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Performance of 2 commercial real-time polymerase chain reaction assays for the detection of *Aspergillus* and *Pneumocystis* DNA in bronchoalveolar lavage fluid samples from critical care patients

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ABSTRACT

This article investigates the performance of 2 commercial real-time polymerase chain reaction (PCR) assays, MycAssay™ *Aspergillus* (Myc^{Asp}Assay) and MycAssay™ *Pneumocystis* (Myc^{PCP}Assay), on the ABI 7300 platform for the detection of *Aspergillus* (Asp) or *Pneumocystis jirovecii* (Pj) DNA in bronchoalveolar lavage (BAL) samples from 20 patients. Operationally, patients enrolled were clustered into 3 groups: invasive aspergillosis group (IA, 7 patients), Pj pneumonia group (PCP, 8 patients), and negative control group (5 patients). All the IA patients were Myc^{Asp}Assay positive, whereas 12 non-IA patients returned negative PCR results. Furthermore, 7 of 8 PCP patients were Myc^{PCP}Assay positive, while 9 non-PCP patients were PCR negative. In conclusion, these data provide an early indication of the effectiveness of both the Myc^{Asp}Assay and Myc^{PCP}Assay on the ABI 7300 platform for the detection of either Asp or Pj DNA in BAL from patients with deep fungal infections.

1. Introduction

Fungal pathogens are a major cause of morbidity and mortality among critical-care patients, such as those in medical intensive care units (Cushion, 2010; Kontoyiannis & Bodey, 2002). The incidence of invasive aspergillosis (IA) in these patients is increasing (Marr et al., 2002) and is associated with a high mortality rate (Meersseman et al., 2007). Although less common, other fungal pathogens such as *Cryptococcus*, *Fusarium*, *Scedosporium*, *Zygomycetes*, and *Pneumocystis* are also emerging as aetiological agents in these settings. In particular, *P. jirovecii* is a ubiquitous fungus that can cause significant respiratory disease in immunocompromised hosts, with a mortality rate of 35–55% in patients without AIDS (Roblot et al., 2002), as compared with 10–20% in patients with AIDS (Pulvirenti et al., 2003).

Difficulties and/or delays with diagnosis due to nonspecific clinical manifestations, poor diagnostic sensitivity of conventional approaches, and nonspecificity of radiological imaging are some of the reasons why most invasive fungal infections (IFI) are only proven at autopsy (Hope et al., 2005; Sing et al., 2000). Currently, validated routine diagnostic tests, including culture and direct microscopy, are characterized by poor sensitivity and specificity or require long time for identification.

During the last 2 decades, many DNA-based methods have been developed to detect and identify fungal pathogens in a rapid, highly sensitive, and specific manner. While many "in-house" molecular assays have been described, fully approved commercial kits are only recently available whose performance, standardization, and validation are established, and, in turn, their use should be widely encouraged. In particular, commercial assays from Myconostica (Manchester, UK) have now been launched for the detection of *Aspergillus* or *P. jirovecii* DNA in clinical samples (BAL and/or serum) by real-time polymerase chain reaction (PCR), employing selected laboratory platforms. Accordingly, a study by White et al. (2011) investigated the analytical performance and the clinical sensitivity and specificity of the MycAssay *Aspergillus* PCR assay (Myc^{Asp}Assay) in serum specimens. By comparing this system to a validated "in-house" *Aspergillus* PCR test and to the commercial galactomannan ELISA (GM-ELISA), these authors demonstrated that the performance of the Myc^{Asp}Assay is comparable to that of the conventional reference assays. More recently, in a single-centre prospective study, Torelli et al. (2011) investigated the efficacy of the platform for DNA detection in BAL samples, demonstrating that the performance of Myc^{Asp}Assay is comparable to that of GM-ELISA. In particular, they showed that a single PCR-positive result in BAL fluid is associated with a high probability of disease and that the sensitivity

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of detecting IA approaches 100% when Myc^{Asp}Assay PCR is conjunctively used with BAL GM assay, whereas a single PCR-negative result in BAL fluid is sufficient to rule out the suspicion of IA.

In line with these investigations, Hauser et al. (2011) reported the first prospective multicentre evaluation of the performance of the MycAssay *Pneumocystis* PCR assay (Myc^{PCP}Assay) in terms of *P. jirovecii* DNA detection in BAL, when compared to the reference immunofluorescence test (IF). Not only did the Myc^{PCP}Assay have a performance comparable to that of the IF assay, but it was even more sensitive, being capable of detecting additional positive individuals. This raises the possibility that several subjects may have a low level of infection or colonization and that the frequency of infection may be underestimated, at present, due to the poor sensitivity of existing diagnostic tools.

In all the studies mentioned above, the Myc^{Asp}Assay and Myc^{PCP}Assay were used with the Smart Cycler II (Cepheid, USA), the real-time platform originally used for the development and validation of the kits. No data are currently published regarding the performance of Myconostica diagnostic kits with respect to other instruments commonly available and used for routine diagnosis in most clinical microbiology laboratories. To address this issue, in a retrospective study, we investigated the performance of the Myc^{Asp}Assay and Myc^{PCP}Assay in detecting *Aspergillus* or *P. jirovecii* DNA in BAL samples from critical-care patients, by means of the ABI 7300 instrument (Applied Biosystems, Foster City, CA, USA). The analytical and clinical performance of the 2 assays were compared to reference diagnostic tests, including culture, GM-ELISA, and IF; when possible, additional molecular analysis by real-time PCR, nested PCR, and sequencing was also performed.

2. Material and methods

2.1. Subjects enrolled

In this retrospective study, a total of 20 patients of the Policlinico Hospital of Modena were enrolled. On the basis of the clinical diagnosis and according to the European Organization for Research and Treatment Cancer/Mycoses Study Group (EORCT/MSG) criteria, fungal infections were classified as Proven/Probable IA or PCP. Operationally, the 20 patients were clustered into 3 distinct groups, namely, the invasive aspergillosis (IA) group, *Pneumocystis* pneumonia (PCP) group, and control group (CTRL). One of the 20 subjects was enrolled twice into the study, while for another patient also a lung biopsy was available and included in the study; moreover, 5 subjects were enrolled as negative controls. Overall, 22 clinical samples were investigated. The conventional diagnostic tests, conducted in accordance with the local requirements, were culture and galactomannan ELISA (GM, Platelia Aspergillus; Bio-Rad Laboratories, Redmond, WA, USA) for the search of *Aspergillus* and immunofluorescence (IF, MeriFluor *Pneumocystis*, Meridian Bioscience, Cincinnati, OH, USA) for Pj detection.

The present study was approved by the local ethics committee.

2.2. Samples

Twenty-one samples were obtained from direct bronchoalveolar lavage (BAL) procedures required for clinical assessment, while 1 sample was obtained by lung biopsy. For the BAL samples, the volume of sample available for the DNA extraction ranged from 1 to 2 mL. Following collection, the samples were stored at −20 °C prior to DNA extraction.

2.3. DNA Extraction

Fungal DNA was extracted from the samples using the MycXtra Fungal DNA Extraction Kit (Myconostica) following the manufacturer's instructions. DNA was extracted from liquid BAL samples directly, while viscous samples were preprocessed using the NALC-NaOH reagent in the BD BBL MycoPrep Specimen, Digestion/Decontamination Kit (BD Diagnostic Systems, Oxford, UK) following the manufacturer's instructions. DNA extracts were immediately amplified with the specific real-time PCR kit or stored at −20 °C prior to amplification.

2.4. Molecular testing

The 22 samples were assessed by Myc^{Asp}Assay and by Myc^{PCP}Assay following the manufacturer's instructions. The real-time PCR (RT-PCR) was carried out on the ABI 7300 platform, using 10 μL of total DNA template in a final reaction volume of 25 μL. Each kit contained positive and "no template" controls. PCR positivity was determined using a threshold of 39 cycles.

In parallel, when possible, analysis by sequencing as well as by *Aspergillus* spp. real-time PCR Q-PCR Alert Kit (RT-PCR^{Asp}) or *P. jirovecii* nested PCR Alert kit (nested PCR^{PCP}) from Nanogen Advanced Diagnostics (Italy) was conducted, according to the manufacturer's recommendations.

2.5. GM-ELISA and immunofluorescence

GM-ELISA (Platelia Aspergillus; Bio-Rad Laboratories) and IF (MeriFluor *Pneumocystis*, Meridian) tests were performed according to the manufacturer's recommendations for testing BAL samples or respiratory tract specimens. In particular, in the GM-ELISA assay, the cut-off value for BAL specimens was 1.

2.6. Statistical analysis

Mean, standard deviation, and range were calculated for continuous variables, while frequency distribution was computed for categorical variables. Student's *t* test was used to assess differences in Ct values between PCP and IA patients. Diagnostic performance of the different tests was evaluated by receiver operating characteristic (ROC) curve analysis, and estimations of the areas under the curve (AUC) and of their 95% confidence intervals (95% confidence interval [CI]) were calculated. Sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) were calculated as well. The MedCalc software version 12.1.1 was used (Mariakerke, Belgium).

3. Results

3.1. Demographic and clinical characteristics of the patients enrolled in the study

The population studied was composed of 20 patients, with age ranging from 23 to 79 years, with a mean of 60 ± 14 years. Most (90%) of the patients were males, mainly belonging to the Oncology/Haematology/Infectious Diseases Sections, and 2 subjects were HIV-positive. On the basis of the clinical diagnosis and according to the EORTC/MSG criteria, the 20 patients were operationally clustered into 3 groups (Table 1), named as the invasive aspergillosis (IA) group, *Pneumocystis* pneumonia (PCP) group, and control group (CTRL), consisting of 7, 8, and 5 individuals, respectively. In all the subjects, single specimens of BAL fluids were collected with the exception of patient 8, who received 2 subsequent lavages, and patient 5, in whom a lung biopsy sample was also available for assessment.

3.2. Clinical diagnosis with respect to IFI

The 20 patients enrolled had been investigated for biomolecular markers of invasive mycoses. All the results obtained by conventional diagnostic assays (culture, GM-ELISA, and IF) are detailed in Table 2.