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FULL PAPER

Evaluation of Real Time PCR *Aspergillus* spp. in bronchoalveolar lavage samples

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SUMMARY

The present study investigated the improvement in the diagnosis of invasive pulmonary aspergillosis (IPA) adding a molecular test on bronchoalveolar lavage (BAL) to the routine diagnostic approach including microscopy, culture and galactomannan (GM) immunoassay. A total of 133 BAL samples were retrospectively tested for the *Aspergillus* DNA: 112 samples were from immunocompromised patients at risk of invasive fungal infection and 21 were from patients not at risk and without clinical evidence of IPA. The latter samples were used to identify the cut-off of positivity for the molecular test.

Applying the cut-off quantity of 50 copies/reaction, the PCR test had 90% sensitivity and 97% specificity and resulted the most sensitive, specific and accurate among those evaluated. The statistical analysis showed that the probability that a patient is not affected by IPA is 99% when the three tests (PCR, GM and culture) are concordantly negative.

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INTRODUCTION

Rapid advances in transplant medicine and cancer treatment have led to a prolonged survival and to an increase in the number of immunocompromised patients (Chen *et al.,* 2013). This trend has led an increased incidence of opportunistic infections, including invasive mycoses, in transplant and cancer units and in medical and surgical wards (Pappas *et al.,* 2010). Despite the availability of new antifungal drugs, opportunistic fungal infections have high morbidity and mortality rates (Schelenz *et al.,* 2015). Conventional methods, such as direct microscopy and culture, followed by identification of the pathogen, are still considered the gold standards despite the fact that they have a low sensitivity and are time-consuming to perform (Arvanitis *et al.,* 2014). More recently non-cultural tests such as Galactomannan (GM) and 1-B-D glucan in serum and other biological fluids were recognised as important diagnostic tools and are now implemented in standard practice (De Pauw *et al.,* 2008). Non-cultural tests demonstrated 50%-95% sensitivity and 40%-96% specificity depending on the type of patient, biological material used for testing, and fungal species (Hachem *et al.,* 2009). In addition, the GM test can be influenced by interfering agents such as β-lactamic antibiotics, plasmatic infusion solutions or other cross-reactive species of fungi (Barton *et al.,* 2013).

GM assay in serum is reported to have little value in non-neutropenic patients, while it shows high sensitivity if tested on bronchoalveolar lavage (BAL) samples from these individuals, especially in solid organ transplanted subjects and in critical patients in the intensive care unit (Luong *et al.,* 2011; Avni *et al.,* 2012).

Molecular methods have not yet been recognised as diagnostic criteria for the identification of invasive fungal infection (IFI) by EORTC/MSG (European Organization for Research and Treatment of Cancer/Invasive Fungal Infection Cooperative Group and the National Institute of Allergy and Infection Diseases Mycoses Study Group) because of the lack of standardisation of the extraction and amplification techniques (De Pauw *et al.,* 2008). However, several recent studies have evaluated their application (White *et al.,* 2010, Arvanitis *et al.,* 2014; Ibanez-Martinez *et al.,* 2017).

The present study investigated the improvement in the diagnosis of invasive pulmonary aspergillosis (IPA) adding the molecular test on BAL to the routine diagnostic approach including microscopy, culture and GM immunoassay.

Key words:
Aspergillus spp., RT-PCR, Bronchoalveolar lavage.

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MATERIAL AND METHODS

Study design

The study, approved by the Ethical Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan (Italy), is a retrospective analysis of BAL samples by

Aspergillus spp. RT-PCR test. The BAL samples, collected from patients undergoing bronchoscopy for monitoring or diagnosis in the period 2013-2015 were stored at -20°C.
Testing was performed as an anonymous case-control study to evaluate the performance of the *Aspergillus* PCR assay in comparison to the routine methods (GM and culture). Clinical information was collected during the routine diagnostic process and used to solve the discrepancies between methods. IPA was classified as probable or possible according to the EORTC-MSG criteria (De Pauw *et al.,* 2008).
A total of 133 BAL samples were retrospectively tested for the *Aspergillus* DNA: 112 samples from immunocompromised patients at risk of IFI and 21 from patients not at risk, without clinical evidence of IFI and with negative culture and GM, were used to identify the cut-off of positivity for the molecular method. A mean quantity of 6.26±10.89 copies/reaction was detected and the value of 50 copies/reaction (equal to the mean value plus 3 standard deviations) was arbitrarily chosen as the cut-off value to identify the positive sample. Microscopic examination was performed after Gram or Giemsa staining.
A total of 100 and 300 µL of the BAL samples were seeded on Sabouraud Dextrose Agar plates incubated at 32°C for 20 days, and on chocolate agar and Columbia 5% sheep blood agar incubated at 37°C for five days. The isolated filamentous fungi were identified according to macro and microscopic morphological features.
GM test was performed using Platelia *Aspergillus* EIA-Ag (Bio-Rad Marnes la Coquette- France) according to the manufacturer's instructions and samples were considered positive when the optical density index was >0.5 in two determinations.
PCR test was performed on DNA extracted by EZ1 semi-automatic extraction platform using the kit EZ1 DPS Virus (QIAGEN). In the first step, 200 µl of the BAL samples were combined with an equal volume of the lysis buffer ATL (QIAGEN) solution and 10 µl of proteinase K was added to the mixture then incubated for 30 min at 55°C. PCR reaction was performed according to the manufacturer's instructions using *Aspergillus* spp. ELITe MGB Kit (ELITech Group S.p.A.) targeting the rDNA18S region and the 7500 fast Dx real Time Instrument amplification platform (Thermo Fischer Scientific). Each sample was tested only once.
Swabs of the surfaces were tested to evaluate the background signal of the environmental contamination in the working area (12±15.74 copies/reaction) and to verify if signals were under the established cut-off. During all analytical process, we included a negative and a positive control to verify environmental contamination and efficiency of amplification.

Statistical analysis

The results of microscopy were excluded from the analysis because of the high number of negative results. The agreement among PCR, GM, and culture methods was evaluated using Cohen's kappa (k) coefficient (Armitage *et al.,* 2002). The diagnostic performance of each method was evaluated by calculating sensitivity, specificity, positive (PPV) and negative (NPV) predictive values, and the area under the curve (AUC) of the ROC (receiver operating characteristic) curve (Armitage *et al.,* 2002). In addition, the three tests were jointly analysed using multiple logistic regression models to calculate the overall AUC. Statistical analysis was performed with Stata14 (StataCorp., 2015).

RESULTS

BAL samples from 112 immunocompromised patients at risk of IPA (mean age 51.3 years, range 7-83) were examined. Among these, 80 subjects were waiting for a solid organ transplantation (73 lung, four liver, three kidney), 6 patients had hematological malignancies, 7 were hospitalized in the intensive care unit, and 19 in medical wards for pulmonary complications following cancer or autoimmune disorders. Two patients were lost to follow-up and of the remaining 110, 21 were defined as probable/possible IPA according to the EORTC-MSG criteria. (De *Pauw et al.,* 2008).
Results of PCR, GM and culture of BAL are reported in *Table 1*. A total of 64 BAL from patients not affected by IPA were negative for all three tests (PCR, GM and culture). All eight patients positive for all the tests had a diagnosis of probable/possible IPA. The other 13 patients with IPA were positive for one or two tests. Two of them were positive for molecular test only. Five patients (two patients with only positive culture, one with positive PCR only and two with both culture and PCR positive) were not affected by IPA, although they were colonized by *Aspergillus spp.* In colonized patients or in case of contamination, the quantity values were around cut-off value or greatly elevated. On the contrary, in case of probable IPA the quantity values ranged from 59 to 8000 copies/reaction.

Table 1 - *Results of PCR, GM and culture of BAL from 110 patients.*

PCR	*GM*	*Culture*	*Patients with probable IPA*	*Patients without IPA*	*Total number of patients*
NEG	NEG	NEG	0	64	64
NEG	NEG	*POS*	0	2	2
NEG	*POS*	NEG	1	20	21
POS	NEG	NEG	2	1	3
NEG	*POS*	*POS*	1	0	1
POS	NEG	POS	2	2	4
POS	*POS*	NEG	7	0	7
POS	*POS*	*POS*	8	0	8
			21	89	110

IPA, invasive pulmonary aspergillosis.

Table 2 - *Performance of BAL PCR, GM and culture assays in the diagnosis of IPA. Specificity, sensitivity, positive (PPV), negative (NPV) predictive values and area under the curve (AUC) of the three tests. Absolute numbers in parentheses.*

	Performance, %, 95% CI (n° of samples)				
	Sensitivity	*Specificity*	*PPV*	*NPV*	*AUC (%)*
PCR	90, 70-99 (19/21)	97, 90-99 (86/89)	86, 65-97 (19/22)	98, 92-100 (86/88)	94, 87-100
GM Cut-off >0.5	81, 65-97 (17/21)	78, 67-86 (69/89)	46, 29-63 (17/37)	95, 87-98 (69/73)	79, 70-89
GM Cut-off >0.9	81, 65-97 (17/21)	85, 76-92 (76/89)	57, 37-75 (17/30)	95, 88-99 (76/79)	83, 74-92
Culture	52, 30-74 (11/21)	96, 89-99 (85/89)	73, 45-92 (11/15)	89, 81-95 (85/95)	74, 63-85

CI: confidence interval.
PPV: positive predictive value.
NPV: negative predictive value.
AUC: area under the curve of the ROC (receiver operating characteristic) curve.

The overall agreement between PCR and culture was moderate (k=0.58, 95% CI: 0.38-0.78), between culture and GM was slight (k=0.19, 95% CI: 0.01-0.36), and between GM and PCR fair (k=0.34, 95% CI: 0.16-0.53). Performance of PCR, GM and culture assays is reported in *Table 2*.
PCR showed the best performance with sensitivity and specificity of 90 and 97% respectively. GM test had both sensitivity and specificity around 80%, and an elevated NVP (95%) and a low positive predictive value (PPV) (46%) when a cut-off >0.5 was considered. GM specificity increased to 84% considering a cut-off >0.9 with no change in sensitivity or NPV. Culture had a high specificity (96%) but very low sensitivity (52%) and a NPV of 89%.
The best discriminant test was PCR (AUC=94%), followed by GM (*Table 2* and *Figure 1*). The combination of two tests, GM plus culture calculating the AUC-ROC showed a diagnostic performance of 88% (IC 79-96%) and PCR plus culture of 95% (IC 90-100%), while using all three tests the AUC increased to 99% (*Figure 1*).

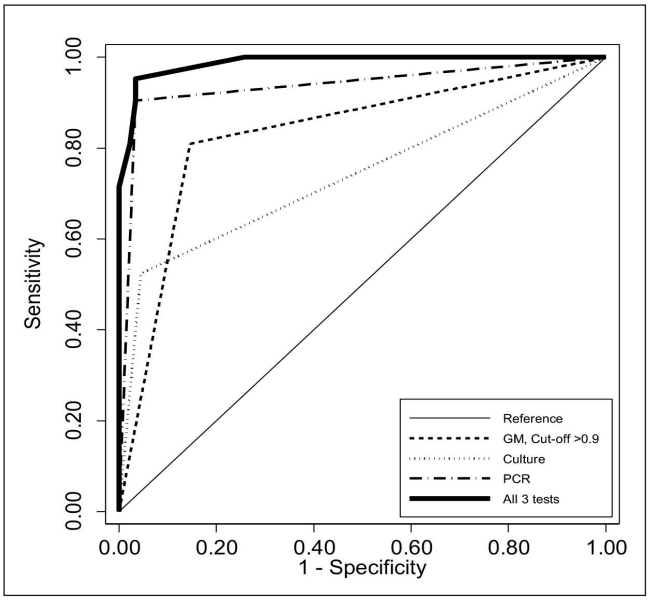

Figure 1 - *ROC (receiver operating characteristic) curves of three tests, considered positive/negative.*

DISCUSSION

This study investigated the value of the detection of *Aspergillus* DNA in BAL for the diagnosis of IPA in immunocompromised patients.
The literature reports a wide range of sensitivity, from 36% to 100% (Khot *et al.,* 2009; Tuon *et al.,* 2007; Luong *et al.,* 2011), of BAL PCR in the diagnosis of IPA. Applying the cut-off quantity of 50 copies/reaction, the PCR test in our experience had 90% sensitivity. False negative results (4%) were probably due to the analysis of BAL sampled from an area far from the lesion (Taremi *et al.,* 2015) or to the poor quality of samples.
False positivity is a recognised limitation of the PCR assay, 8%-12% of false positivity is reported in the literature (Khot *et al.,* 2009; Tuon *et al.,* 2007; Luong *et al.,* 2011; Loeffler *et al.,* 1999), linked to the procedure (e.g. contamination from the environment) or to clinical factors (e.g. colonisation).
In our experience, the false positive rate was only 2% and it might be the consequence of the control of the environmental contamination of working area and the subsequent decontamination, and the use of negative controls in each run (Loeffler *et al.,* 1999).
Our data showed a GM sensitivity of 81% and specificity of 78% when a cut-off >0.5 was considered. Specificity increased to 84% considering a cut-off >0.9. As is well known (Levy *et al.,* 1992), the sensitivity of culture was low, only 52%.
A moderate agreement between PCR and culture was observed. As reported in the literature, the fair agreement between GM and PCR (k=0.34) could be attributed to beta-lactamics, other fungi, and patients' altered intestinal permeability (Luong *et al.,* 2011; Maertens *et al.,* 2009; Beirao *et al.,* 2013). The NPV of culture, GM and PCR were 89%, 95% and 98%, respectively. Statistical analysis using multiple logistic regression models shows that the probability a patient is not affected by IPA is 99% when the three tests (PCR, GM and culture) are concordantly negative. The molecular test has the advantage of also detecting the nucleic acid release from cells damaged by antifungal treatment, while it seems not to distinguish between colonisation and invasive infection. We observed that in colonised patients or in case of contamination, the quantity values could be around the cut-off value or great-

ly elevated. On the contrary, in case of probable IPA the quantity values ranged from 59 to 8000 copies/reaction. Therefore, RT-PCR can be more helpful than conventional PCR because it provides quantitative information on the fungal burden that can be used to distinguish between infection and simple colonisation (Sanguinetti *et al.,* 2003). In conclusion, the molecular method was the most sensitive, specific and accurate among those evaluated. Despite its limited sensitivity, culture remains necessary for the interpretation of the exact aetiology and for *in vitro* antifungal susceptibility testing. The combined use of PCR and GM tests increases the probability of excluding IPA reducing the duration of empirical treatment and facilitating a rapid diagnosis supporting a pre-emptive therapy.

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