Novel immunologic classification of aspergillosis in adult cystic fibrosis

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Background: Patients with cystic fibrosis (CF) demonstrate a wide range of hypersensitivity responses to *Aspergillus*, beyond allergic bronchopulmonary aspergillosis, which require classification.

Objective: This study integrated 2 new methods of *Aspergillus* detection—sputum galactomannan (GM) and real-time PCR—alongside established serologic markers, to reclassify aspergillosis in CF.

Methods: A total of 146 adult patients with CF had serologic tests (ImmunoCap total IgE, specific *Aspergillus fumigatus* IgE, and specific *A fumigatus* IgG), sputum real-time *Aspergillus* PCR, and sputum GM. Patients were classified by using latent class analysis.

Results: Both RT-PCR and GM were more sensitive than culture in detecting *Aspergillus* in sputum (culture 37%, RT-PCR 74%, and GM 46%). Intraassay and interassay reproducibility of PCR and GM was excellent. Latent class analysis of triazole-naive patients identified a nondiseased group and 3 disease classes: class 1 (n = 49, 37.7%) represented patients with or without positive RT-PCR but no immunologic response to *A fumigatus* and negative GM (nondiseased); class 2 (n = 23, 17.7%) represented patients with positive RT-PCR, elevated total and specific *A fumigatus* IgE/IgG, and positive GM (serologic allergic bronchopulmonary aspergillosis); class 3 (n = 19, 14.6%) represented patients with or without positive RT-PCR, elevated *A fumigatus* IgE (not IgG), and negative GM (*Aspergillus* sensitized); and class 4 (n = 39, 30%) represented patients with positive RT-PCR, elevated *A fumigatus* IgG (not IgE), and positive GM (*Aspergillus* bronchitis).

Conclusions: Three distinct classes of aspergillosis in CF were identified by latent class analysis by using serologic, RT-PCR, and GM data. This novel classification will facilitate improved phenotyping, pathogenesis studies, and management evaluations. (J Allergy Clin Immunol 2013;132:560-6.)

Key words: Aspergillus fumigatus*, cystic fibrosis, bronchitis, allergic bronchopulmonary aspergillosis, polymerase chain reaction, galactomannan*

Aspergillus fumigatus causes significant morbidity in patients with cystic fibrosis (CF). Allergic hypersensitivity in CF forms an immunologic spectrum from lone IgE-mediated sensitization to allergic bronchopulmonary aspergillosis (ABPA), affecting up to 65% and 15% of adult patients with CF, respectively.[1,2] Consensus guidelines for the management of ABPA do not embrace patients with *Aspergillus* sensitization and/or colonization.[3] Most available evidence points toward a positive link between greater lung function decline and sensitization to *A fumigatus*, but there are no randomized studies of the effect of anti-inflammatory or antifungal agents.[4-7] The effect of *A fumigatus* colonization is more debatable, and defining colonization is nonstandardized.[8-11] The prevalence of *A fumigatus* in CF sputum samples varies widely with culture technique, from 6% to 57%.[12,13] Therefore, a major obstacle in assessing the clinical impact of colonization or infection is the reliable detection of *Aspergillus* from sputum.[14] Positive sputum culture has not been linked to the development of ABPA or sensitization and is not included in diagnostic criteria. An additional separate clinical entity has also recently been proposed, "*Aspergillus* bronchitis," but is based on observations in just 6 patients persistently growing *A fumigatus* who had improvements in clinical status and lung function following antifungal therapy.[15] No serologic or sputum markers have been found that distinguish patients with colonization from those sensitized or with *Aspergillus* bronchitis/infection.

A diagnostic classification system of *Aspergillus* hypersensitivity and colonization, beyond ABPA, is needed to establish the longitudinal clinical effects of these syndromes and allow future studies of genetics and clinical trials of treatment. Classification requires accurate *Aspergillus* detection and measures of immunologic response. The primary aim of this study was to validate 2 new methods to detect *Aspergillus* in CF sputum—real-time quantitative PCR (RT-PCR) for *Aspergillus* DNA and galactomannan (GM) antigen —and integrate these tests with standard serologic analysis to develop a classification system of

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Abbreviations used
ABPA: Allergic bronchopulmonary aspergillosis
CF: Cystic fibrosis
CV: Coefficient of variation
GM: Galactomannan (a measure of *Aspergillus* growth)
ABPA-S: Serologic ABPA
SPT: Skin prick test

aspergillosis in CF. Secondary aims were to compare baseline clinical differences between classes and monitor changes in body mass index, lung function, and pulmonary exacerbation rates over a 2-year prospective period.

METHODS

Study design

This was a single center, 2-year prospective observational cohort study of adult patients with CF. The study was approved by the South Manchester research ethics committee (07/Q1403/70).

Patients and sample processing

Patients were enrolled from the Manchester adult CF center during outpatient consultations, and all gave written informed consent. Patients enrolled were aged 18 years or older and had a confirmed diagnosis of CF by genetic testing and/or sweat testing. Enrollment was deferred if they had an exacerbation of pulmonary symptoms requiring additional therapy.

Demographics. Patient baseline demographic details were recorded including CF comorbidities, chronic pulmonary infections, inhaled/oral treatments, and lung function. Baseline lung function (FEV_1 and forced vital capacity % predicted) was obtained from patients' annual clinical assessment records, and the best lung function achieved in the year prior to recruitment was recorded. All lung function was performed postbronchodilator, by experienced staff according to European Respiratory Society guidelines.[16] Lung function was recorded again 2 years after enrollment by using the same method. Body mass index was recorded at baseline and 2 years after enrollment. Total days of intravenous antibiotics were prospectively monitored over 2 years as a measure of pulmonary exacerbation rates.

Sputum collection, culture, and DNA extraction. Sputum samples were noninduced, with a minimum volume of 2 mL, refrigerated at 4°C, and processed within 24 hours of collection. An equal volume of Sputasol (Oxoid Ltd, Basingstoke, United Kingdom) was added to each sputum sample, vortexed, and incubated at 37°C for 30 minutes. Sputum culture was then performed according to modified Health Protection Agency National Standards Method BSOP57 (10 μL rather than 1 μL inoculum).[17] The remaining sputum sample underwent further homogenization using sonication as described previously.[18] Culture was repeated after sonication, and 600 μL of sputum was transferred to a sterile microcentrifuge tube for GM detection. DNA was extracted from the remainder of the sample by using the fungal DNA extraction kit MycXtra (Myconostica, Manchester, United Kingdom). Ten sputum samples were split to investigate PCR intraassay reproducibility. Thirty patients gave 2 samples within 9 months to assess reproducibility and chronicity of infection/colonization.

RT-PCR. Samples were prepared in a high efficiency particle arrest-filtered biosafety cabinet. *Aspergillus* DNA was detected and quantified by using a commercial RT-PCR assay, MycAssay Aspergillus (Myconostica), which targets a portion of the 18S ribosomal gene. Cycle threshold (C_t) values less than 38 were positive. Forty DNA extractions were processed twice to investigate PCR interassay reproducibility.

Galactomannan. The Platelia *Aspergillus* enzyme immunoassay (Bio-Rad, Marnes-La-Coquette, France) was used to detect GM in 300 μL sputum samples fully homogenized by Sputasol and sonication (optical density index ≥0.5 positive). Twenty samples were processed twice simultaneously to investigate intraassay reproducibility. Twelve samples were processed daily over 5 days, half were stored at −20°C and half were stored at 4°C between testing, to determine interassay reproducibility.

Allergy tests. Serum samples were tested for eosinophil count, total circulating IgE, specific *A fumigatus* IgG, and 5 specific fungal IgE (sIgE) allergens using the ImmunoCap assay (Phadia, Uppsala, Sweden): *A fumigatus*, *Cladosporium herbarum, Penicillium chrysogenum* (*notatum*), *Candida albicans*, *and Alternaria alternata*. Type 1 immediate hypersensitivity skin prick tests (SPTs) using standard methods were carried out not only to the same panel of fungal allergens but also to 5 common aeroallergens: grass pollen mix, tree pollen mix, *Dermatophagoides pteronyssinus*, and cat and dog dander (Allergopharma, Reinbeck, Germany).[19,20]

Statistics

Data are expressed as means ± SD, and SPSS version 16.0 (SPSS, Inc, Chicago, Ill) was used to compare results from culture, RT-PCR, and GM testing. Results with a P value of less than .05 were considered statistically significant. Latent class analysis was performed by using M*plus* version 6.11 (Muthén & Muthén, Los Angeles, Calif) to detect disease entities on the basis of statistical patterns of association and likelihood between results, which, in turn, gives each patient a probability of membership for each class. Patients on triazole antifungals were excluded, serologic data were log transformed, and both RT-PCR and GM results were converted to binary data. Patients were assigned to classes by using modal probability and then classes were profiled and labeled. Latent classes were compared to look for any differences in demographic, treatment, or clinical characteristics by using Pearson χ^2 tests, ANOVA, and Mann-Whitney U tests.

To create a diagnostic algorithm for future patients, canonical discriminant analysis was performed by using STATA version 11 (StataCorp LP, College Station, Tex). The aim was to first produce linear combinations (canonical variables) of test values that maximally separated the groups. These variables were then used to generate a diagnostic algorithm, using discriminant functions.

RESULTS

One hundred fifty patients consented to participate; 146 completed the study. Baseline clinical characteristics are shown in Table I.

Culture and RT-PCR

Thirty-nine (27%) patients grew *A fumigatus* by culture and 1 additionally grew *A flavus*. In contrast, 108 (74%) patients were RT-PCR positive for *Aspergillus* species. All culture-positive samples were RT-PCR positive; 38 patients were both PCR and culture negative. There was no correlation between colony-forming units and PCR C_t (Spearman correlation coefficient $r = -0.284$). RT-PCR reproducibility was excellent, with an intraassay coefficient of variation (CV) of 1.5% and interassay CV of 1.1% (see Table E1 and the RT-PCR reproducibility section in this article's Online Repository at www.jacionline.org).

Galactomannan

Sixty-eight (46%) patients were GM positive. Of the 108 RT-PCR positive samples, 66 were GM positive and 42 were GM negative. GM reproducibility was excellent, with an intraassay CV of 5% and interassay CV of 9% (see Table E2 and the GM reproducibility section in this article's Online Repository at www.jacionline.org).

Serology and SPTs

SPTs were performed in 115 of the 146 patients; 31 patients either failed to attend appointments or did not consent to testing.