Universidade Federal do Rio Grande do Sul
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Programa de Pós-Graduação em Ciências Pneumológicas

Melissa Orzechowski Xavier

Aplicações e limitações do método de detecção do antígeno galactomanana para o diagnóstico de aspergilose

Porto Alegre, 2008.

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Porto Alegre, 19 de dezembro de 2008.

A Comissão Examinadora, abaixo assinada, aprova a Tese "Aplicações e limitações do método de detecção do antígeno galactomanana para o diagnóstico de aspergilose", elaborada por Melissa Orzechowski Xavier, como requisito parcial para a obtenção do grau de Doutor em Ciências.

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Prof. Dr. Luiz Carlos Severo - UFRGS – Orientador

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"Põe quanto tu és no mínimo que fazes."

Fernando Pessoa

Resumo

XAVIER, Melissa Orzechowski. **Aplicações e limitações do método de detecção do antígeno galactomanana para o diagnóstico de aspergilose.** 2008. 90f. Tese (Doutorado) – Programa de Pós-Graduação em Ciências Pneumológicas. Universidade Federal do Rio Grande do Sul, Porto Alegre.

O Platelia® *Aspergillus* EIA é um teste de ELISA sanduíche para diagnóstico precoce de aspergilose em pacientes neutropênicos que se baseia na detecção de um antígeno (galactomanana) da parede celular de *Aspergillus* spp. O trabalho objetivou avaliar a eficácia deste teste em outros hospedeiros suscetíveis à aspergilose e ainda, avaliar a interferência de potenciais falso-positivos no Platelia® *Aspergillus* EIA, como outras micoses sistêmicas e um antimicrobiano produzido a partir de fungos (piperacilina-tazobactam). Quatro experimentos foram realizados para contemplar os objetivos propostos. Amostras de lavado broncoalveolar de 60 pacientes transplantados de pulmão provenientes da Santa-Casa Complexo Hospitalar de Porto Alegre foram colhidas durante um período de aproximadamente 2 anos e testadas para detecção de galactomanana. Os pacientes foram classificados em aspergilose comprovada, provável e possível, colonização ou exame de vigilância de acordo com critérios do EORTC. Utilizando os casos comprovados (5) e prováveis (6) como positivos, foi calculada a curva ROC que demonstrou valores de sensibilidade de 90,9% e especificidade de 90,6% em um ponto de corte de 1,5. A eficácia do Platelia® *Aspergillus* EIA foi avaliada também em pingüins em cativeiro. Soros de 35 animais foram incluídos no estudo, 9 com aspergilose, 3 com malária, 2 com caquexia e 21 saudáveis. Os soros foram testados por imunodifusão dupla e ELISA sanduíche, resultando em valores de sensibilidade de 33% e 100% e especificidade de 96% e 0, respectivamente. A reação cruzada de outras micoses sistêmicas no Platelia® *Aspergillus* EIA foi avaliada a partir de 120 amostras de soro de pacientes com paracoccidioidomicose, histoplasmose, criptococose por *Cryptococcus neoformans* e criptococose por *C. gattii*. Todas as micoses foram responsáveis por resultados falso-positivos no ELISA sanduíche, sendo de 50%, 67%, 66% e 36,6% a taxa de positividade de cada micose, respectivamente. Em adição, 5 lotes de piperacilina-tazobactam foram testados em concentração de uso clínico (45mg/ml) para avaliação de interferência no Platelia® *Aspergillus* EIA. Destas, apenas uma resultou em valores maiores do que o ponto de corte (0,5), sendo submetida a sucessivas diluições até mimetizar concentrações plasmáticas do fármaco alcançáveis no soro humano, as quais resultaram em valores menores que 0,5,

sendo consideradas negativas. Concluindo, utilizando um ponto de corte maior do que indicado pelo fabricante para uso em neutropênicos, a eficácia do teste foi comprovada para utilização em amostras de lavado broncoalveolar de pacientes transplantados de pulmão. Por outro lado, no hospedeiro animal testado, pingüins, o teste apresentou especificidade nula, não possuindo aplicabilidade como ferramenta diagnóstica para aspergilose neste grupo. Quanto aos fatores de interferência no Platelia® *Aspergillus* EIA avaliados, a alta taxa de resultados falso-positivos referentes à infecção por outras micoses sistêmicas reflete na necessidade de interpretar um teste positivo dentro do contexto epidemiológico do paciente. Por outro lado, as piperacilinas-tazobactam disponíveis no mercado brasileiro não interferiram no resultado do Platelia® *Aspergillus* EIA. No entanto como a variabilidade de galactomanana existe entre lotes, ainda é aconselhável que as amostras para realização do ELISA sanduíche sejam colhidas antes da próxima administração do fármaco.

Palavras-chave: galactomanana; aspergilose; transplante pulmonar; pingüins; reação cruzada; piperacilina-tazobactam.

Abstract

XAVIER, Melissa Orzechowski. **Applications and limitations of a galactomannan detection method in the diagnostic of aspergillosis.** 2008. 90f. Tese (Doutorado) – Programa de Pós-Graduação em Ciências Pneumológicas. Universidade Federal do Rio Grande do Sul, Porto Alegre.

Platelia® *Aspergillus* EIA is a sandwich ELISA to the diagnostic of aspergillosis in neutropenic patients. It detects an antigen (galactomannan) from *Aspergillus* cell wall. Here it was evaluated the performance of this test in other susceptible hosts and the interference of potentials false-positives factors in Platelia® *Aspergillus* EIA. Systemic mycosis and an antimicrobial produced from molds (piperacillin-tazobactam) were tested. Four experiments were executed to study conduce. Bronchoalveolar samples from 60 lung transplant recipients from Santa Casa-Complexo Hospitalar de Porto Alegre were collected during almost two years and tested for galactomannan detection. Patients were classified in proven, probable or possible aspergillosis according to EORTC criteria, or in colonization or surveillance. Considering proven (5) and probable (6) as true positive cases, ROC curve was calculated and showed 90.9% of sensitivity and 90.6% of specificity with 1.5 as optimal cutoff. Platelia® *Aspergillus* EIA efficacy was also tested in captive penguins. Sera from 35 animals were included in the study, 9 with aspergillosis, 3 with malaria, 2 with cachexia and 21 healthy. Samples were tested by double immunodiffusion and sandwich ELISA, resulting in sensitivity values of 33% and 100% and specificity of 96% and 0, respectively. Cross reaction in Platelia® *Aspergillus* EIA was evaluated with 120 serum samples of patients with paracocciddioidomicosis, histoplasmosis, criptococosis due to *Cryptococcus neoformans* and criptococosis due to *C. gattii.* False-positive results were observed in all mycosis, with rates of 50%, 67%, 66% and 36,6%, respectively. In addition, 5 piperacillin-tazobactam batches were tested, in a concentration of clinical use (45mg/ml), to evaluate it interference in Platelia® *Aspergillus* EIA. Those, only one showed positive value, and had been retest after serial dilutions until plasmatic concentration, resulting in value lower than 0.5, negative. In conclusion, with a higher cut-off than the indicated from the manufacturer, the efficacy of bronchoalveolar samples tested in Platelia® *Aspergillus* EIA for the diagnostic of aspergillosis in lung transplant recipients was proved. Controversially, in penguins, the test specificity was zero, showing non applicability as a diagnostic method for aspergillosis in this group of risk. Interference in Platelia® *Aspergillus* EIA due to other systemic mycoses shows the necessity

to interpret a positive result after the evaluation of patient epidemiologic context. Finally, piperacillin-tazobactam available in the Brazilian market did not correspond to false-positive results in Platelia® *Aspergillus* EIA. However, given that variability occurs between distinct batches, still is indicating to collect samples for galactomannan detection before the next administration of the drug.

Key words: galactomannan; aspergillosis; lung transplantation, penguins; cross-reaction; piperacillin-tazobactam.

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LBA – Lavado broncoalveolar

TC – Tomografia computadorizada

EIA – Ensaio imunoenzimático

FDA – *Food and Drug Administration*

EUA – Estados Unidos da América

CRAM – Centro de Recuperação de Animais Marinhos

ELISA – *Enzyme Linked Immuno Sorbent Assay*

ID – *Immunodiffusion*

USA – *United States of America*

OD – *Optical density*

PPV – *Positive predictive value*

NPV – *Negative predictive value*

IA – *Invasive aspergillosis*

GM – *Galactomannan*

ANVISA – Associação Nacional de Vigilância Sanitária

HIV – Vírus da Imunodeficiência Humana

CMV – Citomegalovírus

RX – Raio-X

RNM – Ressonância Magnética

SNC – Sistema nervoso central

UFC – Unidades formadoras de colônia

KOH – Hidróxido de potássio

AIDS – Síndrome da Imunodeficiência Adquirida

EORTC –*European Organization for Research and Treatment of Cancer*

ROC – *Receiver Operating Characteristic*

Sumário

1 INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA

1.1 ASPERGILOSE EM MEDICINA

As infecções fúngicas têm crescido significativamente em importância na prática clínica nas últimas três décadas, em especial como decorrência do aumento do número de indivíduos imunossuprimidos, criticamente enfermos ou submetidos a procedimentos médicos invasivos. Entre as micoses sistêmicas de importância médica, predominam aquelas causadas por leveduras do gênero *Candida* e por fungos filamentosos do gênero *Aspergillus*. A incidência de infecções de corrente sangüínea causadas por espécies de *Candida* vem decrescendo em muitos centros, em parte como resultado de um maior uso de antifúngicos imidazólicos, especialmente fluconazol[1]. Paralelamente, como o fluconazol não é ativo contra espécies de *Aspergillus*, a importância da aspergilose invasiva está em elevação conforme dados da literatura que demonstram um incremento de 3 a 20 vezes na incidência da aspergilose invasiva nas últimas duas décadas[2].

Enquanto numerosos estudos nos últimos 10 anos têm documentado as características de candidemia no Brasil[3-10], os dados sobre aspergilose invasiva são bastante escassos em nosso país, principalmente devido à dificuldade no diagnóstico precoce da doença. Estudo prévio realizado na Santa Casa Complexo Hospitalar de Porto Alegre demonstrou que 82% dos casos de aspergilose invasiva não foram diagnosticados antes da autopsia[11]. Esta realidade é semelhante em diversos países[12]; estima-se que 20-80% dos casos de aspergilose invasiva não sejam diagnosticados *ante mortem*, muitas vezes não havendo suspeita da infecção por parte da equipe assistente[1,13-15].

1.1.1 Aspergilose invasiva em pacientes transplantados de pulmão

Como a neutropenia prolongada e a terapia com corticosteróides são os principais fatores de risco para aspergilose invasiva, a doença incide particularmente em pacientes hematológicos em tratamento quimioterápico, bem como transplantados de medula óssea[15-17]. Pacientes submetidos a transplante de órgãos sólidos são também propensos à aspergilose invasiva, especialmente transplantados de pulmão, nos quais a incidência da infecção chega a 16% (média 6-8%)[13,18-21]. Vários fatores propiciam um risco maior para aspergilose invasiva em transplantados de pulmão, incluindo piora nos

mecanismos de defesa pulmonar, lesão isquêmica à via aérea, alteração na função alveolar fagocítica, comunicação direta do órgão transplantado com o ambiente externo e um grau elevado de imunossupressão[22]. De fato, a incidência de aspergilose invasiva é maior entre transplantados de pulmão do que em qualquer outro grupo de pacientes submetidos a transplante de órgãos sólidos. A mortalidade nesse grupo chega a 60-74% e estima-se que ao menos 9% das mortes após transplante de pulmão sejam diretamente atribuíveis à aspergilose[20].

A aspergilose em receptores de transplante pulmonar pode ser dividida em três formas clínicas: 1. Colonização da via aérea é caracterizada pelo isolamento de *Aspergillus* em cultivo de secreções brônquicas, na ausência de evidência clínica, radiológica, endoscópica ou histopatológica de lesão no trato respiratório inferior; 2. Casos que se apresentem com cultura positiva de lavado broncoalveolar (LBA), associados à lesão endobrônquica (na ausência de evidência de invasão do parênquima pulmonar) são classificados como traqueobronquite; 3. Por último, o isolamento fúngico associado a evidências radiológicas ou histológicas de invasão tecidual caracteriza aspergilose invasiva. A forma invasiva é conhecida como aspergilose pulmonar invasiva quando se limita ao pulmão, ou por aspergilose disseminada quando houver angioinvasão e disseminação por via hematógena para outros órgãos[23].

Pacientes com colonização respiratória por *Aspergillus* são usualmente assintomáticos e apresentam bom prognóstico, apesar de não se descartar a possibilidade de evolução para uma forma clínica mais grave, que ocorre em aproximadamente 3% dos casos[13,19,23]. A traqueobronquite acarreta lesões ulcerativas no local de anastomose, podendo haver necrose, formação de tecido pseudomembranoso, deiscência da linha de sutura e estenose da via aérea. Geralmente cursa com quadro assintomático, podendo haver febre, tosse e hemoptise em casos mais graves, que podem progredir para a forma invasiva. Sua incidência é maior nos primeiros seis meses pós-transplante[13,19,23]. Aspergilose invasiva cursa com infiltrado pulmonar, podendo levar a cavitações. Esta forma apresenta um prognóstico desfavorável, geralmente evoluindo para o óbito. Embora a maioria dos casos ocorra no primeiro ano pós-transplante[11,16,21], casos esporádicos podem ocorrer depois de decorridos três anos[24,25].

A detecção precoce da doença reduz a mortalidade associada à aspergilose invasiva[26-29], no entanto o diagnóstico definitivo desta condição é limitado por várias razões[30]. Culturas de secreções respiratórias possuem sensibilidade diagnóstica muito baixa: *Aspergillus* é recuperado em cultivo do escarro e do LBA em apenas 8-34% e 45-

62% dos pacientes com aspergilose invasiva, respectivamente. A confirmação diagnóstica usualmente requer avaliação histopatológica, no entanto, quadros de neutropenia ou trombocitopenia marcadas impedem a realização de procedimentos cirúrgicos invasivos nestes pacientes. Biópsias transbrônquicas, por outro lado, são associadas a uma freqüência elevada de resultados falso-negativos. Culturas de sangue, líquor e medula óssea raramente são positivas para *Aspergillus*. A tomografia computadorizada (TC) de tórax em alta resolução é um recurso auxiliar de grande importância no diagnóstico precoce de aspergilose pulmonar invasiva em indivíduos neutropênicos, particularmente na presença de um halo de necrose envolvendo nódulo pulmonar, o chamado "sinal do halo"[17,31,21]. No entanto, este sinal é menos encontrado em não-neutropênicos, e aspergilose pulmonar invasiva em pacientes transplantados de pulmão frequentemente se manifesta do ponto de vista radiológico por áreas de consolidação em placa[21].

Frente às dificuldades no diagnóstico de aspergilose invasiva, muitos pacientes são tratados empiricamente com drogas antifúngicas, o que inevitavelmente resulta em um grande número de pacientes expostos a terapias de custo elevado, associadas a considerável toxicidade e interações medicamentosas. Há, portanto, uma clara necessidade de se aprimorar o diagnóstico destas condições.

1.2 ASPERGILOSE EM VETERINÁRIA

Ao contrário da medicina humana, a incidência da aspergilose em veterinária não está somente relacionada com indivíduos imunossuprimidos. Apesar do déficit no sistema imune agir como um importante fator predisponente às formas graves desta micose, a suscetibilidade varia significativamente entre as espécies animais, muitas vezes acometendo indivíduos imunocompetentes e desencadeando diferentes formas e apresentações clínicas de acordo com o hospedeiro[33].

Em ruminantes, a aspergilose é responsável principalmente por mamites e alterações reprodutivas, culminando com placentite e aborto entre o 6º e 8º mês de gestação[34-38]. Eqüinos imunocompetentes são suscetíveis a guturocistite por *Aspergillus* spp. que pode levar o animal à morte devido a hemorragia por ruptura da carótida[33,39]. Com relação aos pequenos animais, casos de aspergilose são mais relatados em cães, mesmo sem comprometimento de sistema imune, os quais apresentam como principal forma clínica a aspergilose sinonasal[33,36,40]. Apesar de a aspergilose possuir relevância clínica em animais domésticos, e/ou econômica em animais de produção, conforme

descrito acima, o grande destaque desta micose em veterinária está relacionado a aves, principalmente em cativeiro[33].

Características anatômicas e fisiológicas peculiares às aves tornam-as muito suscetíveis à infecção por *Aspergillus* e ainda facilitam a reprodução fúngica[33]. Dentre estes fatores destacam-se a ausência de epiglote e escassez de epitélio ciliado no trato respiratório que facilitam a penetração dos conídios no trato respiratório inferior; ausência de diafragma dificultando a expulsão dos conídios infectantes do organismo através do reflexo da tosse; ausência de macrófagos alveolares, primeira linha de defesa contra os conídios de *Aspergillus*; e, ainda, presença de polimorfonucleares (heterófilos) com mecanismo de ação diferente e menos efetivo do que os neutrófilos de mamíferos. Em adição, as aves apresentam sacos aéreos, estruturas pouco vascularizadas, responsáveis pelo armazenamento de ar, sem função de troca gasosa, as quais propiciam um habitat ideal para o desenvolvimento e reprodução de fungos do gênero *Aspergillus*[33].

Em estabelecimentos de produção avícola, a aspergilose é considerada a infecção fúngica de maior ocorrência, levando à altos índices de mortalidade, que podem ultrapassar 50% do lote em aves jovens nos primeiros dias de vida, podendo também acometer embriões no interior dos ovos[33,41,42]. Aves silvestres e marinhas, algumas com elevado valor ecológico, são especialmente suscetíveis à infecção por *Aspergillus* spp., o que acarreta sérios prejuízos em zoológicos e centros de reabilitação[33,36,41,43-46].

A apresentação clínica da aspergilose em aves pode ser diversa, dependendo do sítio anatômico inicial da infecção. A doença pode ocorrer no cérebro levando à sinais clínicos neurológicos como ataxia e torcicolo, na pele causando dermatite necrótica granulomatosa, e nos olhos desencadeando ceratite geralmente unilateral. No entanto, o trato respiratório inferior é o principal sítio anatômico da micose, que pode ser classificada como aguda ou crônica e localizada ou sistêmica[33,41,44,46-48].

A forma aguda ocorre principalmente em aves domésticas jovens, aves silvestres e marinhas, a partir da germinação de conídios em um órgão vital, ou da formação de múltiplas lesões simultaneamente. Esta forma causa uma rápida e massiva colonização fúngica, com formação de granulomas miliares nos tecidos acometidos e curso clínico geralmente menor que uma semana, podendo ocorrer morte aguda em 48 horas. Já a forma crônica acomete principalmente aves adultas e se caracteriza por granulomas no trato respiratório que tendem a disseminação para órgãos adjacentes, apresentando um

curso clínico de semanas a meses de duração[41,44,46,48]. A siringe, órgão fonador das aves localizado próximo à bifurcação da traquéia, é um local freqüentemente acometido pelo fungo, devido ao estreitamento do lúmen e ao padrão de turbulência do ar que permite que os conídios escapem do fluxo expiratório[48]. Esta forma traqueal da aspergilose é caracterizada por lesão localizada e acúmulo de secreção e restos necróticos na região, levando à obstrução parcial ou total da via aérea, com dispnéia evidente e morte aguda[41,46-48].

Em casos de aspergilose sistêmica, a disseminação fúngica geralmente ocorre via sacos aéreos ou via hematógena, com a formação de trombos vasculares contendo hifas fúngicas, e as lesões são comumente encontradas em trato respiratório, fígado, serosa do trato gastrointestinal e vísceras abdominais, podendo ou não ter sinais clínicos aparentes, como redução gradual da função respiratória, plumagem eriçada, apatia e anorexia[41,46,47].

1.2.1 Aspergilose em pingüins

Os pingüins são aves marinhas especialmente suscetíveis à aspergilose, e que frequentemente desenvolvem a forma sistêmica e disseminada da doença[41,47,49-51]. Estas aves têm sofrido significativamente os efeitos da ação humana no meio ambiente. Das 17 espécies, quatro estão ameaçadas de extinção e sete estão classificadas como vulneráveis na lista vermelha da União Internacional de Conservação da Natureza e dos Recursos Naturais, devido à pesca predatória, as alterações climáticas e principalmente à poluição crônica dos mares[52-57].

Em cativeiro, zoológicos e aquários, fatores como manejo, transporte e mudança de habitat, contribuem para o aumento da suscetibilidade dos pingüins à infecção por *Aspergillus*[41,46,47]. O agravante em centros de recuperação decorre da contaminação por petróleo, lesões traumáticas, desidratação e desnutrição, que culminam com a debilidade geral do indivíduo[47,49,51,57]. De fato, estudos sobre aspergilose em pingüins em cativeiro demonstram taxas de mortalidade de 50 a 88,9% em zoológicos[50,59-61] e de 79 a 83% dos exemplares em centros de recuperação[57,58,62].

O principal elo da aspergilose em pingüins com a aspergilose humana é justamente a dificuldade no diagnóstico precoce da doença, culminando com ineficácia do tratamento e conseqüente morte do indivíduo infectado[33,46,47]. Os sinais clínicos são inespecíficos, como apatia, anorexia e dispnéia, e ainda muito tardios quando presentes.

É comum morte súbita sem sinal clínico evidente, o que dificulta ainda mais a formulação de um diagnóstico presuntivo[33,41,46,50]. Sinais radiológicos também aparecem somente em casos muito avançados, quando não há mais reversão do quadro clínico e TC não está disponível na maioria dos locais de cativeiro de pingüins[41,46,47]. Em adição, a debilidade do animal enfermo impede a realização de procedimentos invasivos como endoscopia e biópsia, muitas vezes definidores do diagnóstico de aspergilose[46,47].

A pesquisa de anticorpos anti-*Aspergillus* é ainda considerada o principal método diagnóstico para aspergilose em pingüins[46,47,63]. No entanto apresenta limitações, como ocorrência de falso-negativos em indivíduos debilitados sem condição de formação de anticorpos a um nível detectável pelo exame e falta de padronização das técnicas para uso em aves[63,64]. Em adição, um exame sorológico positivo não diferencia infecção de contato prévio[65,66]. Assim, a necessidade de um novo teste, não invasivo, e que se baseie no diagnóstico direto, independente da resposta imune do hospedeiro é evidente, e ainda, seria de extrema aplicabilidade para controle da aspergilose nestes animais em cativeiro.

1.3 NOVOS MÉTODOS DIAGNÓSTICOS EM ASPERGILOSE INVASIVA

Na tentativa de sobrepor as dificuldades referidas, novos métodos para o diagnóstico precoce da aspergilose invasiva em pacientes humanos de alto risco têm sido desenvolvidos. Resultados promissores provêm da detecção de antígenos de *Aspergillus* em espécimes clínicos através de métodos imunológicos em amostras clínicas de fácil obtenção, como soro e LBA[17]. O principal deles, considerando facilidade de execução e acessibilidade no Brasil é a detecção da galactomanana através do método de ELISA sanduíche.

1.3.1 Detecção de galactomanana

A galactomanana é um polissacarídeo termoestável componente da parede celular de fungos do gênero *Aspergillus*, composto por uma estrutura ramificada com uma cadeia linear de α-manana e cadeias curtas de β(1,5) galactofuranose. Este antígeno é liberado na circulação sanguínea durante o crescimento das hifas nos tecidos do hospedeiro e, por ser hidrossolúvel, pode ser encontrado em diferentes amostras

clínicas, como líquor, LBA e urina[67-69]. O limiar para a detecção do antígeno em amostras clínicas depende do método utilizado; entre os métodos disponíveis, destacam-se a aglutinação em látex, o radioimunoensaio, o ELISA por inibição da absorbância e o ELISA sanduíche[14,17]. Entre estas, a técnica de ELISA sanduíche é a mais promissora, por ser a mais sensível, detectando baixas concentrações de galactomanana (0,5-1 ng/ml) em amostras clínicas.

ELISA sanduíche fornece resultado rápido, em aproximadamente 3 horas, e está disponível comercialmente (Platelia® *Aspergillus* EIA - BioRad). O teste consiste em um imunoensaio que utiliza anticorpo monoclonal de rato (EB-A2) o qual reconhece os epítopos β(1,5) galactofuranose da molécula de galactomanana, e requer ao menos quatro destes epítopos livres para efetuar sua ligação e formar o complexo antígeno-anticorpo[70]. Este teste está disponível na Europa desde a década de 90, tendo sido aprovada pelo *Food and Drug Administration* (FDA) para uso nos EUA em 2003[71] e em 2007 pela Agência Nacional de Vigilância Sanitária para uso no Brasil[72].

Quando monitorada de forma seriada, a galactomanana antecipa o diagnóstico de aspergilose invasiva em um intervalo de 6 a 14 dias em indivíduos neutropênicos[69]. Devido à provável liberação intermitente do antígeno, o teste deve ser realizado duas vezes por semana, e confirma o diagnóstico ao apresentar resultados positivos em pelo menos duas amostras consecutivas. O ponto crítico está no tratamento do soro com calor em presença de EDTA, o que permite dissociar os imunocomplexos e precipitar as proteínas séricas que poderiam interferir no teste. Outra dificuldade relaciona-se ao ponto de corte utilizado[67]. Enquanto a escolha de um ponto de corte mais elevado resulta em maior especificidade, perde-se em sensibilidade; ao contrário, um menor ponto de corte se associa a maior sensibilidade, com perda em especificidade. Até recentemente, o ponto de corte aceito utilizado na Europa era de 1,5 ng/ml, enquanto nos EUA o valor aceito era de 0,5 ng/ml[15,73]. Porém, em 2005 Verweij et al.[74] confirmaram os benefícios na redução do ponto de corte na Europa para 0,5, que resultou em um aumento do intervalo da mediana de 1 para 10 dias entre a positividade do teste e o diagnóstico de aspergilose invasiva. No momento, 0,5 ng/ml é o valor aceito ambos na Europa e nos EUA[67]. Embora galactomanana possa ser testada no LBA, o limiar para positividade no LBA é consideravelmente menos estudado do que no soro[67]. Utilizando-se um ponto de corte de 1,0 a 1,5, a maioria dos estudos encontrou que a sensibilidade do exame de detecção de galactomanana no LBA variou entre 85-100%[75-77].

Alguns fatores prejudicam o desempenho do teste de detecção da galactomanana. A taxa de resultados falso-negativos oscila entre 8 e 10% e está relacionada a quadros de encapsulação da infecção, presença de anticorpos anti-*Aspergillus* (formando imunocomplexos que impedem a ligação do antígeno no EB-A2), ou exposição a agentes antifúngicos (como profilaxia). A freqüência de resultados falso-positivos usualmente varia entre 8-14%, sendo as principais causas o uso de agentes quimioterápicos citotóxicos (promovendo dano às mucosas intestinais), enfermidade do enxerto *versus* hospedeiro, anticorpos auto-reativos, infecção por outros fungos como *Penicillium* e *Paecylomyces*, bacteremias por *Pseudomonas* spp., *Escherichia coli*, antibióticos de origem fúngica (piperacilina-tazobactam; amoxicilina com ácido clavulânico) e contaminação no laboratório, entre outros fatores[1,14,17,25,67,70,71,78].

A maior parte dos estudos com galactomanana tem sido realizada em pacientes neutropênicos, sobretudo aqueles submetidos a transplante de medula óssea. Em contrapartida, estudos em transplantados de pulmão são ainda escassos[22,25,79]. Da mesma forma, são poucos os estudos com detecção de galactomanana em medicina veterinária. Apesar de ter sido realizado em distintas espécies, até o momento, os resultados obtidos não foram promissores. Em cães, não foi comprovada a superioridade do teste de ELISA sanduíche (Platelia® *Aspergillus* EIA) em relação a um método de ELISA indireto no diagnóstico de aspergilose sistêmica e sino-nasal[80]. Comparação semelhante entre testes diagnósticos para placentite e aborto por *Aspergillus* foi realizada em um estudo incluindo fêmeas bovinas com problemas reprodutivos, demonstrando que a detecção da galactomanana pelo ELISA sanduíche nestes casos apresenta baixa sensibilidade[38]. E, em aves, o único estudo descrito com detecção de galactomanana pelo Platelia® *Aspergillus* EIA foi realizado a partir de soros de aproximadamente 200 falcões, apresentando alta especificidade, porém sensibilidade de somente 12%[81].

1.4 CUSTO-EFICÁCIA DOS NOVOS TESTES DIAGNÓSTICOS

No Centro de Câncer da Universidade do Texas, EUA, a aspergilose invasiva correspondeu a um gasto financeiro de 633 milhões de dólares no período de um ano[1]. Neste mesmo contexto, foi realizado um estudo na Austrália durante quatro anos, onde foram observados 4.583 casos de pacientes internados com diagnostico de aspergilose,

que totalizaram um custo de hospitalização de aproximadamente 43 milhões de dólares[2]. Na realidade brasileira, os dados sobre a epidemiologia e gasto com a aspergilose invasiva nos pacientes hospitalizados são escassos. Uma avaliação foi realizada em um estudo comparando o uso do voriconazol com o uso da anfotericina B deoxicolato no tratamento da aspergilose, tendo estipulado um custo por paciente de 63 a 73 mil reais[82]. De fato, em grandes centros hospitalares as despesas com antifúngicos para tratamento ou prevenção de infecção por fungos filamentosos com destaque para *Aspergillus* em pacientes hospitalizados, constituem-se em uma das maiores na lista de todos os medicamentos utilizados. Em contraste com tais gastos com drogas antifúngicas (tanto para uso profilático quanto terapêutico) e custos associados a hospitalizações e potenciais complicações relacionadas a diagnósticos tardios, os investimentos com recursos diagnósticos são ainda irrisórios[74,83]. Deve-se ainda acrescer que tanto a melhora clínica mais rápida assegurando melhor qualidade de vida ao paciente como a redução de letalidade decorrente da aplicação mais precoce de métodos mais eficazes de diagnóstico têm valor incalculável para o paciente e para a comunidade.

Os custos com terapia antifúngica profilática para aspergilose em pingüins em cativeiro, também são relevantes. Esta profilaxia é indicada para todos os pingüins juvenis e/ou debilitados (abaixo do peso de referência para a espécie ou contaminados por petróleo), e ainda em períodos pré e pós-transferência de animais[57,84-87]. A detecção precoce da doença através de métodos diagnósticos eficazes gera benefícios diretos e indiretos, à medida que melhora a eficácia terapêutica e o prognóstico, levando a uma redução da mortalidade dos animais, o que representa um valor ecológico imensurável.

Este estudo busca avaliar a aplicabilidade do teste de detecção de antígeno para o diagnóstico de aspergilose em diferentes grupos de risco, e avaliar no contexto brasileiro fatores responsáveis por falso-positivos no Platelia® *Aspergillus* EIA, como outras infecções fúngicas sistêmicas incluindo paracoccidioidomicose, e antimicrobiano piperacilina-tazobactam, ainda não estudado no Brasil.

2 OBJETIVOS

2.1 Avaliar a eficácia do teste de detecção da galactomanana (ELISA sanduíche – Platelia® *Aspergillus* EIA) no diagnóstico de aspergilose invasiva em transplantados de pulmão, a partir de amostras do trato respiratório (LBA);

2.2 Avaliar a eficácia do Platelia® *Aspergillus* EIA no diagnóstico de aspergilose invasiva em pingüins em cativeiro;

2.3 Avaliar a potencialidade de reação cruzada de outras infecções fúngicas sistêmicas no teste de detecção da galactomanana (ELISA sanduíche);

2.4 Avaliar a interferência do antimicrobiano piperacilina-tazobactam, disponível no mercado brasileiro, no Platelia® *Aspergillus* EIA.

3 ARTIGOS COMPLETOS

3.1 "Galactomannan detection in bronchoalveolar lavage for the diagnosis of invasive aspergillosis in lung transplants recipients"

Artigo sob as normas da revista *Transplantation*.

Galactomannan detection in bronchoalveolar lavage for the diagnosis of invasive aspergillosis in lung transplants recipients

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ABSTRACT

One hundred and seventeen bronchoalveolar (BAL) samples from sixty lung transplant recipients were prospectively evaluated in Platelia™ *Aspergillus* EIA in the diagnosis of invasive aspergillosis. Five cases were classified as proven, 6 as probable, and 35 as possible aspergillosis. Another 12 samples were obtained from colonized patients, and 59 BAL samples were obtained during routine surveillance. The best cut-off was determined by receiver operating characteristic (ROC) analysis. Using 1.5 as a cut-off value, galactomannan testing in BAL had the following performance: sensitivity 90.9%, specificity 90.6%, and positive and negative predictive values of 48% and 99.1%, respectively. Galactomannan detection in BAL fluid is a promising tool for the diagnosis of invasive aspergillosis in lung transplant recipients. In order to avoid false-positive results, a higher test cut-off should be applied to BAL samples, in comparison to sera.

Keywords: *Aspergillus*, galactomannan; lung transplant recipients, diagnosis, bonchoalveolar lavage.

INTRODUCTION

Aspergillosis is an important disease in lung transplant recipients with an incidence ranging from 6-16% (1). It is estimated that 9% of all deaths after lung transplantation are attributed to invasive aspergillosis (2). Several factors are responsible for a higher susceptibility of these patients, in comparison to other solid transplant recipient. Direct communication of the transplanted organ with air that carries *Aspergillus* conidia, ischemic injury of the respiratory tract, decrease in the mucociliary clearance, and limitation in alveolar macrophages action are the main factors associated with in the occurrence of an invasive *Aspergillus* disease(2).

Three main clinical presentations of aspergillosis can be found in lung transplant recipients. Colonization – not properly a disease – is characterized by a positive culture for *Aspergillus* in the absence of any clinical, bronchoscopical or radiological signs. Tracheobronchitis courses with localized lesions that usually occur near the anastomosis. The third presentation is invasive pulmonary aspergillosis (IPA), a condition that is associated with mortality rates of 60-80% (3).

The diagnosis of aspergillosis is primarily limited by the low sensitivity of methods based in culture. In addition, detection of antibodies is impaired by the use of immunosuppressive therapy, and radiological signs are usually non-specific. For many patients, samples for histopathology are hard to obtain (1, 2, 3). Thus, there is a need for a non-invasive technique to diagnose invasive aspergillosis in these patients.

A direct diagnostic test (sandwich ELISA) has been developed to diagnose invasive aspergillosis. The test detects galactomannan (GM), a polysaccharide released from *Aspergillus* cell wall during hyphae growth in tissues. Galactomannan detection has been widely tested using serum from neutropenic patients, with a high sensitivity and specificity (1). However, the test has limited sensitivity to diagnose invasive aspergillosis in non-neutropenic hosts, a population at which angioinvasion is uncommon. Broncoalveolar lavage (BAL) seems to be a better sample for these patients because the antigen can be detected earlier in the local of infection (1, 4). Here, we evaluate prospectively the performance of galactomannan detection in BAL samples from lung transplant recipients to aspergillosis diagnostic.

MATERIAL AND METHODS

Patients and samples

One hundred and seventeen BAL samples from 60 consecutive lung transplant recipients were included in the study. The study was conducted in a single centre, Santa Casa-Complexo Hospitalar, Brazil. Samples from patients who had undergone bronchoscopy for surveillance or suspected infection or rejection during March 2007 to November 2008 were prospectively analyzed. Samples from patients already receiving systemic antifungal drugs for any reasons were excluded. The study received independent ethical approbation from the institutional and national review boards. Patients' notes were reviewed to record variables such as demographic data, underlying diseases, prior *Aspergillus* colonization, and type of transplant (single or double).

All BALs were performed by trained physician from the lung transplant team. In summary, these procedures were performed at room temperature by instilling 0.9% sterile saline solution using a syringe through the working channel of the bronchoscope. The total volume of saline solution instilled into the lung was typically 150 ml, and 50 to 100 ml of BAL fluid was recovered. Samples were sent to the Mycology Laboratory, cultured in Sabouraud dextrose agar, and stored at 4ºC from galactomannan detection within 48 hours.

Immunosuppressive regimen

The immunosuppressive regimen used was cyclosporine, azathioprine and prednisone, or mycophenolate mofetil in association with tacrolimus and prednisone.

Definitions

Invasive aspergillosis was classified as proven, probable or possible, according to updated European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria for the diagnosis of invasive fungal diseases (5). These criteria were slightly modified to better suit the lung transplant population. Accordingly, all lung recipients were considered positive to the 'host factor' criteron. In addition, any new radiological finding at chest imaging or new lesion at bronchoscopy were accepted as the 'clinical criteria'. Since this study aimed for the evaluation of the performance of galactomannan testing in BAL, a positive galactomannan result was not accepted as a component in the 'microbiologic' criterion. Therefore, all patients classified as having proven or probable invasive aspergillosis had positive culture for *Aspergillus*. Diagnosed were also reviewed with the lung transplant team, and a consensus was reached for all cases. Cases of proven and probable aspergillosis cases

were further classified as tracheobronchitis or IPA. Tracheobronchitis was classified as ulcerative or mild, the latter characterized by mucosal hyperemia, limited purulence in the absence of bacterial infection or bronchial stenosis. Patients with *Aspergillus* spp. cultured from respiratory tract samples that did not fulfill the criteria for invasive aspergillosis or *Aspergillus* tracheobronchitis were considered to have colonization.

Galactomannan antigen testing

A commercial kit was used for galactomannan detection (Platelia *Aspergillus* EIA, Bio-Rad, USA). BAL samples were processed according with manufacturer's instructions for serum samples. Briefly, 100 µl of Platelia treatment solution (4% ethylenediamine tetraacetic acid solution, EDTA) were added in 300 µl of BAL sample, homogenized and heated until 120ºC for 6 min in a heat block, followed by centrifugation at 10,000 X *g* for 10 min. Next, 50 µl of the supernatant and 50 µl of the horseradish peroxidase (HRP)-labeled monoclonal antibody (EBA-2) were incubated in antibody-pre-coated microplates for 90 min at 37°C. The plates were washed five times, after which they were incubated with 200 µl of substrate chromogen reaction solution for 30 ± 5 min in the dark, at room temperature. The reaction was stopped with 1.5 N sulfuric acid solution and, finally, the plates were read at an optical density at 450 nm (OD 450) with a reference filter of 620/630 nm. Positive, negative, and cut-off controls were incorporated in each assay.

Data analyses

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated from BAL GM testing in reference to proven and probable of invasive aspergillosis cases (from now on referred to as 'positive cases'), using the total number of samples in the study. The optimal cut-off for BAL GM testing was determined by receiver operating characteristic (ROC) analysis. Categorical data were compared using the chi-square or Fisher Exact test and expressed by two-by-two contingency tables. Mann-Whitney test was used for continuous variables. All statistical analyses were performed using SPSS software version 11.04 (Statistical Package for the Social Sciences, Chicago, USA). *p* values of <0.05 were considered significant.

RESULTS

Demographic and clinical characteristics of the 60 patients included in the study are described in table 1. From the 117 samples studied, 5 (4.3%) were classified as proved, 6 (5.1%) as probable, 35 (29.9%) as possible aspergillosis. Twelve samples

(10.3%) represented colonization. Most samples (50.4%) were obtained during routine surveillance. Considering only patients with proven / probable / possible aspergillosis, most infections were classified as pulmonary (67.4%) or tracheobronchitis (32.6%).

Table 2 shows more details about the 11 samples from nine patients that were classified as proven or probable cases of aspergillosis. Aspergillosis incidence, calculated from those patients represented a rate of 15%. Almost a half of cases (44.4%) occurred in the first six months of transplantation, and 33.3% were observed one year after surgery. Invasive pulmonary aspergillosis was the mainly clinical presentation, representing 63.6% of cases. Most patients with tracheobronquitis presented with the ulcerative form of the disease (75.0%), and 25.0% of cases were classified as mild. *Aspergillus fumigatus* and *A. flavus* were the most frequent species (45.4% each), followed by *A. niger* (9.1%).

GM indexes for BAL samples ranged from 0.25 to 10. Significant difference ($p<0.0001$) in GM indexes was observed when patients with proven / probable aspergillosis ('positive cases') were compared to 'negative cases' (samples from all other patients). Median GM value for these groups were 3.27 and 0.51, respectively. We also searched for other factors that could potentially increase GM values, leading to false-positive. There was no association between requirement of hemodyalisis and GM indexes ($p=0.395$). However the current administration of piperacillin-tazobactam was correlated with positive GM values ($p=0.027$).

GM indexes were higher in single than in bilateral transplants ($p=0.042$). *Aspergillus* colonization prior transplants procedure was observed in 10 samples (8.5%) and showed non association with GM values ($p=0.421$). *Candida* spp. were isolated in culture alone or in association with other fungi in 47 (40.2%) of the samples, with no association with GM values ($p=0.522$). Fungi not belonging to the *Aspergillus* or *Candida* genus were isolated in only 3 samples (2.6%).

Overall mortality for lung transplant patients was 20% (n=12). Mortality was significantly higher when patients with invasive aspergillosis were compared with controls (55.5% versus 13.7%, respectively; $p=0.002$). GM indexes also differed from patients who died and those who did not (median GM values of 1.03 and 0.54, respectively ($p=0.019$). The odds ratio for death in lung transplant patients diagnosed with aspergillosis was 11.8, in comparison to lung transplant patients who did not have this infection (95% confidence interval 2.9-48.4).

ROC curve (Figure 1) determined 1.5 as the best cut-off, which was associated with a 90.9% sensitivity and 90.6% specificity (Table 3). Applying this cut-off value, we had only one false-negative result, which occurred in a patient who had nebulized amphotericin B for 19 days – in addition, 11 samples from 9 patients resulted in false-positives results. Five out of these 11 cases had *Aspergillus* spp. recovered in culture, and they were classified as colonization. One patient was being treated with piperacillin-tazobactam. The positive and negative predictive values of galactomannan testing using a 1.5 cut-off were 48% and 99.1%, respectively.

DISCUSSION

Galactomannan detection in BAL samples in non-neutropenic patients has been investigated as a promising alternative to the low sensitivity observed when serum samples are tested in these patients (typically ~ 30%) (6, 7, 8). Particularly in lung transplant recipients, few data exist on the performance of Platelia *Aspergillus* EIA in BAL samples (9). To the best of our knowledge, this is the first investigation of BAL GM detection from lung transplant recipients in Brazil.

The overall positive patients (4 proven and 5 probable) found here corresponded to 15% of case studied. This value is higher in comparison to 5.17% (2 proven and 4 probable) reported before (9). Maybe due to hospitalar construction that occurred during the experiment, which is a common cause of aspergillosis increases. However, results from these studies agreed in a sense that invasive pulmonary aspergillosis was the mainly disease presentation in lung transplant recipients. Given this fact, the estimated risk of death were 11 times greater in patients who develop aspergillosis than in negative cases.

A previous study has shown that, using 1.0 as a cut-off, sensitivity and specificity of GM detection in BAL samples from lung transplants recipients were 60% and 98%, respectively (9). In comparison, the best cut-off in our study was defined as 1.5, with a high sensitivity and specificity. It is worth noting that the 1.5 cut-off was associated with a negative predictive value of 99.1%, which allows for the exclusion of the invasive aspergillosis diagnosis with a high degree of certainty.

In a study with non-immunocompromised hosts, GM detection in BAL samples using a cut-off value of 1.5 showed 66.7% of sensitivity and 94% of specificity in the diagnosis of invasive aspergillosis (6). Sensitivity was increased to 100% and specificity was reduced to 88.1%, respectively, using 1.0 as a cut-off. Clancy *et al*. (10)

described the efficacy of Platelia *Aspergillus* EIA in BAL samples from solid transplant recipients with 5 distinct cut-off values that ranged between 0.5 and 2.5. In that study, the optimal cutoff was defined as 2.0. Using a value of 1.5, the authors found 100% sensitivity and 92.1% specificity, a PPV of 45.4% and NPV of 100%, which were similar to our results. Due to the limited PPV observed in these studies, a positive GM test in the BAL fluid would require confirmation by means of a new test, or testing by a different method such as real time polymerase chain reaction (PCR). The low positive predictive value is highly influenced by colonization by *Aspergillus* species, a common finding in lung transplant recipients (10). Actually a significant association was observed between simple transplant and GM values, which could be attributed to previous colonization of the native lung. Colonization with *Aspergillus* species occurred in only 10.3% of cases, in contrast with previous studies showing frequencies as high as 25-30% (1). However, we could speculate that the frequency of colonization may be underestimated in our center, since in many cases cultures were not performed before transplantation. In addition, patients were not routinely screened using *Aspergillus* serology. Five cases of colonization showed false-positives GM results in our study. However, colonization showed negative GM results in other 7 cases, as described previously (9).

Piperacillin-tazobactam is frequently reported as a cause of false-positive results in Platelia *Aspergillus* EIA using serum samples (11-13). It is believed that the use of this antibiotic wound not interfere to a great extent in when GM is tested in the BAL, since the epithelial lining fluid concentration of piperacillin is only 56% of the serum steady state concentrations (14). Given this fact, the observed association between the current receipt of piperacillin-tazobactam and positive GM values ($p=0.027$) should be interpreted with caution, since the drug was administrated only to 8 patients in the study, 2 of whom were cases of proven and one was classified as probable invasive aspergillosis (patients 4, 5 and 6 in table 2). Therefore, higher GM indexes may have explained in these patients by aspergillosis.

The only false-negative case described here was from a probable case of tracheobronchitis. Although evidence of fungal in tissue was not observed at histopathology, the patient had ischemic lesions in bronchus mucosa, proximal to the anastomotic region, and culture was positive for *A. flavus*. Thus, two hypothesis can explain; one is that positive fungal culture corresponded to a colonization and lesions were due to a different cause; or, more likely sounding, that represented truly positive

case that tested negative in the GM assay due to aerosolized antifungal prophylaxis that was initiated nearly 20 days before the sample was tested. False-negative cases like this were also observed by Husain et al. (9). Given this, 3 proven cases under concomitant antifungal prophylaxis may had presented with higher OD indexes than the actual observed results. However, all of these presented with GM OD values higher than 1.5.

Finally, we could conclude for the high applicability of GM detection in BAL samples for the diagnosis of invasive aspergillosis in lung transplants recipients. Using a cut-off value of 1.5, a negative result would allow for exclusion of the diagnosis of aspergillosis with a high degree of certainty. A positive result, however, would require retesting, taking into consideration the clinical context, in addition to radiologic, cytologic and histopathological results. BAL detection of GM, in parallel with others conventional tests, contributes to define the diagnosis of aspergillosis in lung transplant recipients.

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Table 1 – Demographic and clinical characteristics of lung transplants cases included in the study (n=60).

Characteristics	**Median (range) or percent (n)**
Age, years	55 (10-72)
Sex	
Male	51.7 (31)
Female	48.3 (29)
Type of transplant	
Simple	85.0 (51)
Bilateral	15.0 (9)
Underlying pulmonary disease	
Emphysema	40.0 (24)
Idiopathic pulmonary fibrosis (IPF)	31.7 (19)
Lymphangioleiomyomatosis	8.3 (5)
Cystic fibrosis	5.0 (3)
Bronchiolitis obliterans	5.0 (3)
Silicosis	3.3 (2)
Emphysema and IPF	1.7 (1)
Scleroderma	1.7 (1)
Histiocitosis	1.7 (1)
Brochiectasis	1.7 (1)
Hemodyalisis	5.0 (3)
Aspergillus colonization prior to lung transplantation	8.3 (5)
Current nebulization with amphotericin B	6.7 (4)
Current use of systemic amphotericin B	8.3 (5)
Current use of piperacillin-tazobactam	10.0 (6)
Current immunosuppressive regimen	
Prednisone	100 (60)
Cyclosporine	71.7 (43)
Azathioprine	70.0 (42)
Tacrolimus	35.0 (21)
Mycophenolate mofetil (MMF)	25.0 (15)

Table 2 – Data from lung transplant recipients (11 samples from 9 patients) included in the groups of proven or probable aspergillosis.

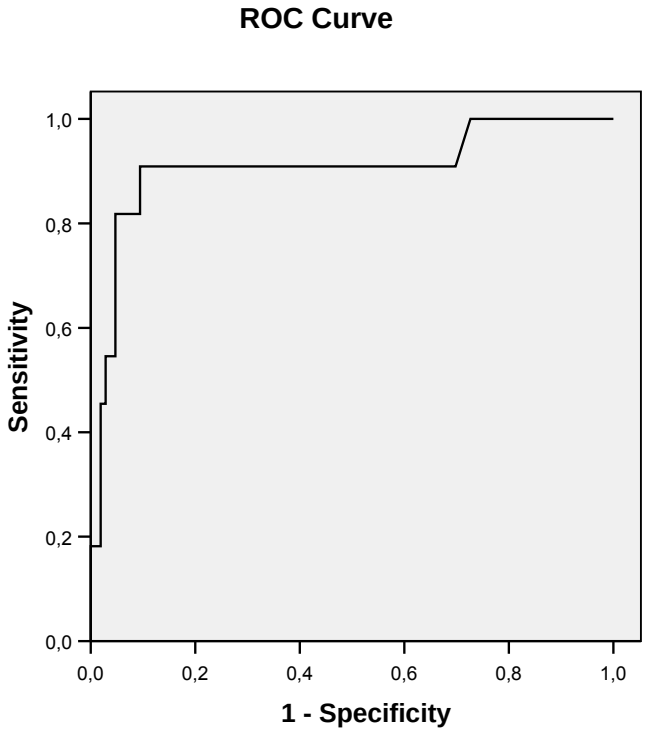
Case	Aspergillosis classification	Aspergillosis presentation	Onset posttransplant (days)	Prior *Aspergillus* colonization	Etiologic agent	Current antifungal	GM index in BAL	Outcome
1	Proven	IPA	20	yes	*A. flavus*	No	10.00	Alive
2	Proven	IPA	180	no	*A. fumigatus*	Nebulized amphotericin B	8.00	Died
3	Proven	IPA	20	yes	*A. flavus*	No	4.17	Alive
4	Proven	IPA	312	no	*A. fumigatus*	Systemic amphotericin B	2.37	Died
5	Proven	IPA	830	no	*A. fumigatus*	Systemic amphotericin B	2.28	Died
6	Probable	TCB	8	yes	*A. flavus*	No	5.00	Alive
7	Probable	IPA	994	no	*A. fumigatus*	No	4.49	Died
8	Probable	TCB	8	yes	*A. flavus*	No	3.27	Alive
9	Probable	TCB	183	No	*A. fumigatus*	Nebulized amphotericin B	2.44	Alive
10	Probable	IPA	472	No	*A. niger*	No	1.48	Died
11	Probable	TCB	23	No	*A. flavus*	Nebulized amphotericin B	0.40	Alive

GM- galactomannan; BAL – bronchoalveolar lavage; IPA – invasive pulmonary aspergillosis; TCB – tracheobronchitis;

Table 3 – Performance of Platelia *Aspergillus* EIA in BAL samples of lung transplant recipients using distinct cut-off values.

Cutoff value	Sensitivity (%)	Specificity (%)
0.5	90.9	49.1
0.7	90.9	68.9
1	90.9	83
1.5	90.9	90.6
2.0	81.8	93.4
2.4	63.6	95.3

Figure 1 – ROC curve from results of GM detection in BAL samples from lung transplant recipients.



3.2 "A comparison of antibody detection by Immunodiffusion technique and EIA galactomannan testing for the diagnosis of aspergillosis in penguins"

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A comparison of antibody detection by Immunodiffusion technique and EIA galactomannan testing for the diagnosis of aspergillosis in penguins

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Abstract

The high mortality of captive penguins due to aspergillosis is mostly associated with difficulties in establishing an early diagnosis. Here we evaluate the efficacy of two techniques for the diagnosis of aspergillosis in penguins. Serum samples from magellanic penguins from the Rehabilitation Centre of Marine Animals (Rio Grande, Brazil) were evaluated by immunodiffusion technique and by a commercial sandwich EIA to detect galactomannan. The study included birds with invasive aspergillosis (n=9), malaria (n=3), cachexia (n=2), and healthy controls (n=21). Immunodiffusion testing showed a low sensitivity (33%) but a high specificity (96%) in the diagnosis of invasive aspergillosis. On the contrary, galactomannan testing was 100% sensitive but absolutely not specific (0%) in the diagnosis of this condition. A search for a better diagnostic test in penguins developing invasive aspergillosis is still ongoing.

Keywords: immunodiffusion, antibody, sandwich ELISA, galactomannan, aspergillosis, penguins.

Introduction

Aspergillosis is one of the most important diseases in captive penguins (Ainsworth & Rewell, 1949, Khan et al., 1977, Xavier et al., 2007). This mycosis frequently presents as an invasive and disseminated disease that may rapidly progress to death (Xavier et al., 2008). The high mortality of invasive aspergillosis in seabirds is attributed mostly to the difficulties to establish an early diagnosis. Both clinical signs and radiological alterations are nonspecific and occur late in the course of infection, therefore limiting the efficacy of antifungal therapy (Redig, 1993, Kearns & Loudis, 2003).

The ability of classic methods such as fungal culture and histopathology to diagnose aspergillosis is hampered by the need to perform invasive procedures to obtain samples (Redig, 1993). Few non-invasive exams are used in zoos for the diagnosis of aspergillosis in penguins. These tests are based on the detection of anti-*Aspergillus* antibodies by immunodiffusion (ID) or by indirect ELISA examination (Redig, 1993, Diebold et al., 1999). However, false-negative results are frequently observed in immunocompromised individuals due to their limited capacity to produce antibodies (Latgé, 1999).

A commercial EIA test to detect galactomannan has been widely used to diagnose invasive aspergillosis in the neutropenic human host (Wheat, 2005). The test uses a rat monoclonal antibody that targets the galactofuranose sides of galactomannan, a molecule released from *Aspergillus* cell wall during growth in tissues (Wheat, 2005; Aquino et al., 2007). Since galactomannan testing is based on antigen detection, it allows for the diagnosis of aspergillosis in immunocompromised hosts which are unable to react by antibodies production.

Very limited data is available on the performance of galactomannan testing in the diagnosis of aspergillosis in non-human hosts (Arca-Ruibal et al., 2006). For instance, invasive aspergillosis is particularly problematic for debilitated seabirds such as penguins (Xavier et al., 2007). Therefore, here we compare the efficacy of antibody detection by the immunodiffusion test (ID) with the commercial sandwich EIA for galactomannan detection in the diagnosis of aspergillosis in penguins from a rehabilitation centre.

Material and methods

This research was conducted from 2006-2008 at the Rehabilitation Centre of Marine Animals (CRAM) (Rio Grande, Southern Brazil). A total of 35 magellanic penguins (*Spheniscus magellanicus*) were studied. Animals were diagnosed with invasive aspergillosis (n=9), malaria (n=3), and cachexia (n=2); 21 healthy penguins were also included as controls.

Blood was collected aseptically from the medial metatarsal vein (1.5-2.0 ml). Samples were initially centrifuged for 5 min at 1500 rpm. Serum was separated and transferred to a sterile tube, which was stored at -20ºC for laboratory processing.

All cases of invasive aspergillosis were caused by was *Aspergillus fumigatus* and were confirmed by necropsy. Cases of malaria were also confirmed by post-mortem examination. Penguins with cachexia arrived at the rehabilitation centre with poor body conditions and died within 24 hours. Healthy penguins were those already rehabilitated and ready to be freed to their natural habitat.

All serum samples were studied by immunodiffusion testing in lamina. Tests were performed according to the manufacturer's instructions. In summary, *Aspergillus* antibody detection was performed using the commercial *A. fumigatus* ID Antigen™ (IMMY) test. A total of 10 µl of sera from patients was distributed in the central well along with positive and negative controls. After 24 hours incubation at room temperature results were observed by an indirect light over a dark background. A serum sample showing precipitin bands with identity with sera control band was considered positive. Galactomannan EIA testing (Platelia™ *Aspergillus*, BioRad, USA) was performed for 30 penguins, including 5 with aspergillosis, 3 with malaria, 2 with cachexia and 20 healthy birds. Because of the small volume of sera obtained for study, tests were performed with 150 µl of sera and 50 µl of treatment solution, instead of 300 µl and 100 µl, respectively, as recommended by the manufacturer. Results were expressed in terms of optical densities (OD) and were considered positive when the value of the ratio index between sample OD and the mean cut-off OD was of $\geq$0.5.

Descriptive statistics were used to summarize the data. Sensitivity, specificity, positive and negative predictive values of the tests were calculated using a two-way frequency table. Culture and histopathology were used as gold-standards for the diagnosis of invasive aspergillosis.

Results

The performance of ID and galactomannan tests in the diagnosis of invasive aspergillosis is shown in Table 1. Sera from most penguins included in this study tested negative in the ID analysis (88.6%). Positive ID results occurred for 3 penguins with aspergillosis and for 1 healthy bird. Negative ID results were obtained from the remaining 6 penguins with aspergillosis, 3 birds with malaria, and 2 with Cachexia, as well as from 20 healthy animals. Conversely, sera from all animals tested positive in the galactomannan test, including sera from non-*Aspergillus* as well as healthy birds (Table 2).

Discussion

It is well known that penguins from rehabilitation centres are more susceptible to aspergillosis than most other animals in zoos or aquariums. These birds frequently present with low weight, sometimes showing oil contamination and suffering from other injuries such as trauma (Redig, 1993, Russel et al., 2003, Silva-Filho & Ruopollo, 2006). Given these circumstances, these animals have an impaired ability to mount an effective antibody response. That explains the low sensitivity of ID testing observed in our study (33%). In addition, ID testing is further limited by the non-quantitative nature of the test, and by the fact that it does not allow for an early diagnosis, since the high molecular height of the IgM antibodies limit their diffusion on the gel (Redig, 1993, Latgé, 1999). Moreover, a positive ID result does not necessarily mean infection, since exposure of birds to *Aspergillus* species is quite common (Grazick & Cockrem, 1995). Despite these limitations, most zoos seem to rely on antibodies detection testes to diagnose invasive aspergillosis in penguins (Redig, 1993, Diebold et al., 1999).

New diagnostic tests are ultimately required to diagnose invasive aspergillosis in penguins. As far as we are concerned, this is the first study investigating the performance of galactomannan testing in this context. Although highly sensitive (100%), the test had no specificity in the diagnosis of aspergillosis. The reason for its poor performance is unknown. A previous study investigating aspergillosis in falcons showed that the test had very limited sensitivity (12%) but was very specific (95%) (Arca-Ruibal et al., 2006). The low sensitivity observed in that study might be related to the use of antifungal therapy or to the localised presentation of the mycosis. On the contrary, false-positive results in our study might be related to the presence of some proteins from penguins not being denaturized during sample processing. That remains however highly speculative, since no investigation was performed to clarify that.

Conclusion

In conclusion, this study shows that galactomannan testing performed disappointingly in the diagnosis of invasive aspergillosis in penguins. An unacceptably high rate of false-positive results occurred. ID testing seems to still hold its place in the diagnosis of this condition, despite showing a very low sensitivity. New diagnostic modalities deserve investigation in the field.

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Table 1 – Sensitivity, specificity, positive and negative predictive values of the immunodiffusion and the sandwich EIA tests in the diagnosis of aspergillosis in penguins.

Diagnostic test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
ID	33	96	75	81
EIA	100	0	17	0

Legend. ID, Immunodifusion; EIA, Enzyme immunoassay; PPV, positive predictive value; NPV, negative predictive value.

Table 2 – Detection of galactomannan by a commercial sandwich ELISA in serum samples of penguins in rehabilitation.

Animal status	Number of penguins	OD index	Mean OD values
Aspergillosis	5	1.76 to >12.5	6.54
Malaria	3	1.46 to 3.59	2.52
Cachexia	2	2.31 to 3.88	3.09
Healthy	20	3.71 to >11.9	7.07

Legend. OD, Optical density.

3.3 "Cross-reactivity of *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Cryptococcus* species in the commercial kit Platelia *Aspergillus* EIA"

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Cross-reactivity of *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Cryptococcus* species in the commercial kit Platelia *Aspergillus* EIA

Running title: Cross reactivity in Platelia *Aspergillus* EIA

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Abstract

Cross-reactivity in Platelia *Aspergillus* EIA was evaluated using 120 sera from patients with paracoccidioidomycosis, histoplasmosis, and cryptococcosis. At a cut-off value of 0.5, positivity rates were 50%, 67% and 50%, respectively. The implications for these findings are discussed.

Keywords: fungal infections, galactomannan, Platelia *Aspergillus* EIA, endemic mycoses, cryptococcosis.

The sandwich enzyme immunoassay Platelia *Aspergillus* EIA (Bio-Rad, France) is a commercial test that has been extensively used for the diagnosis of invasive aspergillosis (IA). The assay detects galactofuranose-containing side chains of galactomannan, an antigen released from *Aspergillus* hyphae during growth in the host (1). In addition to varied sensitivity, the test is limited by false-positive results, mostly due to the use of antibiotics. Like *Aspergillus* species, many other fungi have been shown to produce galactomannan (1-4,6,9). Here we evaluate the cross-reactivity of four important pulmonary fungal pathogens in the commercial galactomannan test – *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, *Cryptococcus neoformans*, and *Cryptococcus gattii*.

A total of 120 serum samples from 102 patients were evaluated. These included 30 samples from patients with each of the following: paracoccidioidomycosis (30 patients), histoplasmosis (n=26), cryptococcosis due to *C. neoformans* (n=28) and *C. gattii* (n=18). Serum from patients not suspected to have fungal disease have been tested in parallel as negative controls (n=12). Platelia® *Aspergillus* EIA testing was performed according to the manufacturer's specifications, using a cut-off value of 0.5. Samples were obtained from the serum bank at the Mycology laboratory (Santa Casa-Complexo Hospitalar, Brazil). Most samples had been stored at -20^{o}C for <5 years. A few samples were in the freezer for more than 10 years, including a 17 year-old serum sample.

All patients with paracoccidioidomycosis had positive serology (immunodiffusion) to *P. brasiliensis*. The infection was diagnosed by microscopy in 27 cases and by culture in Sabouraud agar in 5 patients. Histoplasmosis was confirmed by immunodiffusion, microscopy and culture in 30, 11 and 6 cases, respectively. Cryptococcosis was diagnosed by the Latex-Crypto antigen detection system (Immuno Mycologics, Inc., U.S.A.). *C. neoformans* and *C. gattii* were differentiated after culture in canavanine-glycine-bromothymol blue agar.

The results of this study are summarized in Table 1. Sera from patients not suspected to have fungal disease all tested negative in the galactomannan test, with optical density (OD) indexes ranging between 0.16-0.41. Positive results in the galactomannan test occurred for 50%, 67%, 63%, and 37% of samples from patients with *P. brasiliensis*, *H. capsulatum*, *C. neoformans*, and *C. gattii* infection, respectively. Lower galactomannan indexes were observed for *C. gattii*, in comparison to *C. neoformans* (p=0,017). No correlation was observed between the galactomannan OD index and the Latex titer for *Cryptococcus* species (R^2=0.003; p=0.689).

We looked for other variables that could result in false-positive results in the galactomannan test. Only two patients were on hemodialysis, and none had received

piperacillin-tazobactam or amoxicillin-clavulanate. Other antimicrobial drugs usually do not result in false-positive results, even in the presence of high serum concentrations (8). Although the influence of freezing and thawing on the galactomannan antigen assay is not clear, it is usually believed that long term storage may actually decrease galactomannan levels (1). The impact of serum storage at different freezing temperatures, from -20^{o}C to -80^{o}C is also unknown.

When more than one sample was obtained from the same patient, concordant results were usually observed. Discordant results occurred for one individual with histoplasmosis and in another one with *C. neoformans* infection. In both cases a positive sample was followed by a negative test after antifungal therapy was started, a very well known phenomenon (1). Sera from seven patients with *C. gattii* infection were tested more than once. For six of these patients the same serum sample was run in duplicate and one patient was tested 6 times. Also again, most discordant results were seen after antifungal therapy was started. Due to the retrospective character of our study we were not able to obtain additional clinical information for our patients. Thus, any assumption about reduced galactomannan indexes in patients started on antifungal therapy is speculative at most. Moreover, the impact of the mycosis clinical presentation (e.g., fungemia versus non-disseminated disease) on galactomannan testing could not be evaluated.

Previous studies have described that cross-reactivity might occur with several fungi in the commercial galactomannan test. These include *Geotrichum capitatum* (3) and *Penicillium marfenei* (4), molds that contain galactomannan in the cell wall. Cross-reactivity of *H. capsulatum* and *C. neoformans* has also been described (2,4,6,9). The mechanism of *H. capsulatum* cross-reactivity is not yet elucidated. Although samples from patients with histoplasmosis may give positive results in the Platelia® *Aspergillus* EIA test, samples from patients with aspergillosis are negative in the *Histoplasma* antigen EIA (9). *C. neoformans* cell wall contains galactoxylomannan, an antigen similar to galactomannan which may result in cross-reaction in the galactomannan test (2). However, this was not confirmed in a recent study (5). The hypothesis that genotypic variations could explain the differences observed among studies in *C. neoformans* remains to be tested.

To the best of our knowledge, this is the first study in which reactivity with antibody used in the Platelia assay was tested in patients infected with *P. brasiliensis* and *C. gattii*. These are important pulmonary pathogens, affecting mostly non- immunocompromised hosts. Galactomannan is a cell wall component for both the yeast and the mycelial forms of *P. brasiliensis*. In addition, *H. capsulatum* and *P. brasiliensis* are phylogenetically close-related

fungi (7). Similarly to *C. neoformans*, the presence of an epitope causing cross-reactivity may also occur for *C. gattii*. Actually a previous investigation showed that soluble antigens from one reference strain of *C. gattii* tested positive in Platelia® *Aspergillus* EIA (2).

This study reinforces that caution should be taken when considering a positive galactomannan test in a patient with respiratory infection. The diagnostic of IA using galactomannan may be tricky in patients coming from areas where paracoccidioidomycosis and histoplasmosis are endemic. Cryptococcosis also affects solid organ transplant recipients – a population at risk for IA – and may present with multiple pulmonary nodules. Moreover, these mycoses may differ in terms of response to antifungal drugs. For instance, echinocandins are not active against *Histoplasma* species, and experience using voriconazole for histoplasmosis remains limited (9). Both *Geotrichum* and *Cryptococcus* species are usually susceptible to amphotericin B and azoles, but intrinsically resistant to echinocandins. Conversely, *P. brasiliensis* infection can be treated with sulphonamides. In order to properly interpret the meaning of a positive galactomannan testing, clinical and epidemiological data should be taken into consideration.

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Conflict of Interest

The authors disclose no conflicts of interest in association with this manuscript.

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Table 1. Results of serum galactomannan testing from patients infected with *Paracoccidioides brasiliensis* (n=30), *Histoplasma capsulatum* (n=30), *Cryptococcus neoformans* (n=30), and *Cryptococcus gattii* (n=30).

Agent	Serum galactomannan optical density index*						
	Range	**Mean values**	**95% confidence interval**	**≥2.0**	**≥1.0 to 2.0**	**≥0.5 to 1.0**	**<0.5**
Paracoccidioides brasiliensis	0.23 – 5.47	0.99	0.53 – 1.44	3 (10.0%)	5 (16.6%)	7 (23.3%)	15 (50.0%)
Histoplasma capsulatum	0.24 – 5.38	1.43	0.88 – 1.98	7 (23.3%)	7 (23.3%)	6 (20.0%)	10 (33.3%)
Cryptococcus neoformans	0.18 – >10	1.95	0.95 – 2.94	9 (30.0%)	5 (16.6%)	5 (16.6%)	11 (36.6%)
Cryptococcus gattii	0.18 – >9	1.03	0.38 – 1.68	4 (13.3%)	3 (10.0%)	4 (13.3%)	19 (63.3%)

* Optical density indexes higher or equal than 0.5 are considered positive. High indexes (i.e., > 1.0) increase the test specificity in the diagnosis of invasive aspergillosis (reference 1).

3.4 "Galactomannan detection from piperacillin-tazobactam brands available in the Brazilian market"

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Galactomannan detection from piperacillin-tazobactam brands available in the Brazilian market

Running title: Galactomannan and piperacillin-tazobactam

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Abstract

Piperacillin-tazobactam is a broad spectrum antimicrobial agent that can cause false-positive results in the commercial Platelia *Aspergillus* EIA test. So far, no study has been performed in Latin America to evaluate the clinical implication of this finding. Here we studied the potential for galactomannan detection in piperacillin-tazobactam batches commercialized in the Brazilian market. Five batches from distinct laboratories were tested in duplicate in the Platelia *Aspergillus* EIA according to the manufacturer's instructions. Only one drug showed cross-reaction at a cut-off of 0.5. Human serum was spiked with this particular drug aiming to mimic achievable piperacillin-tazobactam concentrations in the serum. Results were all negative for galactomannan detection, even at high drug concentrations. Results from this pilot study suggest that piperacillin-tazobactam might not be a clinically significant cause of false-positive results in the Platelia *Aspergillus* EIA test in Brazil.

Keywords: piperacillin-tazobactam; aspergillosis; Platelia *Aspergillus*; galactomannan.

Introduction

Galactomannan (GM) detection has gained importance in the diagnosis of invasive aspergillosis (IA) [1, 2]. Several monoclonal antibodies have been developed to detect *Aspergillus* GM molecules, including EB-A2, the actual basis for a sandwich enzyme immunoassay commercial kit (Platelia™ *Aspergillus* EIA – Bio-Rad, USA). Although this test has been available in Europe for roughly a decade, it was not before 2003 that the assay was cleared by the Food and Drug Administration (FDA) for use in the United States [3]. In Brazil, approval was obtained by the national regulatory agency (ANVISA, *Associação Nacional de Vigilância Sanitária*) in late 2007 [4]. Therefore, experience with GM testing in South America is still quite limited.

The ability of GM testing to contribute to the diagnosis of IA is limited by several factors. In addition to varied sensitivity [2], test performance is hampered by the occurrence of false-positive results, particularly occurring in patients taking antibiotics of fungal origin [5, 6]. In many cases, a positive GM result can be obtained by testing directly the antibiotic batch. Piperacillin-tazobactam and amoxicillin-clavulanate are usually implicated as the main contributors to false-positive reactions [6-8]. Since IA mostly affects patients with prolonged neutropenia (and piperacillin-tazobactam is one the first-line options for empirical treatment of febrile neutropenia) cross-reactions might be of clinical significance. However, there is a controversy about the frequency and importance of piperacillin-tazobactam contamination with GM [9-12], with some authors arguing that we are now in use of a more pure drug [13]. Since no such a data is available for South America, here we investigate the relevance of piperacillin-tazobactam contamination with GM by testing the drugs available in the Brazilian market.

Material and Methods

A list of companies manufacturing piperacillin-tazobactam in Brazil was initially obtained at the ANVISA website. We were able to get a vial from 5 out of the 7 branches available in the Brazilian market. These were named drugs A, B, C, D, and E.

Lyophilized drugs were diluted as for clinical use in sterile NaCl 0.9% to a final concentration of 45 mg/ml. Platelia *Aspergillus* test was performed according to manufacturer's instructions. In summary, 300 μL of the reconstituted drug was pre-treated as serum samples. GM testing was performed in duplicate and in two laboratories by distinct scientists who were blinded to the other laboratory results. Sodium chloride was used as a negative control, in addition to the negative control provided in the Platelia *Aspergillus* kit.

GM results were expressed as optical densities (OD) – samples were considered positive when the ratio between the OD observed for the sample and the mean cut-off OD was ≥0.5.

In order to evaluate the potential for false-positive GM results in clinical samples, human serum was spiked with piperacillin-tazobactam using drug samples that showed positive results in the GM testing. Serum used for this experiment was known to be negative for GM (OD of 0.28). Different concentrations of piperacillin-tazobactam were tested, mimicking achievable peak and trough levels after a standard 4.5 g dose: 600 μg/ml, 300 μg/ml, 150 μg/ml and 75 μg/ml [10]. In this experiment, 300 μL of serum was also used as a negative control.

Results

Results of this study are summarized in table 1. Apart from drug B, all drugs tested negative in GM assay. Results were consistent even when tested by different laboratories. Drug B was positive to GM detection in all tested wells, with GM OD indexes ranging from 0.74 to 0.88 (mean values of 0.80). Sodium chloride used to dilute drugs tested negative.

Spiking human serum with different concentrations of drug B resulted in negative GM results (OD of <0.5). Mean GM OD indexes for 600 μg/ml, 300 μg/ml, 150 μg/ml and 75 μg/ml of piperacillin-tazobactam were 0.38, 0.37, 0.37 and 0.21, respectively.

Discussion

To the best of our knowledge this is the first study evaluating cross-reaction in the Platelia Aspergillus EIA test using piperacillin-tazobactam batches commercialized in Brazil. Positive results in the GM test occurred for only one out of the 5 brands tested.

Several studies conducted in other countries have already evaluated the amount of GM contamination in piperacillin-tazobactam vials and the clinical implications for these findings. Results are somehow contradictory, suggesting that variation may occur when brands are compared and even within different batches [6, 9, 10, 12-17]. For instance, Singh et al. [10] tested 3 different batches of piperacillin-tazobactam from a single manufacturer in Pittsburgh and found very high positive indexes in all samples evaluated (OD of >5.2). Similarly, Aubry et al. [6] detected positive GM indexes in 7 out of 10 batches of piperacillin-tazobactam in France. Median GM index in their study was 2.5, ranging form 0.7-7.8. Also in France, Bart-Debasse et al. [5] described positivity in all piperacillin-tazobactam batches tested (n=10), with OD index ranging from 0.9-4.3. Similarly, an Italian study [9] found positive GM index in 26/30 (86%) piperacillin-tazobactam batches evaluated. Some other authors however have

questioned these findings, stating that false-positive results are of limited clinical relevance [12, 15]. In addition, one study tested several piperacillin-tazobactam batches and found that their GM content was reduced over the time of study. The authors speculated about the possibility of a modification in the manufacturing process to have occurred over time, resulting in more purity and less GM contamination [13].

A study on the kinetics of GM decrease after cessation of β-lactam antibiotics showed that median time for a negative Platelia *Aspergillus* EIA test result is 5.5 days. Quite impressively, results may remain falsely positive for up to 30 days [6]. Due to the possibility of cross-reaction with piperacillin-tazobactam in the Platelia *Aspergillus* EIA test some authors have suggested that testing of suspected antibiotic batches remains the only indicator of possible false EIA positivity [5]. Others have hypothesized that EIA false-positivity might be reduced by sampling serum at trough piperacillin-tazobactam levels or prior to the administration of the next dose [10, 11, 13]. In order to minimize false-positive results in the Platelia *Aspergillus* GM test in patients on piperacillin-tazobactam, separate tubes of the central venous line should be used for blood sampling and for administration of β-lactam antibiotics [13].

'Drug B' was the only piperacillin-tazobactam that resulted in a positive reaction in the Platelia *Aspergillus* EIA in this study. Conversely, at achievable concentrations of piperacillin-tazobactam in serum we did not observe any cross-reaction with this antibiotic. Drug concentrations ranging from 75-600 μg/ml resulted all in GM OD of <0.5, which was similar to negative controls.

Some limitations of this study deserve to be mentioned. First of all, only a single batch of each brand of piperacillin-tazobactam was tested – as already stated, marked variation in GM contamination might occur amongst batches. Secondly, it has been already shown that the kinetics of GM may vary according to the duration of antimicrobial treatment, with GM accumulating after time [5, 8]. Also, the amount of GM in the antibiotic batches does not always correlate with GM levels in vivo [13].

In conclusion, this pilot study suggests that treatment with piperacillin-tazobactam might not be an important cause of false-positive results in the Platelia EIA test in Brazil. More data is clearly needed on this field. Ideally, the amount of GM should be checked in each hospital, using different batches of piperacillin-tazobactam.

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Table 1: Optical density indexes of galactomannan as determined by the commercial Platelia *Aspergillus* EIA in five brands of piperacillin-tazobactam commercialized in Brazil.

Piperacillin-tazobactam		**Laboratory 1**			**Laboratory 2**			**Final results**
Drug	**Batch**	**Test 1**	**Test 2**	**Mean**	**Test 1**	**Test 2**	**Mean**	**Mean**
A	133881E	0.50	0.31	0.40	0.22	0.18	0.20	0.30
B	006373	0.88	0.86	0.87	0.74	0.74	0.74	0.80
C	C09341	0.26	0.19	0.22	0.25	0.22	0.23	0.23
D	7100839	0.37	0.40	0.38	0.36	0.30	0.33	0.35
E	0760133	0.20	0.26	0.23	0.16	0.20	0.18	0.20

4 CONCLUSÕES

- A detecção do antígeno galactomanana por ELISA sanduíche (kit comercial Platelia® *Aspergillus* EIA) demonstrou uma eficácia satisfatória (90,9% de sensibilidade e 90,6% de especificidade) para uso em amostras de LBA de pacientes transplantados de pulmão. No entanto, nestes pacientes o valor do ponto de corte a ser utilizado é de 1,5.

- O teste de detecção da galactomanana sérica não demonstrou valor diagnóstico para aspergilose em pingüins, apresentando especificidade nula;

- Infecções fúngicas sistêmicas como criptococose, histoplasmose e paracoccidioidomicose são responsáveis por resultados falso-positivos no diagnóstico de aspergilose a partir do teste de detecção da galactomanana sérica;

- As distintas marcas de piperacilina-tazobactam disponíveis no mercado brasileiro testadas no ELISA sanduíche para detecção de galactomanana não foram associadas a resultado falso-positivo.

5 CONSIDERAÇÕES FINAIS

A aplicabilidade do teste de detecção da galactomanana para o diagnóstico precoce de aspergilose invasiva em pacientes transplantados de pulmão foi confirmada. Tendo em vista que o antígeno em pacientes não neutropênicos é rapidamente eliminado da circulação sanguínea, o teste em amostras séricas é comprovadamente ineficaz para o diagnostico precoce da doença, possuindo baixa sensibilidade. Assim, amostras de LBA são uma alternativa viável para execução do Platelia *Aspergillus* EIA, pois permitem detectar o antígeno no foco de infecção e local da lesão. Nestes casos, o ponto de corte a ser utilizado deve ser aumentado para 1,5, e devido ao valor preditivo positivo ser relativamente baixo (48%), a interpretação de um resultado positivo deve ser realizada com cautela, avaliando o contexto clínico-epidemiológico do paciente.

Por outro lado, o outro grupo de risco avaliado, pingüins em cativeiro, não foi beneficiado com esta nova alternativa de teste diagnóstico. Nestes animais, o Platelia *Aspergillus* EIA apresentou especificidade nula. Assim, novos estudos necessitam ser realizados no intuito de aprimorar a técnica para ser realizada para aves, e/ou ainda, de descobrir outros métodos que possam realmente contribuir com o diagnóstico precoce da aspergilose, o que propicia um tratamento eficaz e conseqüente diminuição da mortalidade.

Tendo em vista que tanto a aspergilose, como a criptococose, a histoplasmose e a paracoccidioidomicose culminam com comprometimento pulmonar, as reações cruzadas ocorridas no *kit* comercial Platelia *Aspergillus* EIA, demonstram que para a interpretação de forma correta do exame laboratorial se faz necessária a avaliação do hospedeiro no seu contexto epidemiológico.

Todos os lotes de piperacilina-tazobactam testados foram negativos para detecção de galactomanana em concentrações plasmáticas alcançadas no soro dos pacientes, indicando que resultados falso-positivos não podem ser atribuídos ao uso deste fármaco. No entanto, cabe ressaltar que o estudo foi realizado com somente um lote de cada laboratório, e devido a variabilidade que pode ser encontrada entre lotes, este resultado não pode ser extrapolado como definitivo e invariável. Com isso, a amostra sérica para realização do Platelia *Aspergillus* EIA deve ser colhida no período antecedente à próxima administração do fármaco, garantindo que este estará em baixas concentrações para análise.

Por fim, o período referente à execução e conclusão da tese de doutorado propiciou a participação efetiva diária no laboratório de micologia da Santa Casa-Complexo Hospitalar de Porto Alegre, laboratório de referência na área, e, em adição, a interação com os

pesquisadores renomados, de reconhecimento nacional e internacional em micologia médica. Isto proporcionou aumento considerável do conhecimento técnico-científico e estabelecimento de um vínculo profissional muito gratificante e de grande valia.

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APÊNDICE

APÊNDICE A - Ficha para colheita de dados dos pacientes transplantados de pulmão incluídos no estudo

Dados de identificação

Nº caso:……..…… Nº pront…… Registro mico…..……

Nome: ……. Idade:…….

Sexo: ( ) M ( ) F Hospital……

Transplante

Data ..... Tipo: ( ) Simples ( ) Bilateral ( ) Cárdiopulmonar

Doença de base

( ) Enfisema
( ) Fibrose pulmonar idiopática
( ) Deficiência de alfa-1 antitripsina
( ) Hipertensão pulmonar primária
( ) Retransplante
( ) Granuloma eosinofílico
( ) Fibrose cística
( ) Sarcoidose
( ) Escleroderma
( ) Silicose
( ) Outra.....……...

Fatores predisponentes associados (últimos 30 dias)

Neutropênico	( ) Sim _____	( ) Não ( ) NI
Enfermidade enxerto X hospedeiro	( ) Sim	( ) Não ( ) NI
HIV	( ) Sim	( ) Não ( ) NI
CMV	( ) Sim	( ) Não ( ) NI
Isolamento prévio de *Aspergillus*	( ) Sim	( ) Não ( ) NI

Regime de imunossupressão .....

Corticoterapia (pulso) (últimos 30 dias)

( ) Sim Droga, data, dose e duração _____
( ) Não ( ) NI

Outro regime para rejeição

( ) Sim Qual _____
( ) Não ( ) NI

Terapia antimicrobiana

Piperacilina-Tazobactam	( ) Sim ( ) Não ( ) NI
Amoxicilina + ác. Clavulânico	( ) Sim ( ) Não ( ) NI

Antifúngico sistêmico (nos últimos 30 dias) ( ) Sim ( ) Não ( ) NI

Quais (droga e dose):_____

Anfotericina B inalada (nos últimos 30 dias) ( ) Sim ( ) Não ( ) NI

Duração, dose e frequência:_____

Dados clínicos (na data do exame)

Terapia intensiva ( ) Sim ( ) Não ( ) NI
Ventilação mecânica ( ) Sim ( ) Não ( ) NI
Sinais Febre (axilar >38ºC) ( ) Sim ( ) Não ( ) NI
Hipotermia (<35,5ºC) ( ) Sim ( ) Não ( ) NI
Tosse ( ) Sim ( ) Não ( ) NI
Hemoptise ( ) Sim ( ) Não ( ) NI
Dor torácica ( ) Sim ( ) Não ( ) NI
Dispnéia ( ) Sim ( ) Não ( ) NI
Outros _____

Avaliação imagem

RX (descrever o exame mais recente):_____
TC tórax: (descrever o exame mais recente): _____
TC ou RNM SNC: _____

Exame Micológico

Exame direto _____
Cultivo: Espécie / UFC / Nº mic_____

Histopatológico (copiar todos os resultados)

Amostra: _____ Data _____
Resultado: _____
Visualização de estruturas fúngicas ( ) Sim quais.....
( ) Não

Fibrobroncoscopia (data mais próxima do exame)

Data _____ Resultado: _____

Aspergilose invasiva ( ) Comprovada ( ) Provável ( ) Possível ( ) Não
( ) Aspergilose Pulmonar Invasiva ( ) Traqueobronquite ulcerativa
( ) Traqueobronquite pseudomembranosa ( ) Colonização

Rejeição aguda
( ) Sim ( ) Não ( ) NI

Rejeição crônica
( ) Sim ( ) Não ( ) NI

Platelia *Aspergillus* EIA®

Data…..…… Valor ……

Evolução

( ) Alta hospitalar Data:……. Duração internação: ……
( ) Óbito Data:……. ( ) Complicações ……

ANEXOS - RELATOS DE CASO

ANEXO A - **“**Invasive pulmonary aspergillosis due to a mixed infection caused by *Aspergillus flavus* and *Aspergillus fumigatus*”

Artigo publicado na *Revista Iberoamericana de Micologia*, 2008; 25:176-178.

Invasive pulmonary aspergillosis due to a mixed infection caused by *Aspergillus flavus* and *Aspergillus fumigatus*

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Summary: Invasive pulmonary aspergillosis is typically caused by a single *Aspergillus* species, most frequently *Aspergillus fumigatus*. Here we report that a lung transplant recipient developed invasive aspergillosis due to a mixed infection caused by *Aspergillus flavus* and *A. fumigatus*. The implications for this rare finding are discussed.

Key words: Aspergillosis, *Aspergillus fumigatus*, *Aspergillus flavus*, Lung transplantation

Aspergilosis pulmonar invasora causada por infección mixta de *Aspergillus flavus* y *Aspergillus fumigatus*

Resumen: La aspergilosis pulmonar invasora es producida típicamente por una única especie de *Aspergillus*, siendo habitualmente *Aspergillus fumigatus*. Se presenta el caso clínico de un receptor de trasplante de pulmón que desarrolló aspergilosis invasora por una infección mixta causada por *Aspergillus flavus* y *A. fumigatus*. Se discuten las implicaciones de la rareza de este caso.

Palabras clave: Aspergilosis, *Aspergillus fumigatus*, *Aspergillus flavus*, Trasplante de pulmón

A 33 year-old man with primary pulmonary hypertension underwent a single right lung transplantation procedure in 1990. His immunosuppressive regimen consisted of azathioprine, cyclosporine and prednisone. Four months after transplantation, the patient was diagnosed with cytomegalovirus (CMV) pneumonia based on the histopathological findings obtained by transbronchial biopsy, and treated with intravenous gancyclovir. Six weeks later he presented with productive cough and severe dyspnea, reporting very limited tolerance to exercises. At admission to the hospital, chest radiograph showed bilateral pulmonary infiltration with areas of pneumonic-type consolidation. Bronchoscopy and transbronchial biopsy were performed.

Hyaline septate branched hyphae were seen in the bronchoalveolar lavage fluid after preparation with potassium hydroxide preparation (KOH 10%). Histopathological studies (haematoxylin and eosin and Gomori-Grocott methanamine-silver nitrate stain) revealed acute suppurative bronchial inflammation. In addition, there was evidence of tissue invasion by hyphae consistent with *Aspergillus* species. Amphotericin B desoxycholate (1.5 mg/kg daily) was commenced, and *Aspergillus flavus* was recovered in culture (performed with Sabouraud dextrose agar with chloramphenicol, and Mycosel agar at 25 ºC and 35 ºC).

The patient died 15 days later despite antifungal therapy in association with broad spectrum antimicrobial drugs. Two courses of steroids also were attempted, without success. At autopsy, the right lung revealed visceral pleura congestion with extensive adhesion. There were multiple yellowish nodules irregularly distributed throughout the lungs, 0.1-1.0 cm in diameter. Some of these nodules had coalesced to form a large reddish mass. Yellowish nodules were observed also in heart and right kidney (Figure 1) and histopathological evidence of tissue invasion by *Aspergillus* hyphae was demonstrated in these nodules. In the mycology laboratory, the lung fragment was processed in the laminar flow hood and cut using sterile technique. Three pieces were removed from a deep pulmonary nodule, and culture showed growth of both *A. flavus* and *Aspergillus fumigatus* in triplicate (Figure 2).

Discussion

Mixed invasive fungal infections are reported infrequently in the literature [1,6]. Previous reports have usually been associated with states of marked immunosuppression, or with the presence of infected medical devices such as central venous catheters. However, the isolation of two different fungal species belonging to the same genus from clinical specimens is particularly rare. Fungaemias probably represent an exception to this general rule [17]. For instance, 2-9% of cases of candidemia have been shown to be mixed [2,8], caused by more

than one *Candida* species. Interestingly, both the clinical presentation and the severity of mixed fungaemias seem to be similar to that seen with monomicrobial fungaemias [8]. In these studies, one or more of the recovered isolates was found to show antifungal drug resistance which might complicate patient management.

Cimerman et al. seem to be the first to report the occurrence of aspergillosis caused by two *Aspergillus* species [5], in a report of osteomyelitis due to *A. flavus* and *A. fumigatus*. *Chalara ellisii* was also recovered in culture. This case was that of a healthy young man who suffered a closed transverse fracture of the femur as a result of a traffic accident. In a review of the literature up to the year 2000, Singh and Husain [15] found that 5% (two of 40) of aspergillosis cases following lung transplantation were associated to mixed infections due to two *Aspergillus* species. However, no details were given about these cases.

In contrast to the invasive forms of aspergillosis, cases of chronic pulmonary aspergillosis are commonly associated with infection by multiple genotypes, particularly in the presence of fungal balls [3,4]. Mycological studies in patients with chronic cavitary aspergillosis and fungal balls usually show the presence of various morphologies and considerable antigenic variability among isolates [4,9-11], which is probably part of a dynamic process consequent to the continual growth and death of fungal elements [14]. Neuvéglise et al. also showed by molecular typing techniques that cystic fibrosis patients can harbour several strains of *Aspergillus fumigatus* over time [12]. The recovery of two isolates belonging to different *Aspergillus* species is however rare.

The precise mechanisms leading to a mixed infection as seen for our patient are unknown. Also unknown is the reason why mixed *Aspergillus* infections are so rare. Our patient had been infected by CMV, an immunomodulatory virus that impairs cellular immunity therefore increasing susceptibility to opportunistic fungal infections [13,16]. Our patient was also potentially exposed to a high infectious load of *Aspergillus* species, since renovations were being undertaken in the hospital area. However, this assumption is only speculative since we do not routinely measure spores in the hospital air, and this case was not part of an outbreak.

Although invasive pulmonary aspergillosis is usually acquired by inhalation of *Aspergillus* conidia, the diameter and surface characteristics of the conidia seem to be important factors in the pathogenesis of aspergillosis. Accordingly, the bigger size of *A. flavus* conidia favours their deposition in the upper respiratory tract, and this species is the main aetiology of *Aspergillus* sinusitis [7]. The rarity of mixed *Aspergillus* infections in cases of invasive pulmonary aspergillosis probably results from the difficulty imposed for the conidia

(mainly *A. flavus* conidia) to reach the pulmonary alveoli during this acute condition. The importance of this and other factors however remain to be elucidated.

In conclusion, we report in this study a rare case of mixed *Aspergillus* infection causing invasive pulmonary aspergillosis. The incidence of this condition is unknown and might be underestimated, considering the low yield of microbiological methods in the diagnosis of this condition. Theoretically, the association of different species in mixed fungal infection could be a cause of treatment failure as, for example, in case that one of the isolates could show resistance to any particular antifungal drug. The importance of multiple sampling to promote greater overall sensitivity to the microbiological methods also deserves further investigation.

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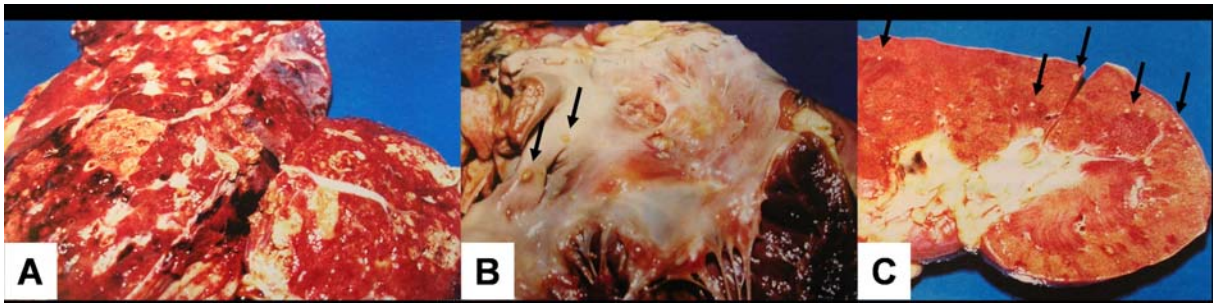

Figure 1. Lesions found at autopsy. Nodular lesions were present in the lungs (A), heart (B), and right kidney (C).

Figure 2. Cultures in triplicate of lung fragments removed after autopsy. The mixed pattern is evident by the repeated growth of both *A. fumigatus* (green to fairly blue colonies) and *A. flavus* (yellow-greenish colonies).

ANEXO B - **“***Aspergillus niger* causing tracheobronchitis and invasive pulmonary aspergillosis in a lung transplant recipient: case report”

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Aspergillus niger causing tracheobronchitis and invasive pulmonary aspergillosis in a lung transplant recipient: case report

Aspergillus niger causando traqueobronquite e aspergilose pulmonar invasiva em transplantado de pulmão: relato de caso

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ABSTRACT

A case of invasive aspergillosis caused by *Aspergillus niger* in a lung transplant recipient is described. The patient presented hyperglycemia starting postoperatively, with other complications such as cytomegalovirus infection. The associated predisposing factors and other implications are discussed. *Aspergillus niger* seems to be a fungal species of low virulence that requires the presence of a severely immunosuppressed host to cause invasive disease.

Key-words: Aspergillosis. Tracheobronchitis. *Aspergillus niger*. Lung transplantation.

RESUMO

Descreve-se um caso de aspergilose invasiva causada por *Aspergillus niger* em um paciente transplantado de pulmão com quadros hiperglicêmicos desde o pós-operatório e outras complicações como infecção por citomegalovírus. Os fatores predisponentes associados e outras implicações são discutidos. *Aspergillus niger* parece ser uma espécie fúngica de baixa virulência, necessitando a presença de um hospedeiro gravemente imunodeprimido para causar doença invasiva.

Palavras-chaves: Aspergilose. Traqueobronquite. *Aspergillus niger*. Transplante pulmonar.

Aspergillus tracheobronchitis and invasive pulmonary aspergillosis are frequent clinical presentations of *Aspergillus* infections in lung transplant recipients[11]. It has been documented that aspergillosis is associated with at least 9% of the deaths following lung transplant procedures. The vast majority of infections are caused by *Aspergillus fumigatus*[8 10], followed by other species such as *Aspergillus flavus*, *Aspergillus terreus* and *Aspergillus niger*. Here, we report the unusual occurrence of *Aspergillus niger* infection following lung transplantation. The implications of these findings are discussed.

CASE REPORT

A 48-year-old woman had undergone right lung transplantation in 1993 due to idiopathic pulmonary fibrosis. Her early postoperative period was marked by the occurrence of hyperglycemia. Acute rejection was suspected five days after the surgical procedure because of oxygen desaturation events. Broad-spectrum antibacterial therapy was started, including amikacin, ceftazidime and vancomycin. She was also started on a three-day course of high-dose methylprednisolone (1,000mg/day). A transbronchial biopsy revealed the presence of hyaline hyphae infiltrating the lung parenchyma. The hyphae were septate, with acute-angle branching, which was consistent with aspergillosis. Lung biopsy culturing revealed growth of *Aspergillus niger*. This mould was also recovered from sputum and bronchoalveolar lavage, both cultivated on Sabouraud dextrose agar with chloramphenicol at 25ºC. She was put on a combination of itraconazole (400mg daily) and amphotericin B (1.5mg/kg/day), which she took for two months. Further cultures for fungi were all negative.

Over the next months, the patient presented cytomegalovirus pneumonia associated with steroid-resistant rejection. She was treated with ganciclovir and the monoclonal antibody OKT3 but developed renal failure, pancytopenia and respiratory failure. She eventually died seven months after the transplant surgical procedure. Autopsy studies revealed foci of bronchopneumonia involving all lobes of the right lung. There was extensive necrosis and suppuration affecting the peribronchial structures, bronchial walls and bronchial mucosa. The right lung parenchyma was diffusely invaded by hyaline hyphae. The left lung showed paraseptal emphysema and extensive bronchiectasis. Septated hyphae showing acute-angle branching were also found invading the left lung parenchyma and the left lower bronchus.

DISCUSSION

Reports of invasive pulmonary aspergillosis caused by *Aspergillus niger* are uncommon in the literature[2 6]. In some of these cases, previous colonization has been

suggested as a potential risk factor for invasive disease[3 4]. The paucity of case reports on invasive pulmonary aspergillosis due to *Aspergillus niger* has been explained by the limited virulence of this species, since *Aspergillus niger* is less likely than *Aspergillus fumigatus* to be associated with invasive disease when recovered from clinical specimens[16]. This has been linked with some physiological, structural and acidophilic characteristics1[13]. Firstly, the size of *Aspergillus niger* conidia (6-7μm) and the presence of strong interspore bridges impair *Aspergillus niger* penetration into the lower respiratory tract. After inhalation, the spores are therefore easily captured and eliminated by the host mucociliary system[13]. Secondly, *Aspergillus niger* is less thermotolerant than *Aspergillus fumigatus*, and its ideal temperature for fungal growth is around 30ºC. This makes germination difficult in the presence of the human body temperature (~37ºC)[1]. The acidophilic nature of *Aspergillus niger* (ideal pH 4.5-4.8) is another limiting condition for fungal pathogenicity[13].

Although *Aspergillus niger* has rarely been associated with invasive infections in lung transplant recipients[2 4], tracheobronchitis is a common manifestation of aspergillosis in these patients, with incidences ranging from 5% up to 14%[2 7 9]. In most cases, this occurs after bilateral lung or right single-lung transplantation14, as described in the present report. *Aspergillus* tracheobronchitis should not be seen as a benign condition, since it might progress or coexist with invasive pulmonary aspergillosis[5], as described in this report.

Similarly to what was demonstrated by other authors[15], our patient developed invasive endobronchial *Aspergillus niger* infection following a state of enhanced immunosuppression. She suffered from marked hyperglycemia, starting in the early postoperative period, which was followed by cytomegalovirus infection. Several courses of high-dose corticosteroid therapy were required, in addition to anti-lymphocytic globulin and OKT3 for organ rejection. These are well-known predisposing conditions for invasive aspergillosis[9 11 12]. The short period of antifungal drug treatment (less than two months) that she received was obviously insufficient to completely clear the infection. Accordingly, *Aspergillus niger* invaded the lung parenchyma and the patient died of invasive pulmonary aspergillosis, which was confirmed by autopsy. This outcome has now been described by several authors[7 9 12.]

In summary, *Aspergillus niger* is a mould of low virulence that rarely causes infection in transplant patients. As shown in this report, states of marked immunosuppression are usually present in transplant patients with *Aspergillus niger* infection.

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ANEXO C - "Invasive *Aspergillus flavus* sinusitis: case report in a patient with biphenotypic acute leukemia"

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Invasive *Aspergillus flavus* **sinusitis: case report in a patient with biphenotypic acute leukemia**

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SUMMARY

Here we report a case of invasive pansinusitis with proptosis of the right eye caused by *Aspergillus flavus* in a immunocompromised patient with acute biphenotypic leukemia without aggressive therapy response.

KEYWORDS: Leukemia; *Aspergillus flavus*; Invasive sinusitis.

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A 17-year-old white man was diagnosed with acute biphenotypic leukemia and underwent chemotherapy with cytarabine, idarubicin and etoposide (7+3+5) followed by high dose cytarabine. After an initial complete remission of short duration he relapse and underwent a second course of induction chemotherapy with metoxantrone and etoposide without response. The patient was pancitopenic and with persistence of blasts; biphenotypic acute leukemia (lineages myeloid and T-lymphoid) demonstrated in a bone marrow biopsy.

He was being treated for febrile neutropenia when developed a fever of 39°C and headache. Physical examination showed edema, hyperemia and proptosis in the right eye with periorbital swelling laterally. Computed tomography (CT) scan of the head revealed opacification of the right maxilar, ethmoidal, sphenoidal and frontal sinuses. Bone erosion was also observed in the medial wall of the right maxilar sinus (Fig. 1). Lung CT scan revealed no abnormalities.

Drained material of the right maxilar sinus was examined and revealed narrow, hyaline, septate hyphae elements, and charateristic dichotomous branching. Fungal culture yielded *Aspergillus flavus*. Microscopic examination of the biopsy obtained from the sinus mucosa showed chronic inflammation and invasion of the submucosa with numerous fungal hyphae consistent with *Aspergillus* (Fig. 2).

Althought precipitating antibody to *A. flavus* antigens was negative, the diagnostic was also confirmed by the positivity in two serum galactomannan immunoassay, latex agglutination (Pastorex *Aspergillus*, Sanofi Diagnostic Pasteur) and sandwich enzime immunoassay (ELISA, 1.34) test (Platelia *Aspergillus*, BioRad, France).

The patient was receiving amphoterecin B at a dose of 1mg/Kg/day since two months before the diagnosis of *Aspergillus* sinusitis, due to an episode of candidemia. After the definitive diagnostic of fungal sinusitis, itraconazole (200mg/daily) was associated to the therapy and surgical procedure was indicated (rhinosinusectomy). However, this aggressive treatment was unsuccessfull, leukemia and fungal infection progressed, clinical status deteriorated and the patient starts to show neurological signs evoluting to death.

Fungal sinusitis, commonly caused by the genus *Aspergillus*, is frequently described in immunocompetent patients and in patients with AIDS as a chronic indolent invasive sinusitis, characterized by a granulomatous response. In neutropenic patients, as observed in our report, the presentation of an *Aspergillus* sinusitis is a fulminant invasive disease where rapidly progressive, gangrenous mucoperiosteitis is frequently fatal[1,4,5,10].

Biphenotypic acute leukemia is an uncommon type of leukemia, which probably arises in a multipotent progenitor cell with capability of differentiating along both myeloid and

lymphoid lineages[3]. Reports of sinusitis by *A. flavus* in patients with leukemia as described here were already observed with concomitant invasive pulmonary aspergillosis[6], as well as rhinosinusitis presentation[9]. In fact, in the largest series of fungal sinusitis described in the literature, *A. flavus* was the mainly etiologic agent, representing 65% (11/17) of all cases[2].

Early diagnosis plays a great role in the treatment efficacy of fulminant sinusitis. Therapy is based in surgical remove of the damaged tissue associated with antifungal administration, where amphotericin B is the drug of choice[1]. Despite this aggressive treatment the outcome death is common, mainly due to the great period between the beginning of the disease and the therapy start, which permit the infection progress to a severe clinical form. Thus, in a series of five rhinocerebral mycosis cases, four patients died although the amphotericin B administration[7], as well as in the largest series of fungal sinusitis where nine from the 17 patients evoluted to death[2]. In the other hand, the development of a severe *Aspergillus* infection in a patient receiving a fungicide drug, amphotericin B as showed in our report, is very uncommon, since this antifungal should prevent the fungal growing and consequently the disease appeared. However, a very similar case was described in the literature[8] suggesting that only the antifungal chemotherapy is not efficient in the control of a fungal infection in neutropenic patients. These both report emphasize the need of preventive measures as installation of ventilation systems with high efficiency particulate airtype filters in rooms of patients included in a risk group[7,8].

RESUMO

Sinusite invasiva por *Aspergillus flavus*: relato de um caso associado a leucemia aguda bifenotípica

Descreve-se um caso de pansinusite invasiva com proptose do globo ocular direito causado por *Aspergillus flavus* em um paciente imunossuprimido com leucemia aguda bifenotípica sem resposta a terapia agressiva.

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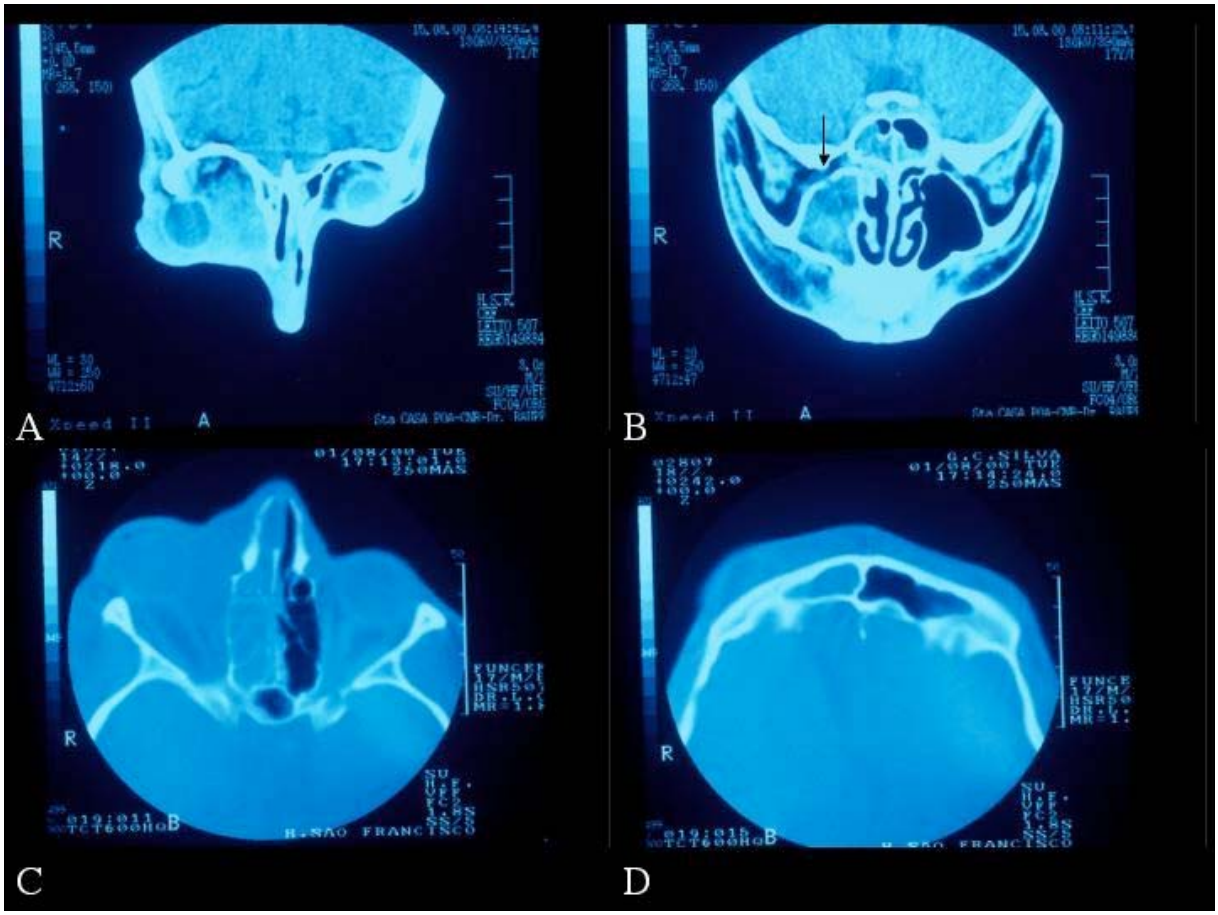

Fig. 1 – CT scan of the head showing opacification of the right maxilar, ethmoidal, sphenoidal and frontal sinuses (A, B, C and D), bone erosion in the medial wall of the right maxilar sinus (B), and proptosis of the right eye (A).

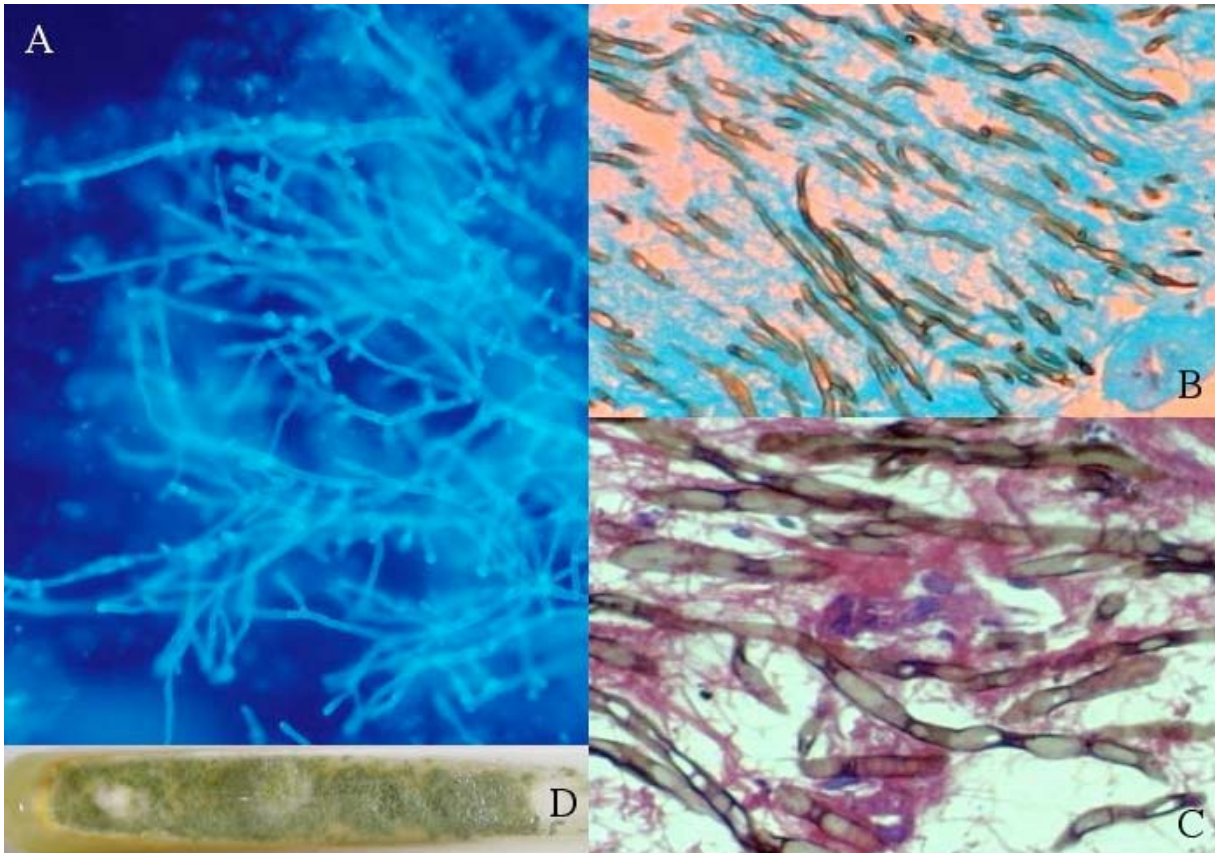

Fig. 2 – Microscopic examination of a sinus mucosa biopsy with calcofluor white (A), with Gomori Methenamine silver stain (B) and with combination of stains Gomori Methenamine Silver and Hematoxilin & Eosin (C) showing septate and dichotomous branching hyphae characteristic of *Aspergillus*. Sabouraud agar dextrose with *A. flavus* (D).