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Diagnosis of invasive fungal infections in haematological patients by combined use of galactomannan, 1,3-β-D-glucan, *Aspergillus* PCR, multifungal DNA-microarray, and *Aspergillus* azole resistance PCRs in blood and bronchoalveolar lavage samples: results of a prospective multicentre study

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ABSTRACT

High mortality rates of invasive fungal disease (IFD), especially invasive aspergillosis (IA), in immunocompromised haematological patients and current diagnostic limitations require improvement of detection of fungal pathogens by defining the optimal use of biomarkers and clinical samples. Concurrent bronchoalveolar lavage (BAL) and peripheral blood samples of 99 haematological patients with suspected IFD were investigated within a multicentre prospective study. Diagnostic performance of a galactomannan (GM) enzyme immune assay (EIA), a 1,3-β-D-glucan assay (BDG), an *Aspergillus* PCR, and a multifungal DNA-microarray (Chip) alone or in combination were calculated. IFD were classified as proven (n=3), probable (n=34), possible (n=33), and no IFD (n=29) according to EORTC/MSG criteria. GM, PCR, and Chip showed superior diagnostic performance in BAL than in blood, whereas specificity of BDG in BAL was poor (48% (14/29)). The combination of GM (BAL) with BDG (blood) showed sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and DOR (diagnostic odds ratio) of 92% (34/37), 93% (27/29), 94%, 90%, and 153.0, respectively. Combining GM (BAL) with PCR (BAL) showed convincing diagnostic potential for diagnosing IA with sensitivity, specificity, PPV, NPV, and DOR of 85% (17/20), 97% (28/29), 94%, 90%, and 158.7. Addition of the DNA-microarray resulted in further detection of two mucormycetes infections. In 1 out of 15 *Aspergillus* DNA-positive samples a triazole

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resistance-mediating *Cyp51A* mutation was found. Combination of biomarkers is superior to their sole use in diagnosing IFD, particularly IA. Integrating blood and BAL samples into a diagnostic algorithm is an advantageous approach. **T. Boch, CMI 2016;22:862**

Introduction

Invasive fungal disease (IFD), particularly invasive aspergillosis (IA) represents life-threatening complications in patients with haematological malignancies receiving intensive chemotherapy or undergoing allogeneic haematopoietic stem cell transplantation [1]. Early diagnosis is associated with improved survival. Antifungal therapy (AFT) is usually started empirically or pre-emptively, based on host factors, fever or a chest CT-driven approach [2].

The current diagnostic reference standard encompasses positive culture and/or histology findings, but displays low sensitivity. Conventional culture turns positive with a considerable time lag or at a later stage of infection, if at all [3]. Only positive culture results from sterile sites, excluding bronchoalveolar lavage (BAL), and/or histological examination provide definitive proof of IFD. As a consequence, a proven early diagnosis is rarely achieved.

Modern diagnostic efforts aim at detection of fungal cell wall components or nucleic acids. Galactomannan is a cell wall component of *Aspergillus* spp. and its detection via Platelia *Aspergillus* enzyme immunoassay (EIA) is part of the diagnostic criteria for IA. GM testing in BAL is promising and has been examined in multiple studies [4]. However, its sensitivity in blood and performance in the diagnostic work-up during antifungal prophylaxis or therapy are unsatisfactory [5]. The optimal cut-off value remains under discussion [6,7].

1,3-β-D-glucan (BDG) is a cell wall component of many different fungal pathogens, with some distinct exceptions (e.g. *Mucorales* spp.). Thus, analysing BDG solely in blood is seen to be controversial for diagnosis of IA [5]; however, valid data for investigating BDG in BAL from haematological patients are missing [8]. Only BDG in serum has been implemented in the revised European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) guidelines.

For non-*Aspergillus* moulds serology-based markers are inadequate. Molecular identification of fungal DNA by PCR or DNA-microarray technologies is feasible and can broaden the spectrum of detectable fungal pathogens [9,10]. For the diagnosis of IA, many PCR-based approaches have been described [11,12]. Diagnostic performance is best in samples from the site of infection such as BAL [13], and is reduced under AFT [14]. Combination of PCR and GM has been described to be beneficial [15]. Our group has previously published a DNA-microarray designed to detect DNA of 15 different fungal species, among them *Mucorales* spp. or *Fusarium* spp. [9]. Its purpose is to broaden the spectrum of detectable fungal pathogens beyond *Aspergillus*.

To elucidate the diagnostic performance of the nested *Aspergillus* PCR assay, the DNA-microarray, and the biomarkers (BDG and GM) as single tests and in combination, we report on the results of a prospective, multicentre study evaluating concurrent BAL and blood samples of haematological patients at high risk for IFD.

We integrated these methods in a combined approach to elucidate and potentially improve the diagnostic performance of the current methodologic repertoire. To gain more data about the emerging problem of azole resistance, samples testing positive for *Aspergillus* DNA were analysed for the occurrence of *Cyp51A* triazole resistance mutations L98H/TR34, TR46, and M220 [16,17].

Patients, materials and methods

Patients and study design

Clinical samples (BAL, peripheral blood) from a total of 99 immunocompromised patients were processed for diagnostic purposes after obtaining informed consent. The study population consisted of individuals with underlying haematological diseases (95% (94/99)) or with severe prolonged myelo- and immunosuppression resulting from therapeutic regimens (5% (5/99)), and therefore high probability for fungal infections. All patients underwent the diagnostic work-up of subsequent BAL in case of suspicious lung infiltrates on chest CT scan. Analyses were done according to good clinical practice guidelines and in accordance with the Declaration of Helsinki. The study was approved by the local ethics committee (Ethics Committee of the Faculty of Medicine Mannheim, University of Heidelberg, Heidelberg, Germany). The study was registered at ClinicalTrials.gov (identifier NCT01695512). Patients were treated from 2012 to 2015 in the University Hospitals of Mannheim, Cologne, Essen, Würzburg, Regensburg, Erlangen, Heidelberg, the Prosper-Hospital Recklinghausen and the General Hospital of Frankfurt/Oder. Primary diseases are shown in Table 1.

Patients were classified as proven (n=3), probable (n=34), possible (n=33), and no IFD (n=29) according to EORTC/MSG 2008 consensus criteria by the authors [18]. Another subgroup of patients with proven/probable aspergillosis was defined by culture, microscopic, and positive GM results (n=20).

Clinical samples

Peripheral blood samples were collected by phlebotomy in a sterile tube to a final concentration of 1.6 mg EDTA per mL blood.

Bronchoscopy with BAL was performed according to the discretion of the local physician, and samples were obtained in a sterile tube without conservation media. The mean sample volume was 10 mL. No BAL was performed for study purposes only.

DNA extraction

DNA extraction both for blood (EDTA vials) and BAL samples was performed according to a protocol described elsewhere [11].

Nested Aspergillus PCR

The diagnostic PCR assay was performed as described by Skladny *et al.* [11] from EDTA blood samples.

DNA-microarray analysis

DNA-microarray analysis was performed as described elsewhere [9,10], detecting a variety of fungal pathogens [10].

To detect positive signals, hybridized glass slides were scanned with an Affymetrix GMS 418 array scanner (Affymetrix, Cleveland, OH, USA) using the recommended settings for Cy3 fluorochrome.