*Fumigati*	*A. oerlinghauensis*	+	+	-	4	4	2	N.P.
VA328	2019	North	6	Clinical	Bronchoalveolar lavage	84	M	N.R.	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	4	0.5	1	No mutation detected
VA346	2019	Centre	9	Clinical	Bronchial secretions	67	M	N.R.	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	2	0.5	1	F46Y, M172V, N248T, D255E

VA350	2019	North	6	Clinical	Bronchial lavage	56	M	Bronchiectasis	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	2	0.5	0.5	No mutation detected
VA299CP	2019	Alentejo	10	Environment	Cowshed air	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	+	+	+	4	4	2	TR_{34}/L98H
VA873CP	2019	Lisbon	11	Environment	FRPD fom waste sorting industry' workers	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	+	+	+	4	2	1	TR_{34}/L98H
VA978CP	2019	Lisbon	11	Environment	FRPD fom waste sorting industry' workers	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	-	+	-	1	0.25	0.25	No mutation detected
VA1161CP	2019	Lisbon	12	Environment	Thermal SPA	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	+	-	-		LV		N.P.
V1207CP	2019	Lisbon	11	Environment	FRPD fom waste sorting industry' workers	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	-	-	+	1	0.5	0.5	No mutation detected
VA1209CP	2019	Lisbon	11	Environment	FRPD fom waste sorting industry' workers	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	+	-	+	8	4	1	TR_{34}/L98H
VA1215CP	2019	Lisbon	11	Environment	FRPD fom waste sorting industry' workers	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	-	+	+	1	0.25	0.125	N248K
VA1216CP	2019	Lisbon	11	Environment	FRPD fom waste sorting industry' workers	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	-	+	-	1	0.25	0.25	N248K
VA610CP	2019	North	13	Environment	Hospital air	N.A.	N.A.	N.A.	*Fumigati*	*A. fumigatus* sensu stricto	±	-	-	2	0.5	0.5	No mutation detected

Legend:

N.A.: Not applicable

N.R.: Not referred

N.P.: Not performed

FRPD: Filtering respiratory protective devices

LV: Lost of strain viability

Overall, all isolates considered as resistant or non-wildtype by microdilution grew in azole screening media (Table 5). It was not possible to run the MICs of two isolates (1 *A. fumigatus* sensu stricto and 1 *A. lentulus*), since they were not able to be recovered in culture.

Isolates that showed elevated MICs to azoles were screened by multiplex *Aspergillus* PCR kit for detection of specific mutations, or sequenced the *cyp51A* gene and its promoter.

The overall prevalence of resistance to azoles in *A. fumigatus* sensu stricto was 3.0% (four clinical – VA85, VA303, VA328 and VA346 - and five environmental isolates – VA86, VA95, VA299CP, VA873CP, VA1209CP) (Table 5). Regarding clinical isolates, the amplification of the *cyp*51A gene of the VA85 isolate was inhibited, preventing any conclusions regarding the resistance mechanism involved (high MICs for PCZ and VCZ, intermediate for ICZ). Although displaying MICs higher than the proposed ECVs, no mutations were found in the *cyp*51A gene of VA328 isolate (high MICs for ICZ and PCZ, wild type for VCZ), whereas *cyp51A* sequencing of VA346 isolate (high MIC for PCZ, intermediate for ICZ, susceptible to VCZ) showed the presence of the following mutations: F46Y, M172V, N248T, D255E. In the VA303 isolate (pan-azole resistant), the TR_{34}/L98H mutations were detected. For the environmental isolates considered as non-wild types, the TR_{34}/L98H was also detected in the VA299CP, VA873CP, VA1209CP isolates, conferring pan-azole resistance. The multiplex *Aspergillus* PCR kit M220-G54 and the *cyp*51A gene sequencing allowed the detection of a G54R mutation in the VA86 isolate, conferring *in vitro* resistance to ICZ and PCZ. Although displaying MICs higher than the proposed ECVs for either ICZ, VCZ and PCZ, no mutation was found in the *cyp51A* gene of the VA95 isolate.

The TR_{34}/L98H was the most frequently detected mutation (in 1.4% of the isolates), found in three environmental and one clinical isolates. In all studied cases, data obtained by the real time *Aspergillus* multiplex PCR was corroborated by sequencing of the *cyp*51A gene and its promotor.

The six cryptic species tested showed elevated MICs to the triazoles (Table 5).

4. Discussion

In this study, we analyzed the *Aspergillus* distribution in clinical and environmental products during a two-year period, from 2017 to 2018, and also the patterns of *A. fumigatus* azole-resistance using isolates from the surveillance programme established by our Reference Laboratory, from 2012 to 2019.

The definitive species identification requires specific sequencing analyses of the beta-tubulin or calmodulin genes, which are not available in most laboratories. This lack of recognition may have important consequences as cryptic species often display some level of intrinsic resistance to azoles and other antifungal drugs. Some of the clinical species identified during our study, such as *A. lentulus*, *A. udagawae*, *A. felis*, or *A. tubigensis* have been associated with refractory cases of IA [32-34].

As expected, *Fumigati* was the most frequently isolated section from our clinical samples. The same was observed in several epidemiological studies in other countries [5, 35].

Diagnosis of invasive aspergillosis often relies on suggestive radiological findings and/or positive fungal biomarkers [36]. Therefore, the absence of microbiological data and the actual contribution of *Aspergillus* spp. other than *A. fumigatus* (namely cryptic species) in clinical setting is unknown [37].

A high percentage (37%) of cryptic species was detected in our study in clinical isolates. To the best of our knowledge, this proportion of cryptic species of *Aspergillus* was the highest reported in literature, even doubling what was previously reported in a previous study from our group [38]. In another Portuguese study [15], this value was much lower (37.0% versus 7.5%). These differences may reflect the geographical source of the isolates and their epidemiological status. In both studies, the most frequent cryptic species was *A. welwitschiae*. In a study published by Pinto et al. [15], the 7.5% prevalence of cryptic species was

distributed by the *A. niger* section (3.1%) and by the *A. fumigatus* section (2.2%). We found 84% of cryptic species among our clinical isolates belonging to *Nigri* section, and 5.2% belonging to the *Fumigati* section. In Spain, the percentage of clinical isolates identified as cryptic species belonging to *Fumigati* section was lower (2.2%) [39]. Regarding mixed infections/colonizations, *A. terreus* was found frequently associated with other species, even with a pan-azole resistant *A. fumigatus* (patient #19). In a study published by Zoran et al., [40] posaconazole-resistance in *A. terreus* isolates is higher than 10%. Given the reduced susceptibility of *A. terreus* to amphotericine B [41], the management of those infections may be more difficult. Simultaneous colonizations/infections of *A. terreus* together with other species should be taken into consideration in the evaluation of therapeutic approaches.

Interestingly, several of the identified cryptic species, especially *A. lentulus*, were isolated together with *A. fumigatus* sensu stricto or with other species (Table 4). This has also been reported in other studies [42]. According to several authors [35, 43-45], cryptic *Fumigati* species have been recognized as occasional causes of invasive aspergillosis in 3 to 6% of cases. Noteworthy, in our surveillance study, several of these cryptic species could not be identified by culture morphology, but only through molecular methods, and five isolates could not even be characterized at section level, having been referred as *Aspergillus* sp. (Table 5).

Infections due to *A. lentulus* or other *A. fumigatus*-related species are associated with a particularly high mortality rate, about 60% [37]. These species are often less susceptible to azoles [44, 46], which is also reported in our study. Their actual prevalence may be underestimated because of their lack of recognition by conventional diagnostic approaches.

Our results showed an even higher frequency of cryptic species in environmental isolates, as corroborated by Simpson index value. As already shown in several studies [47, 48, 49, 50], the environment represents the major source of *Aspergillus* isolates causing infections. Exposure to *Aspergillus* may occur at home, in agricultural/animal production, during hospitalization, during leisure activities, or at the workplace. The development of *Aspergillus* infections depends on the interplay between the environment, the host susceptibility and the fungal species. Thus, the knowledge of the epidemiology of these settings may be important given that different fungal species may cause different symptoms during colonization, infection or sensitization and may show varying susceptibilities to multiple antifungal drugs [51].

In 2017, the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) recommend the susceptibility testing of *Aspergillus* on isolates causing invasive disease in patients from regions where resistance is found in surveillance programs [52]. However, this recommendation brings concern regarding the paucity of surveillance programs that perform mold susceptibility testing. Thus, this study aimed to present an estimate of the frequency of azoles resistant isolates of *A. fumigatus* in Portugal. According to our data, the estimated overall frequency of resistance in *A. fumigatus* sensu stricto was of 3.0%. Such value differs from previous Portuguese studies. Amorim et al., [53] reported a <1% prevalence of itraconazole resistance in 159 isolates of *A. fumigatus* collected from cystic fibrosis patients receiving azole antifungal therapy. More recently, in a study on susceptibility of environmental *A. fumigatus* collected in the North of Portugal [54], 21.8% of isolates were resistant to itraconazole, 38.2% to posaconazole and none of them were resistant to voriconazole. These differences may be explained by the date of collection of the isolates and by the geographical region from where the isolates were obtained.

According to a recent Spanish survey [39], 6.6% of patients carried azole resistant *A. fumigatus* sensu lato. A similar percentage was obtained with our data, 5.1%.

The occurrence of azoles resistant isolates of *A. fumigatus* varies worldwide, from 2.1 to 20% in the UK, 10–12% in the Europe, 10% in Asia, Africa, America and Australia to 1.75% in India, probably due to varying usage of azole fungicides that may select for resistance [28].

Azole-containing plates are easy to use in a routine mycology laboratory, offering the possibility of screening large collections of clinical strains at low cost. When comparing the data obtained by azole screening with data obtained by the reference method, false negatives were rare (only three isolates with MIC > 0.5 mg/L that did not grow on PCZ-agar and one with MIC > 1 mg/L that did not grow on VCZ-agar).

On the other hand, false positives were frequent but in the majority of the isolates considered as having "residual" growth, MICs were below the ECVs values. However, Morio et al. [55] refer that the use of a concentration of 4 mg/L itraconazole, which is above the ECV for *A. fumigatus* and might be a limitation, as some non-susceptible isolates could be missed.

The Clinical Laboratory Standards Institute (CLSI) is developing susceptibility breakpoints for the triazoles and *A. fumigatus* to provide a tool to guide and optimize treatment. Recently CLSI set breakpoints for voriconazole and *A. fumigatus*. These results identified voriconazole MIC values linked to treatment failure (≥2 μg/mL) and further identified susceptibility at concentrations ≤0.5 μg/mL. Regarding posaconazole, Espinel-Ingroff et al. [27] observed an overlap between MICs for non-mutant and mutant isolates was more evident with the ECVs of 0.5 μg/mL and, for that reason, we selected this value as ECV. Seven *A. fumigatus* sensu stricto isolates showed a MIC=2 μg/ml to ICZ and three isolates showed a MIC=2 μg/mL to VCZ. The latter value was considered as intermediate by CLSI. According to the most recent EUCAST guidelines [56], for some organism-agent combinations (as for *A. fumigatus* and itraconazole and voriconazole), results may be in an area, designated as the Area of Technical Uncertainty (ATU), where the interpretation is uncertain. In these cases, results should be reported as resistant with the comment that in some clinical situations (non-invasive infections) itraconazole can be used provided sufficient exposure is ensured. As an example, VA350 isolate showed MIC=2 μg/mL to ICZ but no *cyp51A* mutation was found.

Among our *A. fumigatus* isolates collected from 2012 to 2019 and available for molecular testing, three harbored the TR_{34}/L98H alterations in the *cyp51A* gene. These isolates were collected between 2018 and 2019, which may show an increase in the frequency of circulation of triazole-resistant *A. fumigatus.* The first clinical *A. fumigatus* isolates resistant to azoles detected in Portugal were described in 2018 [14]. According to our retrospective analysis, our first resistant isolate (mutation G54R) was collected in 2015, which reveals that azole resistant environmental isolates emerged in our country earlier than what was believed until now. Sharma et al. [57] also found resistant environmental isolates associated with this mutation. Isolate VA346 showed the presence of the following mutations: F46Y, M172V, N248T, D255E. These mutations (plus E427K) have been described to have different azole susceptibility profiles and to be azole susceptible or resistant, depending on the authors, but in all cases they have higher azole MICs than *A. fumigatus* strains with wild-type *cyp51A* [58]. The same first four mutations were also found (together with E427K) in a pan-azole resistant Portuguese isolate analyzed by Monteiro et al. [54].

Mutation N248K was detected in two isolates that grew in azole screening media but their MICs were lower than the proposed ECVs. As in other studies, N248K amino acid substitutions were previously reported in azole-susceptible *A. fumigatus*, suggesting that these alterations are SNPs that do not confer phenotypic resistance [59].

No mutations were detected in the *cyp51A* gene in the of the triazole-resistant VA95 isolate. As we have only screened for mutations on the *cyp51A*, it is possible that that resistance may be due to additional mechanisms of azole drug tolerance, such as mutations in *hapE* gene, decreased absorption of azole, or increased expression of efflux pumps [60].

Limitations of this study include the fact that the sequencing protocol used does not allow for the complete sequencing of the *cyp51A* gene (reaching only to the codon 365). Thus, mutations occurring at the end of this gene may not be detected. Furthermore, it was not possible to amplify calmodulin or beta-tubulin genes of several isolates, which resulted in an incomplete classification of our set of isolates. The isolates included in this study were not collected systematically from all regions of the country, which may lead to a bias in their geographical distribution. The majority of the isolates were obtained from products that were not accompanied by enough clinical information, making the differentiation between colonization and infection difficult. Finally, when interpreting the resistance percentage reported here, we should also consider that, in clinical practice, a sizeable number of patients have culture-negative aspergillosis. As such, a positive selection bias toward resistance may occur, as sampling procedures are more likely to be undertaken for patients for whom antifungal therapy fails. Considering the before mentioned limitations, it becomes clear

that studies aiming at using a representative set of isolates and that may account for all these potential confounders are much needed, in order to have a more accurate estimation of the resistance profile in our country.

In summary, this study highlights the importance of the implementation of a national surveillance network for *Aspergillus*. These findings are important contributions to raise the awareness to the importance of *Aspergillus* epidemiological distribution and to the surveillance of azole resistance in *A. fumigatus* in different Portuguese institutions, environments and from diverse geographic locations. Understanding the prevalence of azole resistant isolates is important to guide clinical and public health decision-making.

5. Conclusions

The understanding shifts in the epidemiology of *Aspergillus* and of local resistance patterns is needed to manage therapeutic approaches. From all studied *Aspergillus*, a very high percentage of cryptic species was found. In our collection of *Fumigati* isolates, more than 5% of them were cryptic species and from those almost all showed resistance to azoles. From *A. fumigatus* sensu stricto, 3% showed resistance to azoles, being the TR_{34}/L98H the most frequent mutation.

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Apêndice V - MANUSCRITO V. Azole-resistant *Aspergillus fumigatus* harboring the TR34/L98H mutation: First report in Portugal in environmental samples

O apêndice a seguir corresponde a um manuscrito submetido ao periódico Microorganisms. A colaboração que resultou na co-autoria deste trabalho foi realizada durante o período de doutoramento sanduíche desta autora pelo programa de internacionalização PDSE da CAPES, no Departamento de Doenças Infeciosas/ Unidade de Referência de Infeções Parasitárias e Fúngicas do Instituto Nacional de Saúde Dr. Ricardo Jorge, em Lisboa, Portugal.

Neste trabalho, realizei a identificação molecular, o screening para azóis e a microdiluição seriada de parte das estirpes de *Aspergillus*. Além disso, participei da revisão da redação do manuscrito.

microorganisms

Article

Azole-Resistant *Aspergillus fumigatus* Harboring the TR34/L98H Mutation: First Report In Portugal In Environmental Samples

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Abstract: Introduction**:** The frequency in detection of azole-resistant *Aspergillus fumigatus* isolates has increased over the last decade. In Portugal, section *Fumigati* is one of the most frequent, and resistant strains have been found in clinical and environmental contexts. Although several cryptic species within the *Fumigati* section show intrinsic resistance to azoles, one factor driving (acquired) resistance is selective pressure deriving from extensive use of azoles. This is particularly problematic in occupational environments where high fungal loads are expectable, and where there is an increased risk of human exposure and infection, with impact on treatment success and disease outcome. The mechanisms of resistance are diverse, but mainly associated with mutations in the *cyp51A* gene. Despite TR34/L98H is the most frequent mutation described, it has only been detected in clinical specimens in Portugal. Methods: We analyzed 99 *A. fumigatus* isolates from indoor environments (healthcare facilities, spa, one dairy and one waste sorting unit) collected from January 2018 to February 2019 in different regions of Portugal. Isolates were screened for resistance to itraconazole, voriconazole and posaconazole by culture and resistance confirmed by broth microdilution. Sequencing of the *cyp51A* gene and its promoter was performed to detect mutations associated with resistance. Results: Overall, 8.1% of isolates were able to grow in the presence of at least one azole, and 3% (isolated from the air in a dairy and from filtering respiratory protective devices in a waste sorting industry) were pan-azole resistant, bearing the TR34/L98H mutation. Conclusion: For the first time in our country, we report environmental isolates bearing the TR34/L98H mutation, isolated from occupational environments. Environmental surveillance of the emergence of azole resistant *A. fumigatus* sensu stricto strains is needed, to ensure proper and timely implementation of control policies that may have a positive impact on public and occupational health.

Keywords: *Aspergillus fumigatus*; azoles; antifungal resistance; *cyp51A* gene; environment; occupational exposure

1. Introduction

The *Fumigati* section is one of the most prevalent *Aspergillus* sections, in the clinical context as well as in the environment, in Portugal [1-3] and elsewhere [4-11]. They can be isolated from air, water or soil, and can easily contaminate indoor environments. By producing large numbers of small conidia that can become airborne and be inhaled, they can colonize the upper or lower airways producing mycotoxicosis, allergies, and invasive infections [12-19]. Immunocompromised individuals are at a higher risk of developing disease or aggravate pre-existing respiratory conditions. This fact is particularly important in health-care settings, where environmental contamination can result in nosocomial outbreaks of fungal respiratory disease [20-21]. Although infection in immunocompetent individuals is not frequent, exposure to largely contaminated environments as it happens in agriculture, in wood and food (particularly animal) industries, and in waste handling increases the risk of infection.

Exposure to bio aerosols in waste handling and sorting plants and in animal farms has been considered as an occupational health problem. These were the main promoters of several respiratory symptoms, namely decline in lung function, asthma, chronic bronchitis, bronchial hyper-responsiveness, wheeze, and cough. Disposable Filtering Respiratory Protective Devices (FRPD) regularly worn by these workers may constitute a serious occupational hazard since water vapor and sweat are released increasing humidity of the material, and together with high temperature, provide favorable conditions for microorganism's growth. *Aspergillus fumigatus*, has been frequently found on the filters of the FRPD, which can be highlighted as a critical occupational risk [22-24].

Fumigati cryptic species show intrinsic resistance to several antifungals. However, resistance acquisition in *A. fumigatus* sensu stricto is emerging due to selective pressure caused by prolonged azole treatment of chronic aspergillosis patients (that can range from several weeks to years, or even a patient's life time) or due to environmental selective pressure. The mechanisms of azole resistance are often associated with mutations in genes involved in the *A. fumigatus* ergosterol pathway [25], particularly in the *cyp51A* gene which encodes the cytochrome P450 14-α-lanosterol demethylase, the main target of azole antifungals [26]. Point mutations within the *cyp51A* gene (G54, M220) are more frequently associated with prolonged azole prophylaxis/therapy [27-28]. The most common pan-azole resistance mutation is a combination of a 34-bp long tandem repeat in the promoter region and a leucine-to-histidine substitution in codon 98, TR_{34}/L98H [25, 29-30]. This mutation, firstly described in Dutch *A. fumigatus* isolates, is now spread worldwide [31] due to extensive use of azole fungicides in animal, agriculture and processing industries.

The selective pressure on *A. fumigatus* is particularly problematic in environments where the use of azoles is a requirement, such as in agriculture, in preservation industries and sawmills, where an increased probability of emergence of specific occupational health problems has been observed [32-33]. After infection of azole-naïve individuals with these resistant strains, subsequent treatment failure with triazole therapy (the first choice for treatment and prophylaxis of aspergillosis) may occur [34]. Consequently, higher morbidity and mortality rates associated with azole resistance are likely to become a major public health concern [25, 29, 34-35].

Monitoring the emergence of resistant *A. fumigatus* strains to antifungal drugs, particularly to medical triazoles, becomes essential for the adoption of prevention and control strategies with impact in public health. In Portugal, studies have shown that the frequency of azole-resistant *A. fumigatus* sensu stricto

strains is high for itraconazole (ICZ) and posaconazole (PCZ) (up to 92% and 54%, respectively), and lower for voriconazole (VCZ) (up to 3%) [2-3, 35]. The TR_{34}/L98H mutation has been detected in Portugal in clinical specimens but, to our knowledge, it has not yet been found on environmental isolates [2-3].

The objective of this study was to assess the frequency of cryptic species belonging to *Fumigati* section and determine the frequency of azole resistance in *A. fumigatus* sensu stricto strains isolated from environmental samples. These samples were collected from Portuguese health-care facilities (hospitals and health centers) and related healthcare environments (thermal spa), and from occupational environments with high fungal loads (waste sorting plants and dairies), where the presence of these fungi may represent a risk for the development of fungal respiratory disease. Our aim was to improve the knowledge of the *Fumigati* epidemiology in these environmental settings and to understand the molecular mechanisms involved in azole resistance in these isolates.

2. Materials and Methods

2.1. Environmental Sampling

This study was performed using environmental samples collected from January of 2018 to February 2019 in different indoor and occupational environments located in several regions of Portugal, with different sampling approaches (Table 1) in the context of enlarged financed studies focusing on occupational exposure to fungi or indoor air quality assessments [22-23, 36-40].

Table 1. Indoor and occupational environments assessed and applied sampling approaches.

Type and number of indoor/occupational environments assessed	Sampling approaches
Dairy (n=1)	Air impaction
Hospital (n=2) / Health Centre (n=10)	Air impaction
	EDC
	Settled dust
	Surface swab
Thermal spa (n=5)	Air impaction
	EDC
Waste sorting plant (n=1)	FRPD
	MPG

EDC – Electrostatic dust collector; FRPD – Filtering respiratory protection devices; MPG – Mechanic protection gloves.

Indoor air (50 to 250 L) was collected with a Millipore Air Tester (Millipore, Billerica, MA, USA) using a flow rate of 140 L/min, and impacted directly onto culture media plates, according to the manufacturer's instructions [39], for the isolation of *A. fumigatus* (section).

Surface samples were collected by swabbing with a 10 cm × 10 cm square stencil, which was disinfected with a 70% alcohol solution between samplings [39]. Fungal contamination was extracted from the swab by washing with 0.9% NaCl with 0.1% Tween80™, for 30 min at 250 rpm on an orbital laboratory shaker (Edmund Bühler SM-30, Hechingen, Germany) [39]. Wash suspensions were inoculated for *A. fumigatus* isolation as described below.

Pieces with 2 cm^2 (1.4 cm × 1.4 cm) were obtained from Filtering Respiratory Protection Devices (FRPD) and Mechanical Protection Gloves (MPG) [22, 41]. Electrostatic Dust Collectors (EDC) having a surface

exposure area of 0.0209 m (19 × 11 cm) were placed at a minimum 0.93m above floor level, and dust was allowed to settle on the EDC cloth for 13 to 16 days [37]. Fungal contamination was extracted from the FRPD and MPG pieces, and EDC cloths by washing (as described for surface swabs), and suspensions were inoculated for *A. fumigatus* isolation as described below.

Settled dust samples were weighted and 1 g of dust was washed with 9.1 ml of 0.9% NaCl with 0.05% Tween80™, for 60 min at 250 rpm [42]. Wash suspensions were inoculated as below.

2.2. Isolation of Aspergillus Section Fumigati

For *Aspergillus* isolation, 150 µl of the washing suspensions obtained above were inoculated onto malt extract agar (MEA) supplemented with chloramphenicol (0.05%), dichloran-glycerol agar (DG18), Sabouraud dextrose agar (SDA) and SDA supplemented with either 4 mg/L itraconazole (ICZ), 1 mg/L voriconazole (VCZ), or 0.5 mg/L posaconazole (PCZ) [43]. After incubation at 27 °C for 5 to 7 days, fungal species were identified at section level by macroscopic and microscopic morphology using tease mount or Scotch tape mount and lactophenol cotton blue mount procedures and using identification atlases [44-45] (Figure 1). *Aspergillus fumigatus* isolates were selected for further characterization. These procedures followed the algorithm previously suggested to assess the presence of *A. fumigatus* resistant strains [33].

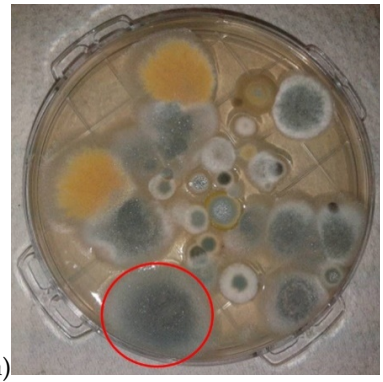
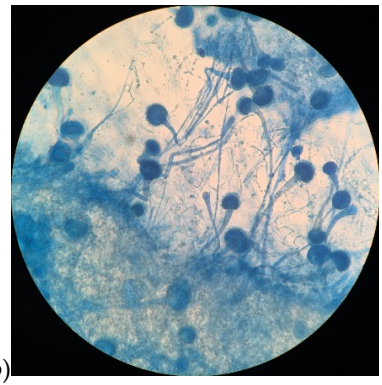
(a)  (b) 

Figure 1. Isolation of *Aspergillus* from air samples: (a) Example of a culture media plate resulting from the collection of indoor air by impaction, and from where *Aspergillus* section *Fumigati* (circled red) was isolated; (b) Example of a microscopic (400x) observation of a lactophenol blue mount of an *A. fumigatus* isolate.

2.3. Molecular Identification of Aspergillus Isolates

Aspergillus fumigatus isolates were confirmed by calmodulin [46] or beta-tubulin [47] sequencing. Briefly, amplifications were performed in a 25 µl volume reaction of Illustra PureTaq Read-to-Go PCR beads (GE Healthcare, Buckinghamshire, UK), containing 15 pmol of the primers cmd5/cmd6 or βtub1/βtub2 (for calmodulin or beta-tubulin amplification, respectively) and 4µl of *Aspergillus* genomic DNA extracted with the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Amplifications were carried with the following thermocycling conditions: 1) for calmodulin an initial denaturation at 95ºC for 10 min, followed by 38 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and a last final extension step of 72°C for 7 min; 2) for beta-tubulin an initial denaturation at 94ºC for 2 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, and a last final extension step of 72°C for 5 min. PCR products were analyzed by electrophoresis through 2% agarose gels and the resultant PCR amplicons were purified using the ExoSAP-IT enzyme system (USB Corporation, Cleveland, OH), according to the manufacturer's instructions. Sequencing of the forward strand was performed with the BigDye terminator v 1.1 Cycle sequencing kit (Applied Biosystems) in the thermal cycler with the following conditions: an initial denaturation at 96°C for 5 sec, followed by 30

cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, followed by one cycle of 72°C for 5 min. The resultant nucleotide sequences were edited using the program GeneStudio™ Professional Edition version 2.2.0.0 and aligned with the program MEGA version 10.0.5. These sequences were compared with sequences deposited in the GenBank database (Bethesda, MD, USA) in order to achieve the identification to species level.

2.4. Screening for Azole-Resistant Isolates

A first characterization of the resistance pattern of the *A. fumigatus* sensu stricto isolates was carried out using screening media made of Sabouraud dextrose agar supplemented with ICZ, VCZ or PCZ [48]. The reference strain *A. fumigatus* ATCC 204305 was used as negative control and the pan-azole resistant strain TR_{34}/L98H (kindly provided by Professor Jacques Meis, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands) was used as positive control. Strains that were not able to grow in the azole-supplemented plates were considered as susceptible to the respective azoles at tested concentrations, according to EUCAST guidelines [48]. On the other hand, isolates that grew in at least one of the screening media were selected for further analysis.

2.5. Antifungal Susceptibility Testing

The M38-A2 protocol from the Clinical and Laboratory Standards Institute (CLSI) was applied for determining the minimal inhibitory concentrations (MIC) for ICZ, VCZ and PCZ [35, 49]. The final concentrations of drugs in the wells ranged from 0.0156 to 8 μg/ml. An internal control strain (*A. flavus* ATCC 204304) with known susceptibility was included in each run as a positive control of the antifungals' potency. Sabouraud dextrose agar plates were inoculated with the final inoculum to check the number of colony-forming units in the inoculum. Plates were incubated at 35ºC and examined after 48h incubation. Absence of visual growth defined the MIC. Breakpoints for mold testing have not been established by the CLSI (recently set breakpoints for voriconazole and *A. fumigatus*). The CLSI epidemiological cut-off values (ECVs) used were 1 mg/L for ICZ, 1 mg/L for VCZ, and 0.5 mg/L for PCZ [50-52]. Isolates with high MICs were tested in triplicate to confirm the obtained results.

2.6. Molecular Identification of Resistance Markers

Azole-resistant isolates were tested by a multiplex real-time PCR which screens for TR_{34}/L98H and TR46/Y121F/T289A mutations found in the *cyp51A* gene and its promoter [AsperGenius® multiplex real-time PCR assay (PathoNostics, Maastricht, The Netherlands) on the RotorGene Q instrument (Qiagen, Hilden, Germany), following the manufacturer's instructions], and/or by sequencing of the *cyp51A* gene and its promoter as described [53-54]. Nucleotide sequences were edited and aligned as described above.

3. Results

A total of 142 environmental samples were studied (Table 2), the majority of which (114, 80.3%) were obtained from the analyzed waste sorting plant.

Table 2. Number of samples screened per sampling approach and type of indoor/occupational environment assessed, and number of samples positive for *Fumigati* section.

Indoor/occupational environment	No. of samples per sampling approach							No. of samples positive for *Fumigati*
	Air	EDC	Settled dust	Surface swab	FRPD	MPG	Total (%)	

								section (%)
Waste sorting plants	0	0	0	0	113	1	114 (80.3)	76 (66.7)
Hospital/Health Centre	13	1	5	1	0	0	20 (14.1)	19 (95.0)
Thermal spa	7	0	0	0	0	0	7 (4.9)	3 (42.9)
Dairy	1	0	0	0	0	0	1 (0.7)	1 (100.0)
Total (%)	21 (14.8)	1 (0.7)	5 (3.5)	1 (0.7)	113 (79.6)	1 (0.7)	142 (100.0)	99 (69.7)

EDC – Electrostatic dust collectors; FRPD – Filtering respiratory protection devices; MPG – Mechanic protection gloves

Aspergillus fumigatus (section) were isolated from all sources studied, particularly from the waste sorting plant (76/114, 66.7%, positive samples) and health care units (hospitals/health centers; 19/20, 95.0%, positive samples), for a total of 99/142 (69.7%) isolates. From these, 93/99 (94.0%) were identified as *A. fumigatus* sensu stricto. For the remaining 6 isolates, identification to species level was not possible as no PCR product for calmodulin or beta-tubulin sequencing could be obtained. Two isolates, obtained from one air sample from a hospital and from a FRPD from a waste sorting worker, were initially classified as *A. fumigatus* based on macroscopic and microscopic characteristics and later identified as *A. sidowii* and *Penicillium* spp., respectively, by calmodulin sequencing and were not considered in the analysis.

Eight out of the 99 *A. fumigatus* sensu stricto isolates (8.1%), obtained from air samples from a diary and a hospital, and from FRPD worn by workers of a waste sorting plant, grew on at least one of the screening media (Table 3). Three isolates (3.0%) were confirmed as resistant to ICZ, VCZ and PCZ, and sequencing of the *cyp51A* gene and its promoter revealed the TR_{34}/L98H mutation in all 3 isolates. In addition, the N248K mutation was also detected in 2 isolates. No other mutations were found.

Table 3. Growth of resistant *A. fumigatus* isolates in different screening media, minimal inhibitory concentrations for ICZ, VCZ and PCZ, and mutations found on the *cyp51A* gene and its promoter.

Isolate number	**Source**	**Azole Screening media**			**Minimal inhibitory concentration (mg/L)**			***cyp51A* mutations**
		ICZ	**VCZ**	**PCZ**	**ICZ**	**VCZ**	**PCZ**	
VA299CP	Dairy air	+	+	+	4	4	2	TR_{34}/L98H
VA610CP	Hospital air	±	-	-	2	0,5	0,5	No mutation detected
VA873CP	Waste sorting plant FRPD	+	+	+	4	2	1	TR_{34}/L98H
VA978CP	Waste sorting plant FRPD	-	+	-	1	0,25	0,25	No mutation detected
V1207CP	Waste sorting plant FRPD	-	-	+	1	0,5	0,5	No mutation detected
VA1209CP	Waste sorting plant FRPD	+	-	+	8	4	1	TR_{34}/L98H
VA1215CP	Waste sorting plant FRPD	-	+	+	1	0,25	0,125	N248K
VA1216CP	Waste sorting plant FRPD	-	+	-	1	0,25	0,25	N248K

ICZ: itraconazole; VCZ: voriconazole; PCZ: posaconazole; -: negative (no growth); ±: residual growth (growth of only one or few small colonies; +: relevant growth (growth similar to positive control).

4. Discussion

The presence of azole resistant isolates in Portugal has been reported both in clinical specimens and in the environment [2-3, 35, 55]. To the best of our knowledge, the TR_{34}/L98H mutations, commonly found in the *cyp51A* gene of azole-resistant isolates, were only reported in clinical specimens in our country. Other mutations in the *cyp51A* gene were reported in Portuguese environmental azole-resistant *A. fumigatus* isolates [3], but none undoubtedly described as being associated with resistance. Thus, in this study, we present the first evidence of environmental pan-azole resistant strains circulating in Portugal harboring the TR_{34}/L98H mutations. These resistant strains were isolated from FRPD worn by waste sorting workers during their working shift and from one air sample from a dairy, which is a strong indicator of the increased risk of disease to which workers from these environments are exposed.

In a recent publication, our research group [56] reviewed the literature on occupational and indoor exposure to *Aspergillus* and potential health effects associated with that exposure. Among the workplaces with high fungal contamination and potentially high levels of mycotoxins, waste sorting plants stand as one of the environments with the highest fungal contamination [57-58]. This is not only due to the type of materials being processed and the consequent availability of nutrients, but also due to the presence of high concentration of indoor dust particles, to the deposition of waste indoors and to the building materials, all of which favor fungal growth and sporulation [56].

Aspergillus is also described as one of the most frequent fungi in animal production farms [58-59]. The higher fungal contamination is probably a result of the higher animal density and confined production, conditions that promote the multiplication of microorganisms. Nevertheless, those are not the only sources of contamination, and animal feed and/or animal litter must be considered as potential sources, as their distribution may generate aerosolized particles that remain in the air or are deposited onto the floor for a long time. Cereal-based feed and wood shavings used in bedding can introduce resistant fungal strains into animal production farms. This may happen as a result of the azole pressure exerted in fungal selection, a consequence of the systemic use of azole and azole-based fungicides in the protection of feed crops [60-61] and in wood processing and preservation [32, 62]. Although correct management and protection equipment may protect workers in these occupational environments, azole residues are still spread through the environment and can have a negative health impact through their toxicity and persistence in the environment, while boosting the development of azole-resistant fungal strains [63-64].

By working in close contact with these materials, waste sorting and animal production workers are at increased risk of cumulative exposure to fungal particles and mycotoxins, which can reach their respiratory systems and result in occupationally acquired respiratory disease [56]. Of particular importance, exposure of these workers to azole-resistant isolates may lead to treatment failure and consequent significant impact on patient management and associated health costs. Therefore, strategies to prevent or minimize the occupational exposure to *Aspergillus* become of paramount importance and should include, but not be limited to, the correct use, storage and elimination of personal protective equipment, particularly FRPDs, and the implementation of continuous assessment and characterization of the *Aspergillus* burden in such environments.

The obtained results also validate the a previously proposed algorithm aiming to support and provide guidance from fieldwork in assessed occupational environments (which sampling methods should be applied) to the bench work (what analysis should be performed) for exposure assessors [33]. Thus, in occupational environments where the azole pressure is high, such as vineyards, sawmills and waste sorting plants, just to name a few, the frequency of cryptic species and of azole resistance of *A. fumigatus* sensu stricto should be assessed as routine to achieve an accurate risk characterization. In high-load environments, such as in the waste sorting unit and the dairy farm assessed, results are most certainly underestimated, since the recovery of the *Aspergillus* section *Fumigati* isolates is a challenging task due to overgrowth of other fungi isolates with fast growth rates, such as *Mucorales* and *Chrysonilia sitophila* [22, 33, 37, 39, 40, 42]. Additionally, isolate recovery relies only on the viable component of the fungal contamination. Thus, the culture-based methods drawbacks should be considered [22, 33].

Several hospital environmental *Fumigati* isolates were also analyzed in this study. Despite no mutations have been detected, monitoring these environments for the frequency of cryptic species (intrinsically resistant to azoles) and for the emergence of resistant *A. fumigatus* strains to antifungal drugs, particularly to medical triazoles, becomes essential for the adoption of prevention and control strategies in order to reduce the mortality associated with these infections in at risk patients.

Azole-resistant *A. fumigatus* strains will probably become more frequent in the future, as a consequence of natural evolution and by selective pressure due to the use of triazoles in medicine and in the environment, with important public health implications. The widespread use of triazoles, in particular, has become the major driver for the clonal expansion of triazole-resistant *A. fumigatus* genotypes, particularly at local level [65]. Therefore, the continued surveillance of *Aspergillus*, both in the clinical and in the occupational environments, at local and regional levels, is of paramount importance for the control of the emergence of resistance and, ultimately, for the prevention of aspergillosis.

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