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ORIGINAL PAPER

Anti-*Aspergillus fumigatus* IgG in patients with bronchiectasis and its relationship with clinical outcome

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Abstract

Aspergillosis is a mycosis, most commonly affecting the airways. This mycosis can worsen the clinical condition of patients with concurrent lung diseases. We assayed for the presence of serum anti-*A. fumigatus* IgG in bronchiectasis patients from a tertiary hospital in south Brazil and evaluated the relationship with clinical outcome. Thirty-one patients with bronchiectasis, without cystic fibrosis, were included. Clinical and epidemiological data were collected from all participants. Positive serological tests were detected in 13% (4/31) of the patients. The mortality rate for the year following the assay was, in the seropositive group, 75% (3/4), whereas in the seronegative group, 15% (4/27). An illustrative case is also shown and discussed. Our study highlights the diagnostic challenge and the possible impact of *Aspergillus* infection on these patients, indicating the necessity of more and larger investigations in the field.

Keywords Fungal disease · Aspergillosis · Immunodiffusion · ELISA · *Aspergillus* IgG

Introduction

Aspergillosis is a disease caused by ubiquitous fungi of the *Aspergillus* genus, commonly with involvement of the lower respiratory tract (Marr et al. 2002; Fang & Latgé 2018). The species *A. fumigatus* sensu stricto, belonging to the *Fumigati* section, is the most pathogenic of the genus and is recognized as the main etiological agent of aspergillosis (Fang & Latgé 2018; Kaur & Singh 2014). The disease has different clinical manifestations related to the host's immunological condition, including allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) and invasive aspergillosis (Máiz et al. 2018).

The airways are constantly exposed to environmental fungi, including *Aspergillus* spp. (Máiz et al. 2018). These pathogens are more able to persist and colonize patients with bronchiectasis, as there is deterioration of the mucociliary clearance system and the presence of thick mucus, which could increase patient risk to develop aspergillosis (King et al. 2007).

Bronchiectasis is defined by the abnormal and irreversible dilation of the bronchial lumen (King et al. 2007; Greenberger 2002), with weakening and destruction of the elastic and muscular layers of the bronchi (Barker 2002; Boyton

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2009). The disease is characterized by a "vicious cycle" of inflammation and infection. The inflammation causes damage to the airways, leading to infection by microorganisms, causing more inflammation and consequent worsening of lung damage (King 2009), resulting in lower quality of life and greater mortality of these patients (Martínez-García et al. 2007; Goeminne et al. 2014; McDonnell et al. 2015).

Aspergillus spp. are isolated from the respiratory tract of bronchiectasis patients with an estimated prevalence of ~ 20%, representing a risk due to its pathogenic potential, including toxin production and the stimulation of host inflammatory cytokines (Máiz et al. 2018; Chotirmall and Martin-Gomez 2018). Thus, patients with previous lung diseases such as bronchiectasis may develop CPA, resulting in persistence and worsening of the lung damage (Denning et al. 2016; Bongomin et al. 2020). Additionally, asthma patients with ABPA without an early and correct treatment, may progress to permanent lung damage such as bronchiectasis (Greenberger 2002; Kosmidis and Denning 2015).

Diagnosis of the association between bronchiectasis and aspergillosis, and whether the mycosis is the cause or the consequence of bronchial damage, is a challenge. Clinical signatures of the infection of *Aspergillus* spp. in this clinical condition are unclear (Tiew et al. 2022), since signs, symptoms and alterations in imaging exams are present in patients with and without colonization or infection with this pathogen (Máiz et al. 2018). However, it is possible that a combination of clinical, microbiological and serological data, such as the detection of IgG antibodies against *A. fumigatus*, could help define the diagnosis (Agarwal et al. 2013; Baxter et al. 2013). Therefore, we evaluated the presence of IgG anti-*A. fumigatus* antibodies in patients with bronchiectasis, from a tertiary hospital in south Brazil, and its relationship with mortality in a year of follow-up. In addition, an illustrative case is presented.

Materials and methods

Study population

A retrospective study was conducted, including data from patients with a previous diagnosis of bronchiectasis seen at the University Hospital "Dr. Miguel Riet Correa Jr." of Federal University of Rio Grande (HU-FURG/EBSERH) in Southern Brazil (Rio Grande/RS), from July 2018 to December 2019. It was part of a project entitled "Clinical impact of fungal and bacterial colonization of airways in patients with bronchiectasis", in which patients were interviewed by applying a questionnaire and then provided respiratory samples (sputum, nasopharyngeal swab, biopsy and/or bronchoscopy) and/or serum samples for laboratory processing.

Inclusion and exclusion criteria

Participants ≥ 18 years, who gave written informed consent, answered the questionnaire, and had blood collection, were included in the study. They had a diagnosis of bronchiectasis confirmed by chest computed tomography, and did not have the criteria for a diagnosis of Cystic Fibrosis (Altenburg et al. 2015; Wielpütz et al. 2016). Patients whose serum sample volume was insufficient to assess the presence of IgG anti-*A. fumigatus*, and those with inconclusive results in that test, were excluded.

Data and variables analyzed

Demographic assessment of patients was based on age and sex. Regarding clinical data, predisposing conditions to aspergillosis (such as COPD, asthma, previous tuberculosis, immunosuppressors, smoking, alcoholism), symptoms, and outcome after 365 days of follow-up were analyzed. In seropositive patients for IgG anti-*A. fumigatus*, the clinical history, clinical evolution and imaging exams were further evaluated through the Medical Archives and Statistics Service of the HU-FURG/EBSERH.

Since specific antibody detection is one of the standard methods for the diagnosis of CPA, the detection of IgG was applied in our population. The laboratory data collected consisted of the results of serological tests for the detection of IgG anti-*A. fumigatus*, both by the Double Radial Immunodiffusion in Gel Agar (IDGA) technique (IMMY—*Aspergillus fumigatus* ID antigen ID control™ Oklahoma, United States), and Enzyme Immunosorbent Assay (ELISA) (BioRad—Platelia™ *Aspergillus* IgG, Marnesla-Coquette, France). In the IDGA test, the results were considered positive or negative, according to the presence of the precipitation line. In the ELISA test, quantitative results were interpreted according to the manufacturer, considered positive, intermediate or negative.

Respiratory samples were cultivated on Sabouraud Dextrose Agar with chloramphenicol and gentamicin and incubated at 30ºC for 30 days with periodic evaluation of fungal growth. *Aspergillus* section *Fumigati* isolated in culture and phenotypically identified had DNA extraction with a commercial kit (High Pure PCR Template Preparation Kit—Roche™). To achieve the identification to species level, PCR assay and partial sequencing of the calmodulin gene (calM) (Hong et al. 2005), and/or partial sequencing of β tubulin gene (benA) was performed (Staab et al. 2009).

To compare *Aspergillus* isolates from respiratory samples of a patient between different years (2013, 2015, 2019), a method (microsatellite markers) that uses short

tandem repeats was chosen. The microsatellite markers method was performed, using the multiplex PCR M3 combination, which amplifies three trinucleotide loci, and has a calculated discriminatory power of 0.9968 (Hong et al. 2005). The three forward primers (STRAf 3A, STRAf 3B and STRAf 3C) (Table 1) were labeled at the 5'end with carboxyfluorescein (FAM), hexachloro carboxyfluorescein (HEX), or dichloro carboxyl fluorescein (NED), respectively. PCR reactions were carried out in a 25 µl volume containing 1 µM of each primer. After that, 15 µL of ultrapure formamide mixture was added in 1 µL of PCR product, and then a denaturation at 95ºC for 3 min was performed in a thermo cycler. Fragment analysis was performed by capillary electrophoresis in a 3500 Genetic Analyzer (Applied Biosystems) instrument, using the molecular weight marker GeneScan 500 ROX Size Standard (Applied Biosystems), and the GeneMapper 6.0 software (Applied Biosystems) was used for data analysis. Sequence analyses were performed using MEGA software version 10.0.5, and obtained sequences were compared with those deposited in the GenBank database (Bethesda, MD, USA).

Data analysis

Data were compiled into a database using the Excel program, and descriptive and frequency analysis was performed. The Mann–Whitney statistical test was performed with SPSS 20.0 (IBM, Chicago, IL US) to compare predisposing conditions (asthma, COPD, tuberculosis, smoking, gender), Chi-square to compare outcome, and Kruskal–Wallis test to compare age of the patients, between groups; considering $p \leq 0.05$ as significant.

Ethical aspects

The study was approved by the Research Ethics Committee in the Health Area of the Federal University of Rio Grande (CEP/FURG), process 23,116.006049–2018-10.

Results

Of 113 patients with bronchiectasis treated at HU-FURG/EBSERH from June 2018 to December 2019, 31 were included in the study according to the inclusion and exclusion criteria. These patients had been diagnosed with bronchiectasis for at least one year before the questionnaire and the serum sample evaluated. About half (52%; 16/31) were men, and the mean age (± SD) was 61 years old (± 11.7).

Fatigue was the main symptom recorded, described by 90% (28/31) of the patients, with shortness of breath in 81% (25/31) and productive cough in 77% (24/31) next most common. Regarding predisposing conditions and/or other risk factors for aspergillosis, 39% (12/31) of these patients had asthma, 23% (7/31) had chronic obstructive pulmonary disease (COPD), and 39% (12/31) had a previous history of tuberculosis (TB), three of them with cavitary sequelae. Smoking and alcoholism were associated factors described in 81% (25/31) and 55% (17/31), respectively. Three patients had HIV infection, and seven reported systemic corticosteroid usage.

The presence of IgG anti-*A. fumigatus* antibodies was identified in 13% of patients (4/31) by the ELISA technique. Three of these patients were men (75%), with a mean age (± SD) of 72 years (± 7.2). Two had associated COPD and two had a history of treated tuberculosis with cavitary sequelae. Of these, only one sample was positive by both ELISA and IDGA. In the remaining three patients, all negative in IDGA, the result of the ELISA was intermediate (Table 2). No statistically significant difference was detected between the seropositive and seronegative groups regarding gender or predisposing conditions ($p > 0.05$). However, patients with positive anti-*Aspergillus* antibody detection were older than those seronegative in immunological assays ($p = 0.02$). All these patients had respiratory symptoms at least 3 months before the study.

The clinical history of the patient with positive results in both serological tests (patient 1) is described below as an illustrative case of how challenging the CPA diagnosis can be and how important the impact of *Aspergillus* infection can be on these patients. The other three patients who were negative on IDGA and with an intermediate result on the

Table 1 Primers description used for DNA sequencing of *Aspergillus* isolates from respiratory samples using a microsatellite markers method

Primer*	Forward primer	Reverse primer	Short tandem repeat
STRAf 3A	GCTTCGTAGAGCGGAATCAC	TACCGCTGCAAAGGACAGT	TCT
STRAf 3B	CAACTTGGTGTCAGCGAAGA	GAGGTACCACAACACAGCACA	AAG
STRAf 3C	GGTTACATGGCTTGGAGCAT	GTACACAAAGGGTGGGATGG	TAG

All primer sequences are given in the 5' to 3' direction. *Source: de Valk et al.(2005).

Table 2 Clinical, epidemiological and laboratory data of patients with bronchiectasis and serum detection of anti-*Aspergillus fumigatus* IgG ($n=4$)

Patient	1	2	3	4
Age	71	82	70	65
Sex	Male	Male	Female	Male
Symptoms	Shortness of breath, fatigue	Productive cough, shortness of breath, fatigue	Productive cough, shortness of breath, fatigue	Productive cough, shortness of breath, fatigue
Other associated conditions	Asthma, COPD, previous TB, recurrent respiratory infections	Recurrent respiratory infections	Recurrent respiratory infections	Previous TB, recurrent respiratory infections
Anti-*Aspergillus* IgG				
ELISA	Positive (13.74 AU/mL)	Intermediate (1.54 AU/mL)	Intermediate (1.46 AU/mL)	Intermediate (1.76 AU/mL)
IDGA	Positive	Negative	Negative	Negative
Outcome	CPA under treatment	Death	Death	Death

COPD chronic obstructive pulmonary disease, *TB* tuberculosis, *ELISA* enzyme linked immunosorbent assay, *IDGA* double radial immunodiffusion in gel agar *CPA* chronic pulmonary aspergillosis, *AU/mL* arbitrary units of antibodies per microliter

ELISA had had productive cough, fatigue and/or shortness of breath for more than six months, and a history of repetitive antibacterial treatments for respiratory infections in the 12 months before the study (Table 2). In the seronegative group, out of 27 patients who answered the questionnaire, 20 had productive cough, 24 had fatigue, 21 shortness of breath, and only 14 reported being treated with antibiotics in the last 12 months. Of the 31 patients, death occurred in 23% ($n=7$) (Supplementary material 1). The mortality rate in the 365 days after serology assay was five times higher in the group of patients in which IgG anti-*A. fumigatus* was detected (75%; 3/4) than in the non-reactive group (15%; 4/27) ($p=0.007$).

Illustrative case

In January 2019, a 73 year old man was diagnosed with CPA based on clinical and laboratory evidence, as will be described. *A. fumigatus* was isolated from his sputum, and serological investigations for aspergillosis were performed.

The patient had HIV infection diagnosed in 2000 and treated for virological suppression. He also had a history of a thymoma resection and radiotherapy in 2002, resulting in lung parenchymal damage as a sequel (fibro-atelectasis in the middle field of the right hemithorax and pleural thickening in the right apex, and deviation of the ipsilateral mediastinum). In 2006, and again in 2009, he was diagnosed and successfully treated for pulmonary infection by *Mycobacterium avium*. Since 2010, he was treated with an inhaled corticosteroid and beta-2 agonist for chronic obstructive pulmonary disease (COPD).

He had experienced recurrent respiratory infections and progressive deterioration of the lung parenchyma on imaging since 2010. Given the previous clinical history, > 10 investigations of sputum/bronchoalveolar lavage culture for mycobacteria infection were performed, all negative after the treatment conclusion (2009).

A. fumigatus was isolated for the first time in a sputum sample in 2013, and a second time in a bronchoalveolar lavage (BAL) sample in 2015. However, in both cases, it was interpreted as contamination or colonization, and no further mycological investigations were performed, nor was any antifungal treatment given. In January 2019, after a new respiratory exacerbation, *A. fumigatus* was again isolated from a sputum sample. All *Aspergillus* isolates collected from this patient were recovered from the biorepository of the Faculty of Medicine's Mycology Laboratory (FAMED-FURG), and they were characterized as being the same strain by microsatellite multilocus genotyping (multillocus genotype 186/165/105). None of the *Aspergillus* isolates collected from other patients shared this multilocus genotype, suggesting that the patient had been persistently infected with the same *Aspergillus* strain, showing the maintenance of this infection.

With these results, the diagnosis of CPA was confirmed (January 2019) and the patient was treated with itraconazole (ICZ, 200 mg oral, every 12 h.), but treatment was not correctly followed by the patient. On May 2019, *A. fumigatus* was isolated once again from another sputum sample. In December 2019, the clinical condition worsened, and then azole therapy (ICZ) was again prescribed. After 6 months of adherence to the correct treatment, the patient showed clinical improvement and stabilization of the radiological images (Fig. 1). At this time, a new anti-IgG test was performed, and the result was negative. The maintenance of the ICZ therapy for more 6 months was initiated (Fig. 2).

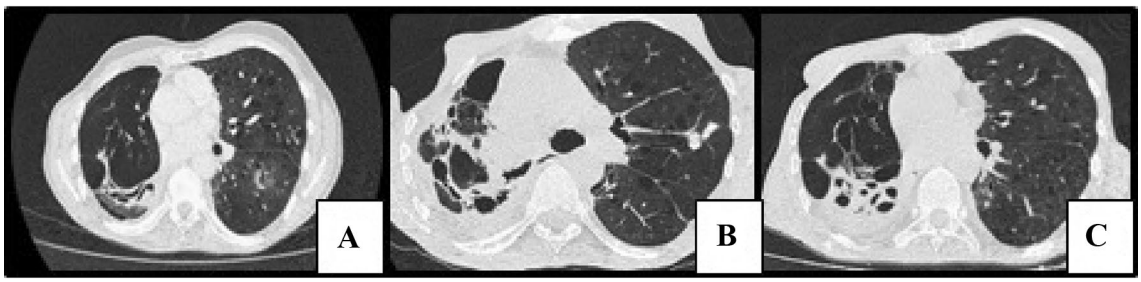

Fig. 1 Imaging exams of the patient: (**A**) 2016—Subtotal atelectasis of the upper, middle and lower lobes and extensive bronchiectasis, (**B**) 2018—Complete atelectasis right upper lobe and lower right lobe, bronchiectasis, bullous areas and cavitation with mucoid secretion, (**C**) 2020—Sequelae in right superior lobe, segmental atelectasis and bronchiectasis

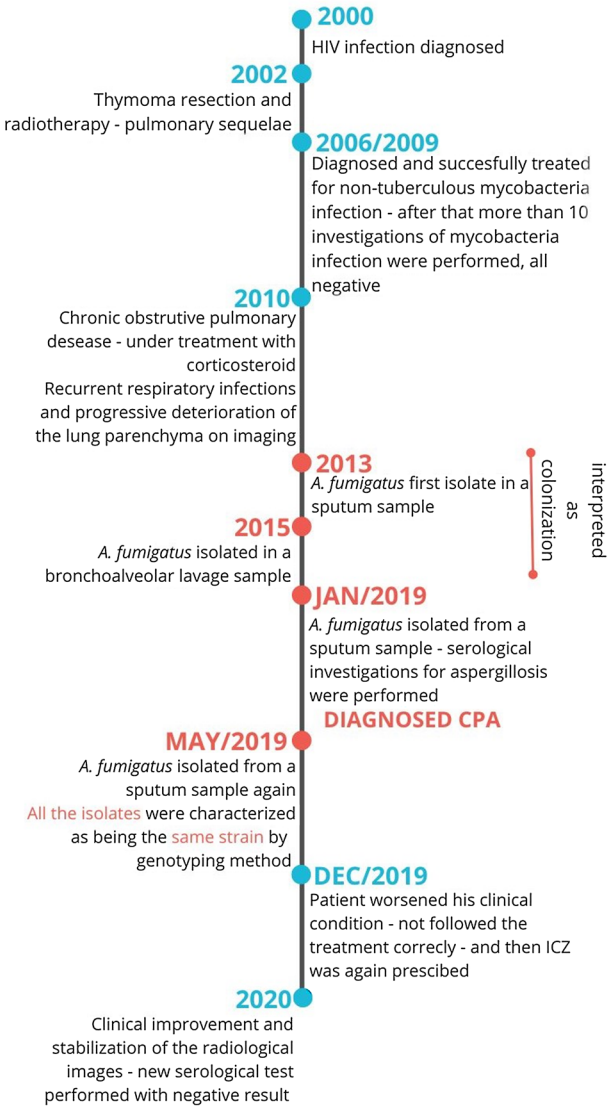

Fig. 2 Timeline of the CPA illustrative case showing the chronologically clinical progression of the disease. The timeline starts when the patient was diagnosed with HIV infection, enumerating other previous clinical conditions, and then the history of isolation of *Aspergillus fumigatus* and the failure of the diagnosis. Lastly, diagnosis confirmation after serology and specific treatment

Discussion

The diagnosis of aspergillosis in bronchiectasis patients is a challenge, given the similarity of symptoms between the underlying disease and its exacerbations. In addition, there is the difficulty of interpreting the isolation of *A. fumigatus* from clinical samples, which can occur due to contamination, colonization and/or infection (Mortensen et al. 2011; Chotirmall et al. 2013; Kosmidis and Denning 2015). Therefore, the use of complementary diagnostic methods, such as the detection of specific antibodies, is recommended in these patients (Denning et al. 2016; 2018; Ullmann et al. 2018). We have found in our cohort 13% (4/31) anti-*A. fumigatus* IgG seroprevalence in patients with bronchiectasis in a tertiary hospital in southern Brazil.

Patients with a positive immunological assay in our study were significantly older than those without antibodies against *Aspergillus* spp. These data are in agreement with studies including patients post-treatment for tuberculosis, in which the prevalence of chronic aspergillosis increases from 25% after one year of treatment to 36% after four years (Denning et al. 2011). Predisposing conditions for aspergillosis, such as asthma, COPD, previous TB are known factors related to a worse prognosis in *Aspergillus* spp. infection (Marr et al. 2002; Kosmidis and Denning 2015; Latgé and Chamilos 2019), however, these conditions were not statistically different between seropositive and seronegative groups in our study, possibly due to the limited number of patients.

Chronic pulmonary aspergillosis was confirmed in one of the four seropositive patients (patient 1), who had antibodies detected by both methods (ELISA and IDGA), and a repetitive isolation of *A. fumigatus* from respiratory samples. In the other three, specific antibodies were detected only by the ELISA test, and given the limitation of the retrospective nature of this study, these patients did not have respiratory sample collection, and did not receive antifungal treatment. The difference in sensitivity between the two serological methods used in our patients has been described previously (Baxter et al. 2013). ELISA is the most recommended test

for purposes of diagnosing aspergillosis, owing to its greater capacity to detect patients with lower concentrations of circulating anti-*Aspergillus* IgG, compared to IDGA (Page et al. 2016; Harada et al. 2018; Latgé and Chamilos 2019; Wilopo et al. 2020). On the other hand, an intermediate result in this ELISA test is not sufficient to support a diagnosis of aspergillosis, thus, repetition of the examination with a new sample, to attain a firmer result, is indicated by the kit manufacturer (BioRad—Platelia™ *Aspergillus* IgG). However, given the retrospective characteristic of this study, the evaluation of new samples from the patients was not possible.

All patients with anti-*Aspergillus* IgG detection in our study had, in addition to bronchiectasis, other classical risk factors for the development of aspergillosis, such as previous tuberculosis with cavitary sequelae, COPD and/or asthma (Denning et al. 2011; Kosmidis and Denning 2015). According to the 2019 Brazilian Consensus on bronchiectasis without CF, the presence of pathogenic microorganisms has been considered a chronic infection and should no longer be seen as simple colonization (Pereira et al. 2019). In this sense, considering that fungal infection can aggravate lung damage, these results suggest that the presence of antibodies, either indicating colonization or disease, may be another risk factor for these patients and should be considered in clinical management. It is also suggested that the importance of *Aspergillus* infection in patients with bronchiectasis may be underestimated (De Soyza and Aliberti 2017; Máiz et al. 2018).

The illustrative case of the patient diagnosed with CPA in 2019 exemplifies the difficulty of diagnosis and the importance of a better investigation. Prior to the serological analysis, *A. fumigatus* was isolated from different respiratory samples over several years, and these findings were not acted on by the physicians. Owing to the persistence of respiratory symptoms and chronic progression of radiological findings, and after another fungal isolation from a respiratory sample associated with a positive ELISA for IgG anti-*A. fumigatus* antibodies, antifungal treatment was started. The patient achieved clinical and radiological improvement, and was the only patient alive in the seropositive group in the year after the examination. The identification of the three isolates from this patient as the same strain by molecular methodology confirmed a persistent infection. A combination of diagnostic methods for patients predisposed to infection by *A. fumigatus* is important, since the presence of this microorganism is linked to increased pulmonary damage and progressive clinical worsening (Denning et al. 2011). In these cases, molecular typing of *Aspergillus* isolates may provide valuable information which can help a clinical decision regarding treatment.

Our results encourage further studies with a larger number of patients, and a multifactorial risk assessment, to confirm the impact of *A. fumigatus* infection as a worse prognostic sign for patients with bronchiectasis. Our contention is that *Aspergillus* seropositivity may be at least a marker of a subset of patients with bronchiectasis who may be destined to do poorly. It is likely that the presence of *A. fumigatus*, either as colonization or infection, acts as another risk factor for an unfavorable prognosis for these patients. In these cases, serological tests could be implemented during follow-up and follow-up visits, as already indicated for other risk groups, such as for patients with tuberculosis cavity sequelae, for the early diagnosis of aspergilloma (Ullmann et al. 2018; Salzer and Cornely 2017). With further support of our findings, seropositivity for *Aspergillus* in bronchiectasis may be a signal for more intensive follow-up of such patients, and more therapeutic intervention, in which antifungal therapy could be a component.

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Declarations

Conflict of interest All authors declare that they have no competing of interest pertaining to this work.

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