Mycopathologia (2012) 174:163–169
DOI 10.1007/s11046-012-9531-1

The Performance of Real-Time PCR, Galactomannan, and Fungal Culture in the Diagnosis of Invasive Aspergillosis in Ventilated Patients with Chronic Obstructive Pulmonary Disease (COPD)

Valério R. Aquino · Fabiano Nagel · Huander F. Andreolla · Fernanda de-Paris · Melissa O. Xavier · Luciano Z. Goldani · David W. Denning · Alessandro C. Pasqualotto

Received: 11 November 2011 / Accepted: 9 February 2012 / Published online: 1 March 2012

Abstract Emerging reports have associated chronic pulmonary obstructive disease (COPD) with invasive aspergillosis (IA), particularly in patients treated with mechanical ventilation and/or corticosteroids. This is a multicentre study in which COPD patients demonstrating a new lung infiltrate while being mechanically ventilated were prospectively evaluated for the presence of IA. From the 47 patients studied, *Aspergillus fumigatus* was recovered in culture in two patients (4.2%). While serum galactomannan (GM) was negative for 94% of patients, GM levels in respiratory samples were >0.5, >1.0 and >1.5 for 74.5, 40.5, and 21.3% of patients, respectively. PCR was positive for 10 patients in the study but did not differentiate *Aspergillus* colonization from infection. The combination of PCR and GM in respiratory samples may be an interesting alternative to diagnose IA in COPD patients.

Keywords Aspergillosis · *Aspergillus fumigatus* · COPD · Galactomannan · PCR

V. R. Aquino · L. Z. Goldani · A. C. Pasqualotto
Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

V. R. Aquino · F. Nagel · F. de-Paris · L. Z. Goldani
Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

H. F. Andreolla · A. C. Pasqualotto (✉)
Molecular Biology Laboratory, Santa Casa de Porto Alegre, Av Independência 155, Hospital Dom Vicente Scherer, Heliport, Porto Alegre 90035075, Brazil
e-mail: pasqualotto@santacasa.tche.br

M. O. Xavier
Universidade Federal do Rio Grande, Rio Grande, Brazil

D. W. Denning
Manchester Academic Health Science Centre, The University of Manchester, Manchester, UK

A. C. Pasqualotto
Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil

Introduction

Chronic obstructive pulmonary disease (COPD) has been recognized in recent years as an emerging predisposing condition to invasive aspergillosis (IA), frequently in association with mechanical ventilation (MV) and therapy with corticosteroids [1–4]. A high mortality rate has been described in COPD patients with IA [1, 2, 4–6], which has been associated with a delayed diagnosis [1]. Even though fungal culture is known for its limited sensitivity in the diagnosis of IA, few studies have evaluated the performance of non-culture-based diagnostic tests in the COPD population [2, 6]. Here, we determined the frequency of IA in mechanically ventilated COPD patients, and compared standard methods in medical mycology to non-culture-based methods, including real-time polymerase chain reaction (PCR) and galactomannan (GM) testing.

Materials and Methods

Study Design

Prospective multicentre cohort study.

Study Period and Participating Institutions

Patients were enrolled during Jan/09–Dec/10 in three intensive care units (ICUs) in two university hospitals in Porto Alegre, Southern Brazil (total 2,000-beds).

Inclusion Criteria

COPD patients receiving corticosteroids who demonstrated a new lung infiltrate (as evaluated by chest X-ray) while on MV in the ICU.

Exclusion Criteria

Chronic pneumonia, recent (<14 days) use of anti-mold drugs, hematological malignancy, neutropenia, and organ transplantation.

Definitions

IA was defined based on the Bulpa criteria [1].

Sample and Assays

Respiratory samples (tracheal aspirates in 45/47) were obtained and processed for microscopy/culture and GM testing (Platelia™ *Aspergillus* EIA, Bio-Rad Laboratories, Marnes-La-Coquette, France). For the purpose of fungal culture, samples were inoculated on Sabouraud agar with chloramphenicol for 10 days at 25 and 35°C. The quality of the respiratory samples was evaluated by considering the number of leukocytes and epithelial cells in the sample—only lower respiratory tract samples were studied. DNA was extracted from respiratory samples using MycXtra kit (Myconostica, Manchester, UK) immediately after receipt and stored at −80°C until analysis in batches. Two commercial real-time PCR assays for *Aspergillus* spp. DNA amplification were performed in duplicate: *Aspergillus* spp. q-PCR Alert kit (Nanogen, Torino, Italy) in an ABI7500 (Applied Biosystems, Foster City, USA) thermocycler and the MycAssay™ *Aspergillus* kit (Myconostica) on the SmartCycler (Cepheid, Sunnyvale, USA). Serum was obtained and tested for GM, *Aspergillus* precipitins, and total IgE levels.

Patient Data

Clinical records were reviewed to document basic demographics, medical illness, smoking history, use of antibiotics, days on MV, microbiological and spirometry results, and length of hospital and ICU stay. Corticosteroid dosages were determined in terms of prednisone equivalents and the cumulative dose of corticosteroids was calculated as the total amount of steroids taken in 30 days preceding study entry. Severity of lung disease was determined using GOLD stage.

Statistical Analyses

Quantitative variables were compared using Mann–Whitney test and categorical data with chi-square/Fisher's exact test. p values of $\leq 5\%$ were considered statistically significant.

Ethical Aspects

The study protocol was approved by the Institutional Ethics Committee in each hospital.

Results

Demographic Data

Table 1 shows baseline characteristics of patients included in the study, and the main findings of this study are summarized in Table 2. A total of 47 patients were prospectively enrolled (female 40.4%). Mean age was 68.6 years (±9.9). Most patients had severe COPD (GOLD stages III/IV in 72.8%), and 12.8% ($n = 6$) had also a history of long-term asthma. Lung function tests were retrospectively obtained for 33 patients and most of these exams (87.9%) showed pure obstructive disease. Cumulative steroid dosages ranged from 100 to 4,125 mg (median 900 mg).

Previous Use of Antibiotics and Bacteriological Results

All patients had received antibiotics in the 14 days before inclusion in the study. At the time patients

Table 1 Baseline characteristics of COPD patients included in the study ($n = 47$)

Variable	Proportion (n)
Sex (female)	40% (19)
Age	68.6 years (average)
FVC (forced vital capacity)	70% of predicted (average)
FEV_1 (forced expiratory volume in 1st sec)	42% of predicted (average)
FEV_1/FVC index	55% (average)
History of smoking	98% (46)
Clinical history of asthma	13% (6)
Cumulative dose of corticosteroids	900 mg (range 100–4,125 mg)
Recent use of inhaled corticosteroids	57% (27)

entered the study, bacteria were recovered from respiratory tract cultures in 42.6% ($n = 20$) of samples, mostly enterobacteria ($n = 12$).

Microscopy/Culture for Fungi

Aspergillus section *Fumigati* was recovered from two patients (4.2%; 200 CFU/mL each). Additional fungi recovered from two other patients included *Histoplasma capsulatum* and *Scedosporium apiospermum*.

Aspergillus Precipitins and Total IgE Levels

Precipitins were positive for three patients, at low titers ($\leq$1:2). Total IgE levels in the population of patients studied varied from 2 to 3,000 IU/mL (median 74 IU/mL). Six patients had asthma and their total IgE levels ranged from 70 to 704 IU/mL (median 125 IU/mL).

GM Results

Most (44/47) serum GM readings were negative (i.e., <0.5). Only three patients had positive GM results at low optical indices (range, 0.51–0.59). In respiratory samples, GM optical indices of ≥ 0.5, ≥ 1.0, and ≥ 1.5 were observed in 74.5, 40.5, and 21.3%, respectively. All patients in the study were on antibiotics—including beta-lactam drugs—and no association was observed between antibiotic use and GM levels in either serum or respiratory samples.

PCR Results

Nanogen PCR was positive for one patient, who had a GM index in the tracheal aspirate of 4.4 and from whom *A. fumigatus* was recovered in culture. She died soon after the diagnosis of IA was made. Nanogen PCR, however, was negative for one another patient who was also *Aspergillus* culture-positive, despite amplification of the test internal control. This particular patient had a GM respiratory index of 1.2 and a total serum IgE of 799 IU/mL—he was discharged without antifungal therapy. Myconostica PCR detected *Aspergillus* DNA in 10 patients, including the two cases that were also *Aspergillus* culture-positive (Table 3).

Combining GM and PCR Results

Neither GM nor PCR results were independently associated with overall in-hospital survival. For instance, mortality in the group of patients with GM respiratory results ≥ 2 was 50.0%, compared to 53.8% in the GM <2 group. Patients with positive PCR results (Myconostica) for *Aspergillus* had a 60.0% mortality, versus 51.4% for PCR-negative patients ($p = 0.730$). Among PCR-positive patients, all patients ($n = 2$) demonstrating GM respiratory levels of >2.0 died, in comparison with 50% of patients who had GM respiratory levels below 2.0 (3/6).

Antifungal Treatment

Only one patient in the study received an antifungal agent with anti-mold activity (the patient with scedosporiosis).

Outcome

Overall in-hospital mortality was 53.2% (25/47). No autopsy was performed.

Table 2 Low frequency of IA in a prospective multicenter cohort study involving critically ill patients with COPD.

Variable	Observation
GOLD staging and steroid dosage	This study enrolled mostly patients with severe COPD taking high doses of steroids. These patients were found in previous studies to be at high risk for IA
Microscopy and culture	Low incidence of IA (4.2%) based on conventional microbiological methods. Other identified fungi included *H. capsulatum* and *S. apiospermum*
IgE levels and *Aspergillus* precipitins	Total IgE levels of >500 IU/mL were observed in 8.5% but no patient had ABPA. Specific IgE against *Aspergillus* was not determined. *Aspergillus* precipitins did not detect IA cases
Serum GM	Zero sensitivity in the diagnosis of IA
GM in respiratory fluids	High frequency of false-positive results using currently accepted cutoff (0.5). Discrimination of IA from other causes of false-positive would probably require a higher cutoff (2.0 or more), preferably in combination with *Aspergillus* PCR
Real-time PCR in respiratory fluids	Myconostica PCR was more sensitive to detect *Aspergillus* DNA, when compared to Nanogen *Aspergillus* PCR kit. Myconostica PCR had a high negative predictive value
Gold standard for diagnosis of IA in COPD patients in ICU	There is no universally applicable gold standard for the diagnosis of IA in COPD patients. Lung biopsy is impossible in this group. Autopsy is frequently not obtained, but confirmatory. Multiple negative tests probably rule out an IA diagnosis, but confirming the diagnosis with individual tests positive is problematic.

Summary of the study main findings (data are presented in more detail in the main text)

ABPA Allergic bronchopulmonary aspergillosis, *IA* invasive aspergillosis, *COPD* chronic obstructive pulmonary disease, *PCR* polymerase chain reaction

Table 3 Results of real-time polymerase chain reaction (PCR) cycle threshold (Ct) values for two commercial PCR kits (Myconostica and Nanogen), in comparison with fungal culture. Only positive results are shown here. PCR internal controls were amplified at all times

Case	Culture	Myconostica PCR		Nanogen PCR	
		PCR result	Target Ct result	PCR result	Target Ct result
1	Positive	Positive	36.3	Positive	32.2
2	Positive	Positive	37.5	–	–
3	–	Positive	37.7	–	–
4	–	Positive	35.6	–	–
5	–	Positive	33.9	–	–
6	–	Positive	36.2	–	–
7	–	Positive	37.8	–	–
8	–	Positive	37.3	–	–
9	–	Positive	35.2	–	–
10	–	Positive	35.0	–	–

Discussion

Recent studies suggest that IA may be frequent in patients admitted to the ICU [1–5]. COPD patients seem to be particularly at risk, which has been associated with previous airway colonization with *Aspergillus* species, in a context of severe structural lung disease, requirement of invasive MV and therapy with corticosteroids [1, 2, 5, 6]. Based on these observations, we selected for inclusion in our study patients who were theoretically at high risk for IA. Even though the minimum frequency of probable IA in our study was only 4.2%, most of the other studies in COPD patients showed similar/lower rates of IA. Gao et al. [3] studied 261 patients with exacerbated COPD and found that only 1.9% had IA. In the study by Guinea et al. [2], 53 cases of IA were found among 14,618 COPD patients requiring hospital admission (incidence 0.4%). On the other extreme, Belgian studies showed IA frequencies as high as 6–24% in ICU patients [5, 6] with some of these individuals also being diagnosed with COPD (7.7–31.4%). Since the

frequency of necropsy in the Belgian studies was very high (70–95%), one might argue that IA is one of the most frequently missed diagnoses in the ICU [7]. However, these were all single ICU-studies so the impact of environmental factors and the possibility of an ongoing outbreak cannot be completely excluded.

A major challenge in the diagnosis of IA in COPD patients relies on how to improve the criteria that has been currently used to define the occurrence of the disease. Most of the studies in the literature have been based on the Bulpa criteria [1] which establishes that the recovery of *Aspergillus* sp. in any patient with severe COPD who is also taking steroids and who presents with a worsening lung disease should be regarded as 'probable IA'. However, it is well known that the vast majority ($\sim$90%) of patients who demonstrate a positive respiratory culture for *Aspergillus* in the hospital are actually colonized by *Aspergillus* and not infected [8]. What most published studies have done so far is to split patients with positive cultures for *Aspergillus* into two distinct groups, and the easiest decision is to regard patients with no sign or symptom of infection as being 'colonized'. However, in the critically ill COPD patient, can we rely on fungal culture to differentiate IA from the several other infectious diseases that might be associated with COPD exacerbation? Probably not, which is due to the fact that (1) standard fungal culture has low sensitivity in this context; and (2) culture does not differentiate colonization (which seems to be common) from infection (that may be quite infrequent). With that in mind, here we attempted to evaluate how newer non-culture-based tests such as real-time PCR and GM could add to the diagnosis of IA in COPD patients in the ICU.

As expected, serum GM was of no value in investigating IA in COPD patients, due to its limited sensitivity in non-neutropenic patients [2]. Recently, several investigators have focused on determining GM concentrations in respiratory fluids in these patients in order to increase test sensitivity [9, 10]. However, it should be noted that no standardized technique has been published for processing respiratory samples, which could impact on GM concentration and sensitivity of microscopy, culture, PCR, and GM. Here, we observed that most COPD patients had high GM concentrations in their respiratory samples. Despite that, we were not able to determine a cutoff to separate infected/non-infected patients, and no particular factor was associated with a 'false-positive' GM result. One of the potential explanations for the high GM titers in respiratory samples resides on the fact that we tested mostly blind tracheal washings (96%) while in other studies most samples were bronchoalveolar lavage (BAL) fluids. BAL fluid is always diluted, and so GM indices might be expected to be lower. When testing sputum samples, other authors have observed higher GM optical indices and reduced specificity in comparison with the BAL fluid [11]. Meersseman et al. found that a GM result of $\geq$0.5 in the BAL fluid was highly associated with IA in ICU patients but median indices were $\sim$4 and $\sim$1.5 for patients with proven and probable IA, respectively [6]. Using the 0.5 cutoff for respiratory samples would probably result in an overestimation of IA cases [10]. This is at variance with the latest FDA approval of GM for respiratory samples in the US with a 0.5 cutoff.

In this study, we showed that PCR was more sensitive than microscopy/culture in determining the presence of *Aspergillus* in the airways. These results were somehow expected, based on previous observations [12]. However, it remains difficult to categorize *Aspergillus* PCR-positive patients into the following groups: (1) allergic disease, including allergic bronchopulmonary aspergillosis (ABPA); (2) chronic pulmonary aspergillosis (CPA); (3) *Aspergillus* tracheobronchitis; (4) *Aspergillus* colonization; and (5) invasive pulmonary aspergillosis. The first can be inferred by the presence of asthma, high IgE levels and eosinophilia, which did not occur in our study. CPA should be suspected in patients with lung cavities and chronic symptoms—such individuals were also not included. Tracheobronchitis was not observed since only two patients were submitted to bronchoscopy. Therefore, the main challenge relies on the differentiation of *Aspergillus* colonization in a critically ill COPD patient from IA. A high GM index in the respiratory tract in a PCR-positive patient could potentially help in that sense. A GM cutoff of $\leq$1.2 was definitively not discriminative, while increasing the cutoff to 1.5–2.0 could be helpful, even though numbers were quite small for irrefutable arguments. We believe that a high GM index (i.e., >2.0) in a respiratory sample that is also *Aspergillus* PCR-positive may increase the post-test probability of IA, making 'colonization' an unlikely diagnosis—an observation that deserves additional investigation (Fig. 1).

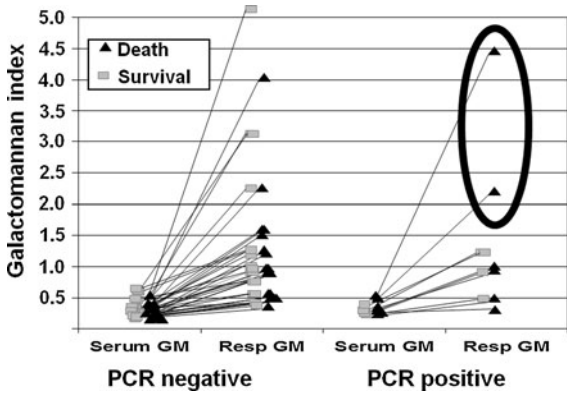

Fig. 1 Distribution of galactomannan (GM) optical indices in the serum and in the respiratory tract, for both *Aspergillus* PCR-positive and PCR-negative patients. A high respiratory GM index in PCR-positive patients (*marked*) might help to differentiate *Aspergillus* infection from colonization

This study is the first comparison of two commercial PCR kits for *Aspergillus* spp. Here we found that MycAssay™ *Aspergillus* kit (Myconostica, UK) was more sensitive than *Aspergillus* spp q-PCR Alert kit (Nanogen, Italy) in detecting *Aspergillus* DNA in respiratory samples, using the same extraction method. Our intention was to have a single extraction method in the study, in order to reduce the number of variables that could potentially have an impact on PCR results. The reasons for such differences in PCR results are not completely immediately apparent. Even though these PCR amplification kits target the same genomic region (18S rDNA) one is based on molecular beacons (Myconostica), while the other relies on Taqman probes (Nanogen)—they must vary in their PCR efficiency. It might also be that Nanogen PCR amplification kit is not suited for the MycXtra Myconostica DNA extraction kit—which is possibly not true since the internal control in Nanogen kit was amplified at all times.

Our study had some limitations that deserve discussion. We showed that fungal culture was far less sensitive than PCR in this study. However, the performance of culture could probably be improved by incubating a larger volume of sample [13]. Since we were aiming for quantitative fungal culture, we used an inoculum of 100 μL. Other measures that could have resulted in better culture yield would include incubation at higher temperatures (e.g., 40–45°C), as well as the supplementation of culture media with additional antibiotics, such as gentamicin. Moreover, despite running the study in three ICUs, patient inclusion was quite slow and we ended up with a limited number of evaluable patients. Many patients were treated in semi-intensive care units and were therefore not enrolled. Most importantly, we were not able to perform autopsy in any patient. Even though no case of proven IA was included in the study, it should be noted that the largest cases series of IA in COPD in the literature also did not include any proven IA case [2].

In conclusion, this prospective multicentre study showed that IA is an uncommon etiology of ventilator-associated pneumonia in COPD patients (minimum incidence 4.2%). The main challenge relies on how to diagnose IA in such patients, based on the limitations of the currently used diagnostic criteria. For instance, one of the patients classified as 'probable IA' in this study was actually colonized by *Aspergillus*, since he did not require any antifungal treatment and was discharged from the hospital in good conditions. PCR was shown to be more sensitive than standard fungal culture, whereas GM testing was of limited sensitivity in the serum, and of low specificity in respiratory samples. The combination of PCR with GM testing in respiratory samples deserves additional investigation, given the known insensitivity of culture for IA in all clinical settings. There is an urgent need to standardize the pre-analytical steps involved in the determination of GM concentration in respiratory specimens.

Acknowledgment We are in debt with our ICU and infection control colleagues, for their help in recruiting patients for this study. We thank Prof. Arnaldo Colombo for his thoughts on this subject. This study was sponsored by independent research grants from Pfizer, Biometrix, and Myconostica.

Conflict of interest Dr Denning holds founder shares in F2G Ltd a University of Manchester spin-out company and has received grant support from F2G as well as the Fungal Research Trust, the Wellcome Trust, the Moulton Trust, The Medical Research Council, The Chronic Granulomatous Disease Research Trust, the National Institute of Allergy and Infectious Diseases, National Institute of Health Research, the European Union, and AstraZeneca. He acts as an advisor/consultant to F2G and Myconostica (now part of Lab21 group) as well as other companies over the last 5 years including Pfizer, Schering Plough (now Merck), Nektar, Astellas, and Gilead. He has been paid for talks on behalf of Merck, Astellas, Novartis, Merck, Dainippon, and Pfizer. In the past 5 years, Dr Pasqualotto has received grant support from Pfizer, Merck, United Medical, Bagó, CAPES, CNPq, FAPERGS, The Fungal Research Trust, Sigma-Tau, and Myconostica. He has been a speaker to or has received travel grants from Pfizer, Schering-Plough (now

Merck), Astellas, United Medical, Biometrix, Merck, and Bagó. All other authors have no conflict of interest to declare.

References

1. Bulpa P, Dive A, Sibille Y. Invasive pulmonary aspergillosis in patients with chronic obstructive pulmonary disease. Eur Respir J. 2007;30:782–800.
2. Guinea J, Torres-Narbona M, Gijón P, Muñoz P, Pozo F, Peláez T, de Miguel J, Bouza E. Pulmonary aspergillosis in patients with chronic obstructive pulmonary disease: incidence, risk factors, and outcome. Clin Microbiol Infect. 2010;16:870–7.
3. Gao X, Chen L, Hu G, Mei H. Invasive pulmonary aspergillosis in acute exacerbation of chronic obstructive pulmonary disease and the diagnostic value of combined serological tests. Ann Saudi Med. 2010;30:193–7.
4. Ader F. Invasive pulmonary aspergillosis in patients with chronic obstructive pulmonary disease: an emerging fungal disease. Curr Infect Dis Rep. 2010;12:409–16.
5. Meersseman W, Vandecasteele SJ, Wilmer A, Verbeken E, Peetermans WE, Van Wijngaerden E. Invasive aspergillosis in critically ill patients without malignancy. Am J Respir Crit Care Med. 2004;170:621–5.
6. Meersseman W, Lagrou K, Maertens J, Wilmer A, Hermans G, Vanderschueren S, Spriet I, Verbeken E, Van Wijngaerden E. Galactomannan in bronchoalveolar lavage fluid: a tool for diagnosing aspergillosis in intensive care unit patients. Am J Respir Crit Care Med. 2008;177:27–34.
7. Anaissie EJ. A bad bug takes on a new role as a cause of ventilator-associated pneumonia. Am J Respir Crit Care Med. 2008;177:1–2.
8. Mortensen KL, Johansen HK, Fuursted K, Knudsen JD, Gahrn-Hansen B, Jensen RH, Howard SJ, Arendrup MC. A prospective survey of *Aspergillus* spp. in respiratory tract samples: prevalence, clinical impact and antifungal susceptibility. Eur J Clin Microbiol Infect Dis. 2011;30:1355–63.
9. Pasqualotto AC, Xavier MO, Sánchez LB, de Oliveira Costa CD, Schio SM, Camargo SM, Camargo JJ, Sukiennik TC, Severo LC. Diagnosis of invasive aspergillosis in lung transplant recipients by detection of galactomannan in the bronchoalveolar lavage fluid. Transplantation. 2010;90:306–11.
10. D'Haese J, Theunissen K, Vermeulen E, Schoemans H, De Vlieger G, Lammertijn L, Meersseman P, Meersseman W, Lagrou K, Maertens J. Galactomannan detection in bronchoalveolar lavage fluid of patients at risk of invasive pulmonary aspergillosis: analytical and clinical validity. J Clin Microbiol. 2012. doi:10.1128/JCM.06423-11
11. Kimura S, Odawara J, Aoki T, Yamakura M, Takeuchi M, Matsue K. Detection of sputum Aspergillus galactomannan for diagnosis of invasive pulmonary aspergillosis in haematological patients. Int J Hematol. 2009;90:463–70.
12. Denning DW, Park S, Lass-Florl C, Fraczek MG, Kirwan M, Gore R, Smith J, Bueid A, Moore CB, Bowyer P, Perlin DS. High frequency triazole resistance found in non-culturable *Aspergillus fumigatus* from lungs of patients with chronic fungal disease. Clin Infect Dis. 2011;52:1123–9.
13. Kirwan MB, Fraczek M, Martin J, Barber PV, Richardson MD, Moore CB, Denning DW. Comparison of *Aspergillus* diagnostic yields from bronchial aspirate, bronchoalveolar lavage and sputa by real-time polymerase chain reaction compared to standard fungal culture. In: 5th advances against Aspergillosis. 2012. Abstract 55.