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Authors
Eigl, Susanne
Hoenigl, Martin
Spiess, Birgit
et al.

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Galactomannan testing and *Aspergillus* PCR in same-day bronchoalveolar lavage and blood samples for diagnosis of invasive aspergillosis

Susanne Eigl[1,†], Martin Hoenigl[1,2,3,4,*,†], Birgit Spiess[5], Sven Heldt[1], Juergen Prattes[2,4], Peter Neumeister[6], Albert Wolfler[6], Jasmin Rabensteiner[7], Florian Prueller[7], Robert Krause[2,4], Mark Reinwald[5], Holger Flick[1], Dieter Buchheidt[5] and Tobias Boch[5,*]

[1]Division of Pulmonology, Medical University of Graz, Graz, Austria, [2]Section of Infectious Diseases and Tropical Medicine, Medical University of Graz, Graz, Austria, [3]Division of Infectious Diseases, Department of Medicine, University of California–San Diego, San Diego, USA, [4]Center for Biomarker Research in Medicine, Graz, Austria, [5]Mannheim University Hospital, Mannheim, Germany, [6]Division of Hematology, Medical University of Graz, Graz, Austria and [7]Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria

*To whom correspondence should be addressed. Martin Hoenigl, MD, Section of Infectious Diseases and Tropical Medicine, Division of Pulmonology, Department of Internal Medicine, Medical University of Graz, A- 8036 Graz, Austria. Tel: +43 316 385 81319; Fax: +43 316 385 14622; E-mail: martin.hoenigl@medunigraz.at

*Tobias Boch, M.D., Dept of Hematology and Oncology, Mannheim University Hospital, University of Heidelberg, D-68167 Mannheim, Germany. Tel: +49 621 383 4115; Fax: +49 621 383 4201; E-mail: tobias.boch@medma.uni-heidelberg.de

†Susanne Eigl and Martin Hoenigl contributed equally

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Abstract

In recent years galactomannan antigen testing (GM) and also *Aspergillus* PCR have become increasingly important for diagnosis of invasive aspergillosis (IA). Whether or not these tests need to be performed with bronchoalveolar lavage fluid (BALF; i.e., primary site of infection), or testing of blood samples is sufficient, remains, however, a matter of debate. We evaluated the diagnostic performance of GM ELISA, and *Aspergillus* PCR by using BALF samples and blood samples obtained at the same day from a total of 53 immunocompromised patients (16 with probable/proven IA and 37 with no evidence of IA according to the revised EORTC/MSG criteria; 38 patients with hematological malignancies were prospectively enrolled at the Medical University of Graz, Austria, 15 patients with mixed underlying diseases at the Mannheim University Hospital). Patients with possible IA were excluded from this analysis. A total of 34/53 (64%) of all patients and 12/16 (75%) of patients with probable/proven IA received mold-active antifungal prophylaxis/therapy at the time of the BALF procedure. Sensitivities of GM and *Aspergillus* PCR were 38% and 44% in BALF, and 31% and 0% in blood, respectively. Best sensitivity (75%) for detecting proven/probable IA was achieved when BALF *Aspergillus* PCR, BALF

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GM (>1.0 ODI), BALF-culture and serum-GM (>0.5 ODI) were combined (specificity 95%). In conclusion, sensitivities of the evaluated diagnostic tests—when interpreted on their own—were low in BALF and even lower in blood, sensitivities increased markedly when diagnostic tests were combined.

Key words: haematological malignancy, fungal, serum, culture, BAL, whole blood, antifungal.

Introduction

Invasive aspergillosis (IA) is an important cause of morbidity and mortality among immunocompromised patients and associated with high mortality rates, especially in the absence of early diagnosis and timely treatment.[1–5] Clinical signs and symptoms of IA as well as radiological findings are often unspecific in the early phase of disease.[5–7] In recent years, antigen testing has become one of the cornerstones of invasive fungal infection (IFI) diagnostics.[8,9]

Galactomannan (GM) is a polysaccharide component of the cell wall of *Aspergillus* spp. that is released into bloodstream by growing hyphae and germinating spores/conidia. In patients with IA GM is measurable in peripheral blood (serum and plasma), bronchoalveolar lavage fluid (BALF), urine, cerebral spinal fluid, or pleural fluid.[10–14] The test, however, has important limitations. As false positive results may occur, factors such as co-medication, underlying diseases, host factors, diagnostic imaging, clinical signs, and former medication of the patient must be taken into account for the correct interpretation of GM levels.[15–17] Furthermore, sensitivity of serum GM may decrease significantly in case of administration of antifungal prophylaxis or empirical/pre-emptive therapy.[18,19]

Aspergillus PCR has been shown to be a very promising method for detection of fungal infection by using BALF or blood from immunocompromised patients,[20–24] especially when combined with the GM test[25,26] and performed early in the course of the infection.[22] While both GM and *Aspergillus* PCR have become increasingly important for diagnosis of IA in recent years it remains unclear whether or not these tests need to be performed with BALF (i.e., primary site of infection) or testing of single blood samples is sufficient to achieve acceptable sensitivity. The objective of this study was to evaluate the diagnostic performance of GM and *Aspergillus* PCR and combinations of these biomarkers by using BALF samples and blood samples obtained on the same day in immunocompromised patients.

Materials and methods

This analysis of prospectively collected data comprised 53 paired routine BALF and blood samples obtained at the same day from 53 adult immunocompromised patients. A total of 38 patients with hematological malignancies undergoing bronchoscopy were prospectively enrolled at the Medical University of Graz, Austria, and 15 patients with mixed underlying diseases (12 patients with hematological malignancy and three patients with other underlying diseases) and suspected IA prospectively enrolled at the Mannheim University Hospital.

Key inclusion criteria were (i) adult patients with (ii) underlying hematological malignancy (Medical University of Graz) or mixed underlying diseases (Mannheim University Hospital) who were (iii) at risk for IA according to attending clinicians (e.g., febrile neutropenia, induction chemotherapy for acute myeloid leukemia, allogeneic stem cell transplantation) and had (iv) a BALF sample obtained in clinical routine. All patients who met inclusion criteria between April 2014 and November 2015 (at the Medical University of Graz) or between July 2013 and May 2014 (at Mannheim University Hospital) and signed informed consent where included in this analysis. IA was graded in accordance with the revised criteria by the European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group (EORTC) and the Mycoses Study Group of the National Institute of Allergy and Infectious Disease (MSG).[27] Patients with possible IA ($n = 13$, all negative *Aspergillus* PCR results in BALF and blood), as well as those who did not have GM and PCR results from both BALF and blood ($n = 6$) were excluded from this analysis.

Conventional mycological culture was routinely and prospectively performed in Graz (Microbiology Laboratory, Department of Internal Medicine) and Mannheim (Institute of Medical Microbiology and Hygiene, Mannheim University Hospital). BALF and serum GM concentrations were prospectively determined in clinical routine by the Platelia EIA (Bio-Rad Laboratories) in Graz (Institute of Hygiene, Microbiology and Environmental Medicine) and Mannheim (Institute of Medical Microbiology and Hygiene, Mannheim University Hospital), respectively. For GM optical density index (ODI) cutoffs of 0.5 (serum and BALF) and 1.0 (BALF) were used to evaluate the diagnostic performance of the test.

All whole blood and BALF samples were initially stored at 4°C and those obtained at the Medical University of Graz were shipped on dry ice using overnight shipping to the Scientific Lab of the University Hospital of Mannheim within

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24 hours of sample collection. At the Scientific Lab of the University Hospital of Mannheim a nested *Aspergillus* PCR assay was performed prospectively in all study samples (and within 48–96 hours of sample collection) according to the protocol of Skladny et al.[28] The mean clinical sample volume for PCR analyses was 3 ml for blood samples and 10 ml for BAL samples. The diagnostic nested PCR assay was performed as described by Skladny et al., according to the protocol for blood and BAL samples.[28] Extraction of DNA was processed via phenol-chloroform extraction according to the protocol of Sambrook et al.[29] Bead beating was not performed. As an internal control, a 138-bp PCR fragment encoded by the human glucose-6-phosphate dehydrogenase gene was amplified in each clinical sample.

Our study was conducted in accordance with the Declaration of Helsinki, 1996, Good Clinical Practice, and applicable local regulatory requirements and law. The study protocol was approved by the local ethics committees, Medical University Graz, Austria (EC-numbers 25–221 and 23–343) and Mannheim University Hospital, Germany (EC-number 2012–320N-MA) and registered at ClinicalTrials.Gov (Identifier: NCT02058316 and NCT01576653).

Statistical analysis was performed using SPSS, version 22 (SPSS Inc., Chicago, IL USA). Negative predictive value (NPV), positive predictive value (PPV), sensitivity and specificity were calculated where applicable. The different diagnostic methods were compared using the diagnostic odds ratio (DOR) method. All DOR values were displayed with 95% confidence intervals (95% CI). Specificity, PPV, NPV, and DOR were calculated for probable/proven IA versus no IA. Cohen's kappa analysis was used for assessment of concordance between GM and PCR.

Results

A total of 53 patients were included in this analysis. Sixteen patients (30%) were classified as proven (n = 2) or probable (n = 14) IA, and 37 patients (70%) as not having IA according to EORTC/MSG 2008. A total of 34/53 (64%) of patients overall and 12/16 (75%) of patients with probable/proven IA were receiving mold-active antifungal prophylaxis/therapy at the time of the BALF procedure (median 2 days, IQR 1–10 days). Patients' characteristics are displayed in Table 1.

For all patients corresponding concurrent BALF, serum and EDTA blood samples were available within a time frame of < 24 h. *Aspergillus* specific PCR from BALF was positive in 7/16 (44%) patients with proven/probable IA and negative in all 37 patients without IA. PCR from concurrent blood samples resulted negative in all 53 patients. GM from BALF was positive in 6/16 patients (cutoff 0.5 ODI; 38%) and in 5/16 patients (cutoff 1.0 ODI; 31%) with proven/probable IA. Same-day serum GM was positive in 5/16 patients (cutoff 0.5 ODI) with proven/probable IA. Cohen's kappa analysis as a measure of concordance between the *Aspergillus* specific PCR and GM in BALF resulted in a value of 0.18 [−0.16 to 0.52] with the strength of agreement to be considered as poor ($\kappa < 0.2$). The respective sensitivity, specificity, PPV, NPV, and diagnostic odds ratio of PCR GM and also conventional BALF culture as single tests as well as of combinations are displayed in Table 2.

Compared to the sensitivity of BALF PCR testing and BALF GM testing (cutoff 1.0 ODI) alone (i.e., 43.8% and 31.3%, respectively), combination of both increased

Table 1. Demographic data and underlying diseases of the study population.

	Study population	Probable /proven IA	No IA	*P*-value*
No. of Patients	53	16/53 (30.2%)	37/53 (69.8%)	–
Sex				*P* > .2
Male	17/53 (32.1%)	6/16 (37.5%)	11/37 (29.7%)	
Female	36/53 (67.9%)	10/16 (62.5%)	26/37 (70.3%)	
Median age (Range)	58 (26 -83)	57 (46-83)	60 (26-82)	*P* > .2
Underlying disease				*P* > .2
AML	17/53 (32.1%)	6/16 (37.5%)	11/37 (29.7%)	
NHL	14/53 (26.4%)	3/16 (18.8%)	11/37 (29.7%)	
MM	10/53 (18.8%)	4/16 (25%)	6/37 (16.2%)	
MDS	3/53 (5.7%)	0/16	3/37 (8.1%)	
CLL	2/53 (3.8%)	0/16	2/37 (5.4%)	
ALL	2/53 (3.8%)	1/16 (6.2%)	1/37 (2.7%)	
Others #	5/53 (13.2%)	2/16 (18.8%)	3/37 (10.8%)	

**P*-value calculated by Fisher exact test, χ^2 test, and Mann–Whitney *U* test.

#included cases of glioblastoma, active tuberculosis (IA), chronic myeloid leukemia, primary myelofibrosis and bronchial carcinoma (no IA).

Abbreviations, ALL = acute lymphocytic leukemia; AML = acute myelogenous leukemia; CLL = chronic lymphocytic leukemia; IA = Invasive Aspergillosis; MDS = myelodysplastic syndrome; MM = multiple myeloma; NHL = Non-Hodgkin lymphoma.

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Table 2. Diagnostic performance of *Aspergillus* specific polymerase chain reaction (PCR), galactomannan enzyme immunoassay (GM) and conventional microbiological culture (culture) in bronchoalveolar lavage fluid (BALF) and blood/serum samples as single tests or in distinct combinations.

IA: *proven/ probable vs. no IA*					
Test (combination) and condition	Sensitivity (n = 16)	Specificity (n = 37)	PPV	NPV	Diagnostic odds ratio
PCR (BALF)	7/16 (43.8%)	37/37 (100%)	7/7 (100%)	37/46 (80.4%)	59.2[3.1–1,1132]
PCR (whole blood)	0/16 (0%)	37/37 (100%)	0/0 (0%)	37/37 (100%)	NA
GM 0.5 ODI (BALF)	6/16 (37.5%)	34/37 (91.9%)	6/9 (66.7%)	34/44 (77.3%)	6.8 [1.4–32.2]
GM 0.5 ODI (serum)	5/16 (31.3%)	37/37 (100%)	5/5 (100%)	37/48 (77.1%)	35.9 [1.8–699.3]
GM (BALF) 1.0 ODI	5/16 (31.3%)	35/37 (94.6%)	5/7 (71.4%)	35/46 (76.1%)	8.0 [1.3–46.9]
Culture (BALF)	3/16 (18.8%)	37/37 (100%)	3/3 (100%)	37/50 (74.0%)	19.44 [0.9–401.9]
GM 1.0 ODI (BALF) and/or GM 0.5 ODI (serum)	7/16 (43.8%)	35/37 (94.6%)	7/9 (77.8%)	35/46 (79.6%)	13.6 [2.4–77.1]
PCR (BALF) and/or GM 0.5 ODI (serum)	10/16 (62.5%)	37/37 (100%)	10/10 (100%)	37/43 (86.1%)	121.2 [6.3–2332]
PCR (BALF) and/or GM 1.0 ODI (BALF)	10/16 (62.5%)	35/37 (94.6%)	10/12 (83.3%)	35/41 (85.4%)	29.2 [5.1–167.5]
PCR (BALF) and/or GM 1.0 ODI (BALF) and/or GM 0.5 ODI (serum)	11/16 (68.8%)	35/37 (94.6%)	11/13 (84.6%)	35/40 (87.5%)	38.5 [6.5–227.1]
PCR (BALF) and/or GM 1.0 ODI (BALF) and/or culture (BALF) and/or GM 0.5 ODI (serum)	12/16 (75.0%)	35/37 (94.6%)	12/14 (85.7%)	35/39 (89.7%)	52.5 [8.5–324.0]

sensitivity to 62.5% with a specificity of 95%. Further addition of GM (serum) increased the sensitivity to 68.8%, while specificity remained unchanged (Table 2). Combination of all three tests (PCR, GM, culture) in BALF and serum GM increased the sensitivity further to 75.0% with an unchanged specificity of 95% [PPV 85.7%, NPV 89.7 and DOR 52.5 (8.5–324.0)].

Of those with probable IA 12/14 (86%) still met the definition after GM was excluded as mycological evidence. After exclusion of the two probable IA cases where mycological evidence was met by positive GM only sensitivities for probable/proven IA (n = 14) were as follows: *Aspergillus* specific PCR from BALF 50%, *Aspergillus* specific PCR from blood 0%, GM from BALF 36% for cutoff 0.5 ODI and 29% for cutoff 1.0 ODI; 31%), and GM from serum 21%.

Discussion

Invasive fungal infections are a critical threat for immunocompromised patients. Early detection is essential for optimal therapeutic success. Diagnostic efforts have been perpetually improved. However, the optimal use of the available diagnostic repertoire is still a matter of debate. In the present study we prospectively analyzed a distinct cohort of immunocompromised patients for whom concurrent BALF and serum samples were collected for GM and *Aspergillus* specific PCR testing. Our main finding was that sensitivities of the evaluated diagnostic tests—when interpreted on their own—were low in BALF and even lower in blood. However, sensitivities increased markedly when diagnostic tests were combined.

Previous studies have shown that both GM and PCR testing play important roles in IA diagnosis.[25,30] The significance of biomarker testing is further outlined by the fact that positive growth of *Aspergillus* spp. in conventional culture is hardly achieved, as also shown in this study. Nevertheless, culture remains essential for detecting other moulds and for broad susceptibility testing, while for azole resistance testing PCR based methods have emerged.[31,32]

The essential concept of this study was the prerequisite of only considering concurrent BALF and blood samples, thus providing same conditions for both assays and types of specimen. Lass-Flörl et al. showed in a direct comparison of an *Aspergillus* specific PCR in blood and concurrent BALF that PCR performance in BALF is less susceptible to prior antifungal therapy.[33] Boch et al. recently showed in a similar approach that GM was significantly more

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valuable as a diagnostic test when performed in BALF compared to same-day blood specimens.[34] The present study expands this concept by adding a simultaneous molecular detection tool, that is, *Aspergillus*-specific PCR. There are other studies of diagnostic performance of GM, PCR, and the benefit of test combination [26,35] with the trend that performance of both tests is generally better in samples from the site of infection, that is, BALF, than in blood. Nevertheless, results vary widely and are hardly comparable among each other. This is mainly due to a plethora of influences such as type and duration of prior antifungal prophylaxis/therapy, underlying disease, or grade of immunodeficiency that might all have a significant impact on the course of a fungal infection and its diagnosis. A study that synchronizes those influences and that includes BALF as specimen from the direct site of infection as well as concurrent blood samples as an example of a readily available clinical sample can therefore add valuable additional information on the diagnostic performance of PCR and GM. In this study blood samples were obtained directly prior or after bronchoscopy.

Our data clearly demonstrate a superior test performance of *Aspergillus* PCR in BALF compared to blood when used as a diagnostic test at a single time point when performed in immunocompromised patients at high risk for IA. Neither proven nor probable IA would have been detected by PCR in blood alone. This poor result might be explained by the high percentage of prior antifungal treatment with consecutively reduced fungal DNA burden in peripheral blood, and that this study used one time PCR testing as opposed to serial screening. Contrary, PCR in BALF turned out to show best test results among all tests and specimens (sensitivity 43.8%, specificity 100%). Overall test performance was reduced but still in the range of data from studies that also included a majority of patients under antifungal treatment.[36] In contrast to the observed discrepancy between BALF and blood for the PCR assay, GM test results in BALF and serum were comparable in our patient cohort, though the sensitivity increased to 43.8% when GM results in BALF and in blood were combined. GM is part of the current EORTC/MSG criteria and is itself a disease defining mycological criterion (defining probable IA) with the inherent difficulty of assessing the performance of an antigen test when it is already used as standard.

The concept of this study focuses on the optimized combination of GM and PCR with special regard to the optimal type of specimen. While GM retained its role in the EORTC/MSG criteria the FDA has recently elevated in its Guidance on Qualification of Biomarker the recommended cutoff for the ODI from 0.5 to 1.0 in BALF.[37] The evaluation of this study has been done with both values. The higher cutoff emphasizes the specificity of the test by simultaneously decreasing the sensitivity. In contrast to GM, PCR assays are still under debate whether they should also be implemented in a revised version of the EORTC/MSG criteria because the standardization process is not yet completed. The nested *Aspergillus* specific PCR assay used in this study is well established and has proven its functionality in various studies including a comparative interlaboratory study among centers from Germany and Austria.[9,38]

Concerning test results from BALF it has to be kept in mind that bronchoscopy and BALF can be biased by the examiner (e.g., volume of lavage fluid). Some physicians tend to avoid or delay bronchoscopy because of the risk of bleeding in the setting of thrombocytopenia or because they consider the invasive procedure to be too strenuous for patients with wasting diseases. Our findings as well as data of other studies[26] support the concept of obtaining specimens from the direct site of infection, for example, BALF, and outweigh the risks of the procedure itself with the significant diagnostic gain. The combination of PCR and GM in BALF resulted in a further increase of combined sensitivity (with a DOR of 29.2) with specificity remaining >90%. Of interest coherence analysis resulted in a poor strength of agreement (Cohen's κ 0.18) between PCR and GM. This direct comparison reveals the advantageous approach of combining fungal DNA detection with detection of fungal cell wall components. Going a step further and adding GM in serum resulted in another upgrade of diagnostic test performance (sensitivity 68.8%, DOR 38.5 [6.5–227.1]). Despite the low sensitivity of conventional culture in BALF (18.8%) as a single test combining all tests in BALF (PCR, GM, culture) with GM (serum) achieved the overall best test performance. In the end, the obvious limitations of each single test within this study could be considerably compensated by this combination. Especially the indispensability of the BALF has been emphasized, which highlights that optimized diagnostics is based on rational choices of diagnostic tools and types of specimen as well.

In conclusion, our study comprising same-day PCR and GM could demonstrate that diagnostic efforts in patients at risk of IA and under antifungal therapy should aim at incorporating test combinations and sampling from the direct site of infection if clinically feasible. Under the influence of antifungal therapy PCR testing of a one-off blood sample is not recommended. The combination of GM in BALF and serum with PCR and culture in BALF showed convincing diagnostic potential among this sensitive patient cohort.

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Conflicts of interest

J. Prattes received travel grant from Pfizer.

M. Hoenigl received research grants from Merck and Pfizer; served on the speakers' bureau of Pfizer, Gilead, Astellas, Basilea and Merck and received travel grants from Astellas, Merck, Gilead, and Pfizer.

A. Wölfler received research grants and speaker honoraria from Merck.

M. Reinwald received travel grants from Merck/MSD and Astellas, and research grants from Gilead Sciences and Pfizer.

D. Buchheidt is consultant to Basilea and Gilead Sciences; received research grants from Gilead Sciences and Pfizer; served on the speakers' bureau of Astellas, Gilead Sciences, Merck Sharp & Dohme/Merck, and Pfizer; and received travel grants from Astellas, Merck Sharp & Dohme/Merck, and Pfizer.

T. Boch received travel grants from Merck/MSD and research grants from Gilead Sciences.

The other authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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