Detection of *Pneumocystis jirovecii* and *Aspergillus* spp. DNA in bronchoalveolar lavage fluids by commercial real-time PCR assays: comparison with conventional diagnostic tests

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SUMMARY

The present study employed two commercial real-time PCR kits, MycAssay™ *Pneumocystis* (PJ-PCR) and MycAssay™ *Aspergillus* (ASP-PCR), for the search of fungal DNA on 44 bronchoalveolar lavage (BAL) fluids from patients at risk of invasive fungal disease. Operationally, on the basis of clinical diagnosis and according to the European Organization for Research and Treatment Cancer/Mycoses Study Group (EORTC/MSG) criteria, patients were clustered in 3 groups: a *P. jirovecii* pneumonia (PCP) group, an invasive aspergillosis (IA) group and a control (CTRL) group, consisting of 8, 10 and 24 patients, respectively. The results were compared to those obtained with conventional diagnostic assays, including BAL culture, galactomannan-ELISA (GM) and immunofluorescence (IF). The PJ-PCR assay returned a sensitivity and specificity of 100% and 94.4%, respectively. The ASP-PCR assay showed a sensitivity and specificity of 80% and 97.1%. When compared to the culture assay, the ASP-PCR showed enhanced sensitivity, and a good level of agreement (kappa = 0.63) was observed between ASP-PCR and GM assays. Overall, our data emphasize the diagnostic usefulness of the two commercial real-time PCR assays, especially in high-risk patients where timing is critical and a low fungal burden may hamper correct and prompt diagnosis by conventional tests.

KEY WORDS: Aspergillus, *Pneumocystis*, BAL fluids, Real-time PCR, Invasive mycoses, Non-culture-based diagnostics.

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INTRODUCTION

Diagnosis of invasive fungal disease (IFD) remains a major challenge because current methods are not sufficiently sensitive or specific, and results are often available too late to be clinically useful. IFD are associated with sig-

nificant morbidity and mortality, especially in immunocompromised or severely ill patients. Air-born infections caused by *Pneumocystis jirovecii* and *Aspergillus* spp. are frequent: *Aspergillus* spp. infections in particular have increased since they are caused by species or subspecies with diminished susceptibility or even resistance to standard antifungal agents (Ostrosky-Zeichner, 2012), as also recently observed in Italy (Prigitano *et al.,* 2014). Pneumonia is the most severe clinical presentation of *P. jirovecii* infection (PCP). It occurs in AIDS patients, where the frequency may be reduced by concomitant highly active antiviral therapy, and in other non-AIDS immunocompromised subjects. In the two categories of patients, the lethality ranges from 10-20% up to 35-55%, respectively (Morris A and Norris KA *et al*., 2012; Monnet *et al.,* 2008; Pulvirenti *et al.,* 2003; Roblot *et al.,* 2002). In AIDS patients, PCP is commonly associated with high pulmonary *P. jirovecii* burdens and microbiological diagnosis is commonly based on microscopic detection of cysts and trophic forms in low respiratory tract specimens. Convewrsely, when occurring in non-AIDS patients, the pulmonary burden is low and microscopic observation may return negative results. In these cases, highly sensitive techniques, such as PCR-based DNA amplification procedures, may represent valid alternative tools for diagnosis.

Among environmental moulds, *A. fumigatus* is recognised as the most frequent cause of invasive aspergillosis (IA). Other species, including *A. flavus*, *A. terreus*, *A. niger* and *A. versicolor*, show increased frequency and are often associated with invasive disease. Several well-known risk factors for IA have been described, including chemotherapy and immunosuppressive treatment in haematological and oncological malignancies, pre-existing colonization by *Aspergillus* or infection by HIV, presence of chronic obstructive pulmonary disease, liver cirrhosis or treatment with corticosteroids (Meersseman *et al.,* 2007). The diagnosis of IA is difficult, but early pathogen identification and prompt initiation of antifungal therapy is critical for a good clinical outcome (Rüping *et al.,* 2008). Currently, the conventional microbiological approach to the diagnosis of IA includes microscopic evaluation and culture-based assessment of respiratory specimens. In addition, commercial kits aimed at detecting fungal antigens have been made available and are increasingly employed. As one of the most commonly used routine tests, Platelia Galactomannan-ELISA (GM) allows the detection of galactomannan, a cell wall polysaccharide component of *Aspergillus*, abundantly released during fungal growth, therefore detectable in patients with aspergillosis. More recently, a chromogenic assay has been proposed for measurement of another early marker of IFD, the (1-3)-β-D-glucan, a polysaccharide component of the cell wall of many fungi, including *Aspergillus* spp. and *P. jirovecii* (Onishi *et al.,* 2012; Persat *et al.,* 2008). Among non-culture diagnostic tests, PCR-based platforms offer powerful tools for early and rapid diagnosis of IFD. The number of publications on PCR-related studies is consistently increasing. Numerous in-house PCR configurations have been described (Alanio *et al.,* 2011; Alanio and Bretagne, 2014; Flori *et al.,* 2004; Heng *et al.,* 2013; Hope *et al.,* 2005) and demonstrate high performance, as underlined by the meta-analysis study (Avni *et al.,* 2012). Currently, PCR-based assays for the detection of fungal DNA have not been included in the European Organization for Research and Treatment Cancer/Mycoses Study Group (EORTC/MSG) guidelines because standardisation and clinical validation have not yet been achieved (De Pauw *et al.,* 2008; White *et al.,* 2010). The recent availability of CE-IVD marked real-time PCR platforms for *P. jirovecii* and *Aspergillus* spp. DNA search may facilitate the use of such a technology, hopefully allowing its large scale employment and subsequent standardization.

The present work employed two commercial real-time PCR-based assays, the MycAssay™ *Pneumocystis* (PJ-PCR, from Myconostica, now a Trinity Biotech company, Kentford, UK) and the MycAssay™ *Aspergillus* (ASP-PCR, Myconostica/Trinity Biotech). Search for fungal DNA was conducted in BAL samples from patients operationally clustered according to clinical, EORTC/MSG criteria and microbiological parameters. The first aim was to evaluate the performance of these PCR-based assays with respect to clinical diagnosis of aspergillosis

and pneumocystosis. The second aim was to establish the sensitivity, specificity and predictive values of these assays compared to those of conventional diagnostic tests, including culture, GM and immunofluorescence (IF).

MATERIALS AND METHODS

Patients and BAL samples

This study investigated BAL samples from patients hospitalized at Modena University Hospital from September 2011 to December 2012. In total, 44 BAL fluids were obtained from 41 patients (2 serial BAL samples were collected from 3 patients at different time points) with fever lasting for ≥ 96 hours, unresponsive to broad-spectrum antibacterial therapy and a high risk of developing IFD. Bronchoscopy was performed in all patients with a fiberoptic bronchoscope under topical lidocaine. BAL was performed by instilling 150 mL of sterile saline solution in a sub-segmental bronchus.

Each BAL sample employed in the present study was the left-over of the material collected for the diagnostic routine analyses conducted at the Microbiology and Virology Unit, Modena University Hospital, according to clinicians' requests. Such residual samples were stored at -80 °C until use.

Operationally, on the basis of clinical diagnosis and according to EORTC/MSG criteria, patients were clustered in 3 groups: a *P. jirovecii* pneumonia (PCP) group, an invasive aspergillosis (IA) group and a control (CTRL) group. In detail, the identification of the PCP patients was made on the basis of clinical and radiological manifestations, positivity at the microscope-based immune-fluorescence test (IF), exclusion of alternative non-infectious lung diseases and response to *P. jirovecii* targeted therapy. To define the IA group, the inclusion parameters were the EORTC/MSG criteria (De Pauw *et al.,* 2008) in the haematological patients. In all the other cases and according to previous studies (Meersseman *et al.,* 2004, 2007; Torelli *et al.,* 2011), we also considered additional host factors, such as chronic obstructive pulmonary disease (COPD), low dosage steroid treatment, and clinical criteria, including symptoms of lower respiratory tract infection (e.g. dyspnea), pleural rub and new infiltrate without an alternative diagnosis. All the cases included in the IA group were diagnosed as probable. The CTRL group consisted of hospitalized patients diagnosed as non-IA and non-PCP, according to clinical and laboratory parameters. The study was approved by the local Ethical Committee (CE53/11).

The routinely preformed diagnostic tests included the IF (MeriFluor Pneumocystis Meridian Bioscience, Cincinnati, OH, USA) for *P. jirovecii*, the GM assay (Platelia *Aspergillus*; Bio-Rad, Marnes la Coquette, France) and the culture-based analysis for *Aspergillus*. For the *Aspergillus* culture, BAL fluid was plated onto Sabouraud glucose (2%) agar (Biolife, Milan-Italy) and incubated for 72 hours; BAL fluid was in parallel plated into Sabouraud liquid broth (bioMerieux, Mercy l'Etoile -France) and incubated until 21 days before subculture onto Sabouraud glucose (2%) agar.

DNA extraction

Fungal DNA was extracted from 2 mL of frozen BAL samples, by means of the MycXtra™ fungal DNA extraction kit (Myconostica/Trinity Biotech), following the manufacturer's instructions. DNA extracts were immediately amplified or stored at -20°C prior to amplification.

Real-time PCR

The presence of *P. jirovecii* or *Aspergillus* spp. DNA in BAL extracts was assessed by MycAssay™ *Pneumocystis* (PJ-PCR assay, Myconostica/Trinity Biotech) or MycAssay™ *Aspergillus* real-time PCR kit (ASP-PCR assay, Myconostica/Trinity Biotech) kits, respectively. In both cases, the platform consisted of a qualitative real time PCR, based on the molecular beacons technology that amplified a specific region of mitochondrial major ribosomal subunit of *P. jirovecii*, or the multicopy 18S rRNA gene of *Aspergillus* spp. Each kit included an internal amplification control (IAC) to detect possible PCR inhibitors in the sample. The amplification process was carried out on the SmartCycler platform (Cepheid, Sunnyvale, California, USA), using the positive cut-off of 39 cycle threshold (Ct) for the PJ-PCR assay, and the positive cut-off of 36 Ct for the ASP-PCR assay, as established by the manufacturer.

Statistical analysis

Mean, standard deviation and range were calculated. Significant differences were evaluated by Fisher test. Diagnostic performance was assessed by receiver operating characteristic curve (ROC) analysis and estimations of the areas under the curve (AUC) and their 95% confidence intervals (95% CI). Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) were calculated as well. The 95% CI for binomial variables was estimated using the Clopper-Pearson exact distribution. To account for the level of agreement to be expected by chance, (Cohen's) kappa was calculated. All statistical analyses were performed using the SPSS software (version 20.0; SPSS, Chicago, IL, USA).

RESULTS

Demographic and clinical characteristics of the patients enrolled in the study

As detailed in Table 1, the population enrolled from different wards was composed of 41 patients, with an age ranging from 19 to 84 years (mean =58±17 years). Males and females were 58% and 42%, respectively. Among all patients, 16/41 (39%) had underlying haematological diseases, while 5/41 (12.2%) were AIDS patients. As detailed in Materials and Methods, the patients were clustered in three groups (Table 1), namely PCP, IA and CTRL groups, consisting of 8, 10 and 24 patients, respectively. Five out of 5 AIDS patients were included in the PCP group. Six out of 16 haematological patients were included in the IA group. All the cases included in the IA group had been diagnosed as probable IFD according to clinical and EORTC/MSG parameters. One patient was diagnosed as having a double infection; therefore, he was included in both the IA and the PCP group. As detailed above, since 3/41 patients enrolled had received two subsequent bronchial lavages a total of 44 BAL specimens was collected and investigated.

PJ-PCR assay: performance, sensitivity and specificity

Table 2 shows the results of the PJ-PCR assay performed in the BAL fluids from the 44 spec-

TABLE 1 - *Demographic and clinical characteristic of enrolled patients*

Characteristic	*PCP patients (n =8)*	*IA patients (n =10)*	*CTRL patients (n = 24)*	*All patients (n = 41)*
Mean age (range)	54 (31-78)	62 (25-81)	61 (19-84)	58 (19-84)
Male sex (%)	5 (62.5%)	4 (40%)	15 (62.5%)	24 (58.5%)
Overall mortality (60 d)	2 (25%)	3 (30%)	7 (29.2%)	12 (29.3%)
Ward				
Haematology	0 (0%)	5 (50%)	9 (37.5%)	14 (34.2%)
Infectious disease	5 (63%)	0 (0%)	0 (0%)	5 (12.2%)
Intensive care unit	1 (13%)	2 (20%)	9 (37.5%)	12 (29.3%)
Nephrology	0 (0%)	0 (0%)	2 (8.3%)	2 (4.8%)
Respiratory disease	1 (13%)	1 (20%)	1 (4.2%)	2 (4.8%)
Oncology	1 (13%)	1 (10%)	2 (8.3%)	4 (9.7%)
Medical ward	0 (0%)	1 (0%)	1 (4.2%)	2 (4.8%)
Underlying disease				
AML	0 (0%)	3 (30%)	5 (20.8%)	8 (19.5%)
ALL	0 (0%)	1 (10%)	2 (8.3%)	3 (7.3%)
Lymphoma	0 (0%)	2 (20%)	1 (4.2%)	3 (7.3%)
AlloSCT	0 (0%)	0 (0%)	1 (4.2%)	1 (2.4 %)
AIDS	5 (63%)	0 (0%)	0 (0%)	5 (12.2%)
COPD	1 (13%)	3 (30%)	2 (8.3%)	5 (12.2%)
SOT	0 (0%)	0 (0%)	6 (25%)	6 (14.6%)
Solid malignancy	1 (13%)	0 (0%)	4 (16.7%)	5 (12.2%)
Other	1 (13%)	1 (10%)	3 (12.5%)	5 (12.2%)

AML = acute myeloid leukemia; ALL = acute lymphoblastic leukemia; AlloSCT = allogeneic stem cell transplant; COPD = chronic obstructive pulmonary disease; SOT = solid organ transplantation. NOTE: the number of patients enrolled was 41; 1 patient was diagnosed as having double infection and thus he was included in the IA and PCP group.

TABLE 2 - *Results of PJ-PCR and ASP-PCR assays.*

Real-time PCR result	*N° of BAL samples from specimens from*			
	PCP (8)	*non-PCP (36)*	*IA (10)*	*non-IA (34)*
Positive	8	2	8	1
Negative	0	34	2	33

imens included in the study. Operationally, the PCP group was analysed with respect to a non-PCP group that consisted of patients from both the IA plus the CTRL group. The real-time PCR tested positive in 8/8 specimens from patients of the PCP group; in particular, the Ct values ranged from 17.9 to 34.5 with a mean of 26.8 cycles (Table 3). The assay tested negative in 34/36 specimens from the non-PCP group (Table 2). Moreover, the Ct values of the two PJ-PCR-positive specimens belonging to the non-PCP group were 37.5 and 37.7; both samples (n. 10 and n. 15 in Table 3) were also IF negative. According to these data, the PJ-PCR assay showed sensitivity, specificity, PPV and NPV (95% CI) of 100% (100-63), 94.4% (99-81), 80.0% (97-44) and 100% (100-90), respectively (Table 4). The corresponding AUC of the ROC curve (95% CI) was 0.97 (0.93-1), as depicted in Figure 1, left panel.

ASP-PCR assay: performance, sensitivity and specificity

Table 2 shows the results of the ASP-PCR assay performed in all 44 specimens. Operationally, the IA group was compared to a non-IA group that included patients from both the PCP plus the CTRL group. The real-time PCR tested positive in 8/10 specimens from patients of the IA group (Table 2) and the Ct values ranged from 25.8 to 35.9 (mean =30.6 cycles; Table 3). The ASP-PCR tested negative in 33/34 specimens from patients of the non-IA group. In particular, the positive result corresponded to a subject from the CTRL group. According to these data, the ASP-PCR assay showed a sensitivity, specificity, PPV and NPV (95% CI) of 80% (98-44), 97.1% (100-85), 88.9% (100-52) and 94.3% (99-81), respectively (Table 4). The corresponding

TABLE 3 - *Results obtained with GM assay, culture, IF and ASP- or PJ-PCR assay in BAL from patients with diagnosis of PCP or IA.*

Patient sample no.	*BAL fluid result*			*Real-time PCR results for the detection of*		*Diagnosis*
	GM OD index	*Culture*	*IF*	*Aspergillus DNA*	*P. jirovecii DNA*	
1	n.a.	*C. albicans*	Positive	Negative	Positive (23.4)	PCP
2	n.a.	Negative	Positive	Negative	Positive (27.9)	PCP
3	0.16	*C. albicans, C. glabrata*	Positive	Negative	Positive (33.6)	PCP
4	0.13	Negative	Positive	Negative	Positive (17.9)	PCP
5	0.02	Negative	Negative	Negative	Positive (31.2)	PCP
6	0.55	*C. albicans*	Positive	Negative	Positive (21.9)	PCP
7	n.a.	Negative	Positive	Negative	Positive (28.6)	PCP
8	1.35	*A. fumigatus*	Positive	Positive (31.0)	Positive (34.5)	IA & PCP
9	0.94	Negative	Negative	Negative	Negative	IA
10	10	*C. glabrata, A. fumigatus*	Negative	Positive (31.5)	Positive (37.5)	IA
11	6.62	*A. flavus, C. albicans*	Negative	Positive (25.8)	Negative	IA
12	6.19	*A. fumigatus*	n.a.	Positive (30.2)	Negative	IA
13	5.87	*A. flavus, C. albicans*	Negative	Positive (29.8)	Negative	IA
14	6.49	Negative	Negative	Positive (34.1)	Negative	IA
15	11.94	Negative	Negative	Positive (35.9)	Positive (37.7)	IA
16	0.53	*A terreus*	Negative	Positive (26.6)	Negative	IA
17	5.22	Negative	Negative	Negative	Negative	IA

n.a.= not assessed.

TABLE 4 - *Performance of PJ-PCR, ASP-PCR and GM assays.*

	Test result (95% CI) by:		
	PJ-PCR	*ASP-PCR*	*GM**
Sensitivity %	100 (100-63)	80.0 (98-44)	100 (100-72)
Specificity %	94.4 (99-81)	97.1 (100-85)	92.3 (98-76)
PPV %	80.0 (97-44)	88.9 (100-52)	87.0 (97-51)
NPV %	100 (100-90)	94.3 (99-81)	100 (100-90)

*For the GM assay, the test was performed in 37 out of 43 BAL specimens.

AUC of the ROC curve (95% CI) was 0.89 (IC 0.76-1), as depicted in Figure 1, right panel.

Comparison among assays: performance, sensitivity and specificity

As detailed in Table 3, BAL culture-positive samples were obtained in 6/10 specimens from the IA group; among the 4 culture-negative specimens, 2 were positive by the ASP-PCR assay (n. 14 and n. 15 in Table 3), with Ct values well above the mean Ct observed in the culture-positive samples. GM assay was performed in 37 out of the 44 specimens investigated, considering a cut-off of 0.5 as indicated by the manufacturer. Ten out of 10 specimens from the IA group returned a GM-positive result; a GM-positive result was also observed in a patient (n. 6) of the PCP group, that was found to be *C. albicans*-positive in BAL culture (Table 3). Furthermore, 3 GM-positive results were observed in the CTRL group (data not shown). According to these data, the GM assay returned a sensitivity, specificity, PPV and NPV (95% CI) of 100% (100-72), 92.3% (98-76), 87% (97-51) and 100% (100-90), respectively (Table 4). Finally, the performance of the ASP-PCR assay showed enhanced (though not statistically significant) specificity and PPV, while the sensitivity and NPV were lower than those of the GM assay. The overall level of agreement between the two assays was good (kappa = 0.63, 95% CI = 0.394- 0.871).

DISCUSSION

New diagnostic markers and techniques aimed at identifying patients at risk and/or with IFD continue to evolve. Recently, commercial platforms based on real-time PCR techniques have become available. Such a bio-molecular approach offers several advantages including high sensitivity, early and rapid results. In ad-

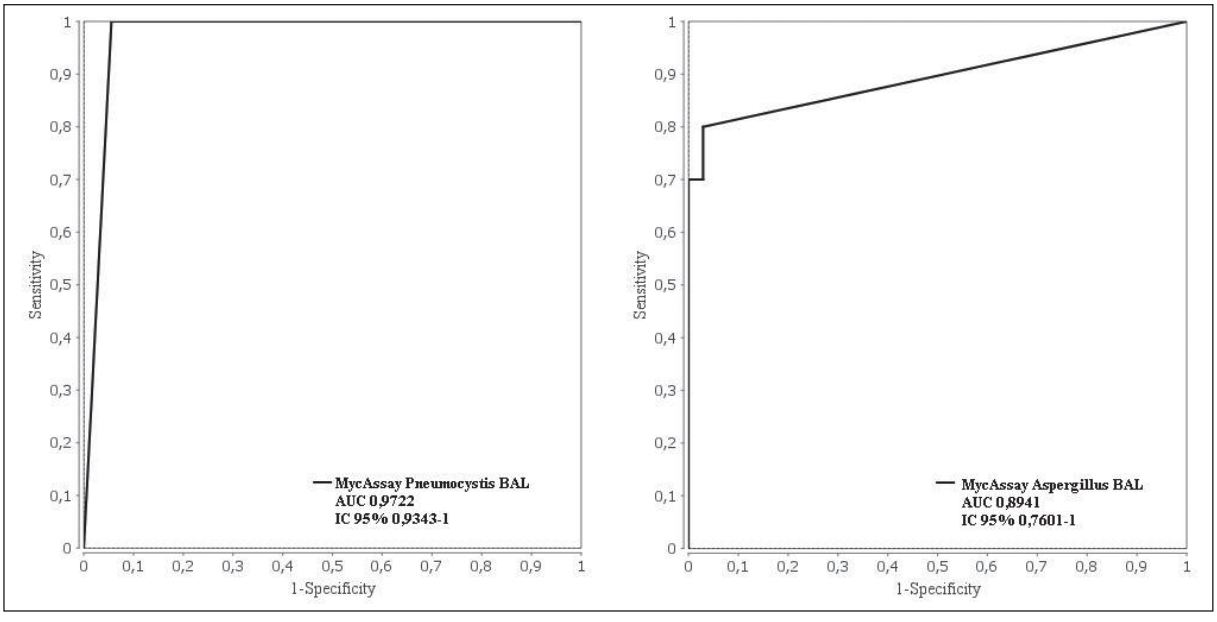

FIGURE 1 - *Receiver operating characteristic curve of PJ-PCR (left) and ASP-PCR assay (right).*

dition, identification at the species level and detection of drug-resistance-associated genes may be obtained. Nevertheless, because of lack of standardization, such PCR techniques have not been included so far among the reference tests in routine diagnosis of IFD. The present single-centre study evaluated the performance of two commercial real time PCR assays, the MycAssay™ *Pneumocystis* and the MycAssay™ *Aspergillus* (Myconostica/Trinity Biotech), in a defined clinical setting of patients at risk of IFD. Concerning the results obtained with the PJ-PCR assay in terms of *P. jirovecii* DNA detection in BAL fluids, very high values of sensitivity and specificity were observed (100% and 94.4%, respectively). These results closely recall those previously described in studies using this same commercial platform (Hauser *et al.,* 2011; McTaggart *et al.,* 2012; Orsi *et al.,* 2012) and are also in line with the range of values for PCR-based platforms described in a meta-analysis (Lu *et al.,* 2011).

Several studies highlight the possibility of identifying by PCR also individuals colonized by *P. jirovecii* and/or are infected at a low burden. Accordingly, our previous study (Orsi *et al.,* 2012) and Hauser's work (2011) reported several unexpected positive results in non-HIV patients. Similarly, our present study found two PCR-positive BAL specimens from non-HIV haematological patients with severe neutropenia and pneumonia. According to routine analysis, those specimens resulted IF-negative. Interestingly, the PCR returned Ct values much higher than the mean Ct observed in BAL from the 8 patients included in the PCP group (37.5 and 37.7 versus 26.8, respectively). In our opinion, these findings together with the clinical outcome (no anti-*P. jirovecii* therapy, no manifested IFD by time), argue for a *P. jirovecii* colonization in the two non-HIV patients. Thus, although not distinguishing between *P. jirovecii* colonization and low burden infection, the PCR-based approach offers additional information that together with a thorough evaluation of clinical, radiological and microbiological findings may facilitate the clinical management of high-risk patients. In this respect, a recent study claims that early diagnosis and treatment are crucial for the survival of PCP patients in cohorts of non-HIV individuals (Asai *et al.,* 2012). Furthermore, the present study observed a BAL sample positive for PCR (Ct 31.2) but IF-negative. This specimen was from a sarcoidosis patient admitted to the Intensive Care Unit with acute respiratory insufficiency unresponsive to broad spectrum antibiotics and high doses of steroids. The radiological picture (despite the co-presence of sarcoidosis) showing bilateral interstitial infiltration was compatible with a PCP. The patient was then successfully treated with trimetropin-sulfometossazole. In our opinion, the negative result obtained with the IF may be ascribed to the well-known low sensitivity of the method. Overall, the present findings argue for the usefulness of PCR-based monitoring in high risk patients. Numerous studies (Alanio *et al.,* 2011; Botterel *et al.,* 2012; Damiani *et al.,* 2013; Maillet *et al.,* 2014; Matsumura *et al.,* 2012) propose in-house quantitative real-time PCR to discriminate between P. *jirovecii* colonization and infection. Such encouraging data, together with the near availability of commercial kits for quantitative real-time PCR will open the way to widescale studies that, in turn, will monitor the efficacy of prophylactic treatment particularly in non-HIV immunocompromised patients.

When we assessed the ASP-PCR assay for the detection of *Aspergillus* spp. DNA in BAL fluid, we found a sensitivity of 80% and a specificity of 97.1%. These results are in accordance with the data reported in the meta-analysis by Avni *et al.* (2012) and with previous studies using the same commercial test (Guinea *et al.,* 2013; Orsi *et al.,* 2012; Torelli *et al.,* 2011). The ASP-PCR was positive in 8/10 BAL specimens from the IA group, with the two ASP-PCR negative specimens also being culture-negative. Furthermore, other two culture-negative BAL specimens returned ASP-PCR-positive results, yet, with very high Ct values, namely 34.1 and 35 (mean Ct value in ASP-PCR-positive specimens =29.15). Despite the limited number of samples assessed, these findings are in line with a previous observation by Torelli and co-workers (2011) who reported a statistically significant difference in Ct average values between BAL culture-positive and BAL culture-negative specimens. Moreover, in the present study, the ASP-PCR assay shows the expected higher sensitivity when compared

to BAL culture, the current reference test. Despite the major drawback of its low sensitivity (Horvath and Dummer, 1996), culture cannot be set aside, since it is the only system allowing antifungal susceptibility testing.

GM assessment in BAL fluids is a very important tool for the diagnosis of IA. In particular, the GM assay has been reported to have a positive impact on the early initiation of appropriate antifungal therapy, and may therefore influence patients' survival (D'Haese *et al.,* 2012; Hoenigl *et al.,* 2014). To date, recommendations for optimal GM cut-off in BAL specimens vary widely (Heng *et al.,* 2013), indicating that no consensus has been achieved yet. In the present study, we adopted the 0.5 cut-off in line with the manufacturer's instructions. Nevertheless, no substantial differences in performance were observed when enhancing this parameter to 1 or 1.5 (data not shown). Another well-described limitation of the GM assay is the occurrence of false positive results in patients with beta-lactam antibacterial therapies, various underlying diseases or specific host factors (e.g. renal failure) (Kedzierska *et al.,* 2007). Also in our hands, 3/24 CTRL specimens were GM-positive while being ASP-PCR-negative. In accordance with the limits of the GM assay, and based on clinical features, these cases have been interpreted as non-IFD. Overall, the MycAssay *Aspergillus* appears to be a good molecular test for the diagnosis of IA, with a performance comparable to that of the GM assay. In agreement with recent studies (Heng *et al.,* 2013; Hoenigl *et al.,* 2014), we support the idea that the simultaneous use of both GM and PCR assays in BAL samples can improve the diagnostic accuracy in patients with suspect IA.

In conclusion, the two commercial real-time PCR assays tested in the present study have a sensitivity equal to or better than the current laboratory standard techniques (IF or culture) and a specificity as high as that of the GM assay. For these reasons, the PJ-PCR is a helpful tool for the diagnosis of PCP, particularly in non-HIV-infected immunocompromised patients, while the ASP-PCR assay is valuable especially in IA high-risk patients who often present a low fungal burden and therefore may not be correctly and promptly diagnosed by conventional assays.

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Conflict of interest

The authors declare that they have no conflict of interest.

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