Improving the diagnosis of invasive aspergillosis by the detection of Aspergillus in broncho-alveolar lavage fluid Comparison of non-culture-based assays

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Improving the diagnosis of invasive aspergillosis by the detection of *Aspergillus* in broncho-alveolar lavage fluid: comparison of non-culture-based assays

Running title : PCR *Aspergillus* in BAL

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ABSTRACT

Objectives: this study aimed to evaluate new tools to diagnose invasive aspergillosis (IA) directly from broncho-alveolar lavage (BAL) samples.

Methods: all consecutive patients with suspected IA who underwent bronchoscopy with BAL were prospectively included. Mycological culture and ELISA detection of galactomannan (GM) were performed on BAL. Two in-house and two marketed PCR assays were used on BAL DNA extracts to detect *Aspergillus* species. Susceptibility testing was performed after culture; marketed PCR assays detected mutations in the *CYP51A* gene associated to resistance.

Results: within 3 years, 1 555 BAL samples were processed, including 413 samples from 387 immunosuppressed patients. IA diagnosis was no-IA, possible, probable or proven IA in 326, 23, 37 and 1 patients, respectively. PCR assays sensitivity for *Aspergillus* detection ranged from 61% to 74%, below GM (87%), but contrasting with 47% for cultures. Combining PCR to EORTC/MSG criteria increased the sensitivity to 100%. Interestingly, tests performance in non-hematological patients ranged from 60% to 75%, and were higher than in hematological patients, and those with prior exposure to antifungals. All 16 isolates of *A. fumigatus* were susceptible; PCR did not detect any resistance marker in the 37 *A. fumigatus* PCR-positive samples.

Conclusion: the molecular detection of *Aspergillus* directly in BAL samples greatly improved the diagnosis of IA, particularly in non-hematological patients.

Keywords : aspergillosis, PCR, BAL, azole resistance

INTRODUCTION

Immunocompromised patients are increasingly frequent, either because of hematological malignancies, or of non-hematological conditions such as solid-organ transplantation, chronic pulmonary diseases, long-term corticosteroid therapy, and stay in intensive care unit (ICU). Despite major progresses in their medical management, invasive aspergillosis (IA) remains a major cause of morbidity and mortality (1). The timely and accurate diagnosis of active *Aspergillus* infection is eagerly required to initiate on time an adequate targeted antifungal therapy (2). However, IA definite diagnosis is still challenging, as clinical and radiological manifestations are most often unspecific. The European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) recommended using microbiological criteria (3,4), such as the histological evidence of tissue infiltration, although rarely obtained, and mycological culture, although positive in only 50% of patients (5,6). This shortcoming has fuelled an interest in non-culture-based diagnostic techniques applied directly in clinical samples, particularly for non-hematological patients, such as antigen detection (galactomannan (GM), beta-1,3-D glucan) by enzyme-linked immuno-sorbent assay (ELISA), or PCR-based assays targeting the identification of *Aspergillus* spp. or of genes coding for triazole resistance. Numerous PCR-based assays able to detect *Aspergillus spp*. DNA with a high performance have recently been developed (7–10). Many centers include now PCR-based algorithms assays in their clinical management. However, data on the relevance of such molecular detection assays in bronchoalveolar (BAL) samples are still poor, preventing their inclusion in disease-defining criteria. Reported sensitivities of PCR assays in BAL have been highly variable, ranging from 36 to 95% (11–14), mainly because of differences in assay characteristics, population evaluated (hematological versus non-hematological patients), and doubts in IA diagnosis.

In addition, the treatment efficacy might be hampered by resistance to antifungals, as it is spreading in Europe (15), as in South-America and Asia (16,17). In France, the incidence of resistant *A. fumigatus* isolates has been reported to be still low in the immunocompromised population (about 1% (18)), whereas in chronically colonized patients the incidence of azole resistant strains can reach 8-12% as observed in our center and others (19). Identification of azole resistance in *Aspergillus fumigatus* is conventionally based on minimal inhibitory concentration (MIC) determination, which necessitates to recover the strain in culture. Triazole-resistant isolates can also be detected by the identification of mutations in the *cyp51A* gene.(20) Several *cyp51A*-based PCR assays have been developed for detecting triazole resistance directly from clinical specimens. Most assays are nested in-house PCR assays.(12,21,22) Simultaneous detection of IA and identification of resistance mutations sparks an increasing interest in clinical settings for an earlier adaptation of antifungal therapy; of note *A. fumigatus* resistant strains have been related to a higher mortality rate, due to a delayed initiation of adequate treatment (23)

. In this context, commercial kits targeting both *A. fumigatus* and *cyp51A* genes have been recently marketed, but their evaluation on clinical samples IA population is still scarce (24,25).

Our study aimed to assess the contribution of two in-house *Aspergillus* real-time PCR assays and of two commercial resistance multiplex assays (AsperGenius®, PathoNostics, Maastricht, the Netherlands; and MycoGenie®, AdemTech, Pessac, France) for IA diagnosis in BAL fluid samples. Additionally, triazole resistance screening by PCR on clinical samples was compared to phenotypic and genotypic characteristics of *A. fumigatus* isolates recovered with cultures.

MATERIALS AND METHODS

Patients and study design

For this prospective study, all consecutive patients with suspected IA who underwent standard bronchoscopy with BAL at Rennes University Hospital (France) were included. Redundant samples for a unique clinical episode were excluded. Patients were classified as having proven, probable, possible IA, or no IA, according to the revised EORTC/MSG case definitions (2).

BAL fluid samples processing

BAL fluid specimens were divided into 3 aliquots to perform culture, GM detection assay and PCR assays, respectively. For culture, 5-10 mL were centrifuged and the resuspended pellet (about 200µL) was directly inoculated on 2 conventional fungal media incubated at 30°C and 37°C for 8 days. Mold isolates from cultures were identified by macroscopic and microscopic examinations. For PCR assays, 1 mL of plain BAL was centrifuged and DNA was extracted from the pellet, using the QIAamp® DNA minikit (Qiagen) according to manufacturer's recommendations. For ELISA, the aliquot was 390 µL, according to manufacturer recommendations.

Susceptibility testing

Itraconazole (ITC) and voriconazole (VRC) susceptibility of *A. fumigatus* strains isolated from IA samples was determined using Etest® strips according to the manufacturer's instructions (bioMérieux, France), after 48h of incubation at 30°C. Azole resistance was assessed for MIC >2mg/mL for ITC and VRC according to EUCAST breakpoints v.8.0.

In-house *Aspergillus* PCR assays

BAL DNA extracts were tested using two real-time "in-house" PCR assays. When discrepant results with other tests (culture or GM) were observed, DNA was retested with both PCR assays.

The first PCR assay ("Af-mito") amplified a 196-bp sequence of *A. fumigatus* mitochondrial gene, as previously described (26). The second PCR assay ("28S") targeted a 67-bp sequence of the *A. fumigatus* 28S rRNA gene, using primers and probe as previously published (27). Each amplification was performed in a 25µL final volume containing 1X TaqMan® Universal PCR MasterMix, 0.5µM of each primer, 0.2 µM of probe and 5µL of DNA sample. Amplification used the following thermal conditions : 2 min at 50°C, 10 min at 95°C and 45 cycles of 15 sec at 95°C and 1 min at 60°C, on a StepOne Plus® instrument (Applied Biosystems).

Commercial *Aspergillus* PCR assays

BAL fluid specimens from IA cases were tested by two commercial multiplex real-time PCR assays, that simultaneously detect *Aspergillus* spp. and the most relevant mutations in the *cyp51A* gene.

MycoGenie® (AdemTech, Pessac, France) is a quadruplex real-time PCR assay which targets *A. fumigatus* (*28SrRNA* gene), TR34, L98H, and an internal control.

AsperGenius® assay (PathoNostics, Maastricht, the Netherlands) is composed of 2 different real-time quadriplex amplification mixtures, targeting *Aspergillus* species and resistance mutations. The species multiplex assay targets the 28S rRNA multicopy gene, allowing the specific detection of *A. fumigatus* complex (Af), *Aspergillus terreus* and *Aspergillus* spp. (Asp sp). The resistance multiplex assay targets the single-copy *Cyp51A* gene of *A. fumigatus*, and can detect the TR34, L98H, Y121F and T289A regions. The distinction between wild-type and mutant *A. fumigatus* strains was performed by melting curve analysis.

Amplification was conducted for 45 cycles on a LightCycler® 480 instrument (Roche), according to manufacturer's instructions. The horizontal threshold was fixed above the background noise and a positive result was defined by a signal detection with a Ct value <45 cycles.

Galactomannan (GM) Platelia *Aspergillus* assay

The sandwich ELISA for BAL GM detection (Platelia *Aspergillus*®; Bio-Rad, Marnes-la-Coquette, France) was performed according to the manufacturer's recommendations, on a 390µL aliquot of BAL sample. For the classification of patients, the BAL was considered as positive when optical density index (ODI) was≥ 0.8, if concomitant serum GM was positive (ODI ≥0.5), and ≥1.0 when serum GM was negative or unavailable.

***Beta-tubulin* sequencing**

Identification of *Aspergillus* species other than *fumigatus* was confirmed by *beta-tubulin* gene typing, using primers *bt2a* and *bt2b* (28). Amplification reaction and subsequent sequencing were performed as previously described (29). Bidirectional sequences were analyzed using Seqscape® software and submitted to GenBank public database by using the BLAST Search program for species identification.

***ITS1-ITS2* sequencing**

Identification of *Scedosporium* from BAL sample was confirmed by *ITS1-ITS2* typing, using the primers *ITS1* and *ITS4*. The protocol was similar as described above (28,29).

Statistical methods.

Data analysis was performed using GraphPad PRISM® v. 5.02 software. Categorial variables were compared using the χ^2 test or Fisher's exact test. A p-value of 0.05 was considered to be statistically significant.

RESULTS

Patient characteristics

As depicted in Figure 1, within 3 years (from January 2012 to December 2014), a total of 1,555 BAL, fluid samples (1,336 patients) were processed, including 413 samples collected from immunocompromised patients with clinical symptoms or radiological signs compatible with invasive fungal infection. Non-redundant BAL fluid specimens of 387 patients at high risk for IA were analyzed in this study.

According to the EORTC-MSG criteria, 61 patients were diagnosed with IA (1 proven, 37 probable, 23 possible cases), whereas the diagnosis of IA was excluded for 326 patients. The distribution of the patients according to their infection status and underlying conditions is presented in Table 1. Twenty-seven out of 61 patients (44%) were hospitalized in intensive care unit and 22 (36%) were in hematology unit.

Diagnostic performance of Aspergillus fumigatus PCR assays

The sensitivity of the 28S PCR assay was higher than that of Af-mito PCR (71.1% versus 60.5%), whereas its specificity for IA was 92.8% (324/349), i.e., lower than that of Af-mito PCR (95.4%, 333/349)(Table 2). It was interesting to note that in the no-IA group, 10 out of the 16 28S PCR positive BAL were from patients with chronic pulmonary disease, as aspergilloma or CNPA in 4 cases. These positive assays are consistent with *Aspergillus* colonization and could not reasonably be considered as false positive results. The combination of both PCRs led to a slight increase of sensitivity (73.7%) with similar specificity (92.3%). By contrast, mycological cultures allowed to diagnose only 18/38 proven or probable IA (47.4%).

In BAL from patients with IA, commercial multiplex assays showed roughly similar results than in-house PCRs. MycoGenie® had the highest sensitivity (73.7%). The AsperGenius® assay had a sensitivity of 60.5% and 68.4% for *A. fumigatus* and *Aspergillus* spp. detection, respectively.

Interestingly, combinations of any PCR assay to current EORTC/MSG criteria (GM detection and culture in BAL) led to an increase of sensitivity (from 92.1% to 97.4 or 100% depending the PCR assay), without significant decrease of specificity (Table 2).

Of the twenty-two samples grown positive for *A. fumigatus*, Af-mito PCR was positive in 19/22 (86%) and 28S PCR in 20/22 (91%) (Table 3). When considering only IA cases, Af-mito and 28S PCRs were positive in 15/16 BAL with positive culture.

Of four BAL from which an *Aspergillus* species other than *A. fumigatus* was isolated, two yielded a positive result with the 28S PCR assay. The cultures were identified as *A. flavus* and *A. ochraceus*, respectively, by microscopic examination and *beta-tubulin* gene sequencing. The sample cultured positive with *A. flavus* also yielded a positive result with the Mycogenie® assay, which targets also the *28S* gene. By contrast, the AsperGenius® assay was negative for *A. fumigatus*, but correctly positive for *Aspergillus* spp. in one case. All PCR assays were positive for a sample grown with *S. apiospermum* (identification confirmed by *ITS1-ITS2* typing from the clinical specimen), suggesting that none of them is 100% specific for *A. fumigatus*.

Resistance screening using commercial PCR assays

A. fumigatus susceptibility to azole was investigated in the 61 samples from IA patients. Mycogenie® PCR assay did not detect any TR34 or L98H mutation in the 37 positive samples for *A. fumigatus* target.

No resistance marker was detected with Aspergenius® assay either. However, we noticed that TR34, L98H, T289A and Y121F regions were successfully amplified in only 16 of the 29 samples with positive *A. fumigatus* detection, regardless of wild-type or mutated allele (Table 4). The mean Ct values for *A. fumigatus* detection in samples for which *cyp51A* amplification failed tended to be higher than that with successful *cyp51A* detection (34.1±4.6 versus 35.8±1.9, p=0.203).

Finally, *in vitro* susceptibility testing could be performed on 11 of the 16 *A. fumigatus* isolates. Only susceptible phenotypes were observed, with ITC and VRC MIC ranging from 0.5 to 1.5 mg/l and 0.032 to 0.5 mg/l, respectively. Susceptibility of *non fumigatus* species cultured from IA BAL samples was also evaluated, yielding to low MIC for *A. flavus* (ITC: 0.5 mg/L, and VRC: 0.38 mg/L). By contrast, *A. calidoustus* had a poor susceptibility to triazole antifungals, with ITC and VCZ MICs at 3 mg/L and 16 mg/L, respectively.

Performance of tests according to the clinical background

As displayed in Table 5, sensitivity of culture combined to BAL GM and Af-mito PCR was significantly lower in patients with hematological malignancy (n=41) compared to the other patients (n=20), with estimated values decreasing from 85% to 43.9%, and 70% to 36.6%, respectively ($p<0.01$, $p<0.05$, respectively). Indeed, the sensitivity of Af-mito PCR was significantly reduced in neutropenic group (30% versus 64.5%, $p<0.05$) compared to non-neutropenic patients. Similarly, a previous exposure to mold-active antifungal agents (as prophylactic or curative treatment) lowered the sensitivity of all tests for *Aspergillus* detection (Table 6), at a statistically significant level for Mycogenie® PCR (40.0% versus 70.7%, p=0.03) and BAL GM assay combined to culture (30.0% versus 70.7%, p=0.005).

DISCUSSION

In this 3-year prospective study, the diagnosis of IA was considered as proven or probable for 38 of 387 immunocompromised patients (9.8%) based on conventional techniques. When PCR was tested directly in BAL samples, its sensitivity was higher than that of culture, but lower than that of GM detection. Mycogenie® PCR assay had a good sensitivity for IA diagnosis (73.7%), followed by Aspergenius® PCR (68.4%). Of the two in-house PCRs, the 28S PCR had the highest sensitivity, compared to Af-mito PCR (71.1% versus 60.5%). This difference was expected, based on *A. fumigatus* genome structure. Indeed while the copy number of the mitochondrial genome relative to the nuclear genome was estimated to be 12 (30), the ribosomal gene was shown to be repeated in 38 to 91 copies, varying according to the strain (31,32). A previous study on AsperGenius® assay in BAL from hematological population, reported a higher sensitivity (84%) (24) and another assay (MycAssay®*Aspergillus*) yielded sensitivity until 95% (12,33); it could possibly be related to a slightly different use of antifungals at this time.

Currently, PCR in BAL is not included in the criteria for classification of IA. Although BAL is frequently sampled in ICU patients, it is rare in hematological patients. In oncological-hematological patients, the increasing use of anti-*Aspergillus* chemoprophylaxis decreases the incidence but also the sensitivity of all diagnostic tests, as shown here. In our study, patients with hematological malignancy and/or allogeneic hematopoietic transplantation were more frequent in the group with possible IA; of note, the rate of patients receiving anti-*Aspergillus* prophylaxis was higher in this group. Conversely, solid organ transplantation was more frequent among patients with proven/probable IA ($p<0.05$). This work clearly demonstrates a superior

value of PCR in BAL for non-hematological immunosuppressed patients. The value of *Aspergillus* detection in endotracheal aspirates of ICU patients was already exemplified by Blot *et al* (34) who proposed a specific algorithm. Here, we observed a high sensitivity with a good specificity for BAL biomarkers such as GM and PCR in non-hematological patients, particularly if combined. Combining PCR to current EORTC criteria increased sensitivity from 92% to 100%. Such increase in sensitivity confirm previous results (35,36).

In hematological patients, despite a moderate sensitivity of each test performed alone, we showed that combination of PCR with EORTC criteria was of particular interest increasing the weak sensitivity of EORTC criteria from 44% to 68%, and particularly an increase from 30% to 50% and from 27% to 54% in patients under antifungals treatment (curative, empirical or prophylaxis) or specifically antifungal prophylaxis, respectively. These findings could lead to the inclusion of PCR among EORTC-MSG IA criteria (37). In our cohort, taking the in-house 28S PCR for the definition of IA would have upgraded 3 episodes from "possible cases" to "probable cases". Indeed, including an earlier and more sensitive method to detect *Aspergillus* in tissues may facilitate the diagnosis of IA, and, in turn, improve its management and clinical outcome. In addition, the high predictive value of simultaneous detection of fungal DNA and of GM antigen could lead to a reevaluation of empirical antifungal therapy for a better adequacy and a limitation of their use (38).

The specificity was excellent with all PCR assays (>92%). It should be noted that among the group of excluded IA, several patients had a chronic pulmonary aspergillosis or impaired pulmonary functions, which are known to increase the frequency of *Aspergillus* colonization. Indeed, of the 16 BAL with positive 28S PCR, we recorded 10 patients with aspergilloma, semi-invasive aspergillosis, or other etiology of chronic respiratory failure. Apart from PCR results, 6 of these patients had a typical imaging (aspergilloma) or a strongly positive detection of *Aspergillus* antibodies and precipitins, leading to a triazole therapy.

Although 28S PCR was described as specific for *A. fumigatus*, we observed cross-reaction with *A. flavus* and *A. ochraceus*, two species already associated with IA in previous studies (6,39,40). Considering this finding, the combination of both techniques appears valuable in case of strongly suspected aspergillosis with negative culture and GM detection. In this setting, as Aspergenius® assay targets both *Aspergillus sp* and *A. fumigatus*, it could have been considered as a relevant tool for IA diagnosis. However, the poor sensitivity observed here is a serious limitation to its use.

Apart ensuring PCR standardization, MycoGenie® and AsperGenius® kits are able to simultaneously detect hot-spot *cyp51A* mutations associated with *A. fumigatus* triazole resistance directly from clinical samples. We did not detect any *cyp51A* mutation in our positive samples, and it was correlated with *in vitro* susceptibility testing results, when available. This finding is consistent with the apparent low azole resistance prevalence in immunocompromised patients in France (around 1%) (18). However, we underlined a substantial poor sensitivity of *cyp51A* gene detection, mainly due to the presence of a single copy *cyp51A* gene in each fungal cell. This limiting factor has been previously raised in several reports using in-house molecular typing methods (22,41). This is especially a concern in samples from immunocompromised patients with a low fungal burden, moreover when an effective antifungal therapy has been initiated before sampling.

Here, we could not strictly rule out any resistant fungi, since these molecular screening focus only on a few hot-spot single polynucleotide polymorphisms, and because several *cyp51A*-independent mechanisms have been reported to contribute to azole resistance (42). In addition, rare cryptic species in *Aspergillus* genus responsible

for IA are increasingly reported. Indeed, attention should be paid to these fungi, with treatment failure likely due to their low intrinsic susceptibility to some antifungals, especially triazole therapy (43–45).

This study is the first study that broadly evaluated the value of several tools for diagnosing IA directly in BAL fluid of immunosuppressed patients, including non-hematological as hematological patients, and clearly demonstrated the value of combining tests, particularly PCR for the detection of *Aspergillus* DNA and ELISA for GM detection. However it has some limitations. First, this is a mono-centric study, which need to be conducted again in other centers. Second, we compared marketed *versus* in-house PCR assays; however, we provided all requested information for allowing to set them up elsewhere. Third, the sample dilution when working on BAL is an inevitable debate. However, while it is hard to circumvent it, this is not a debate for GM determination in BAL that is now include in EORTC/MSG criteria.

Consequently to efficient strategies of antifungal prophylaxis in hematology, ICUs now receive very high risk patients for aspergillosis and need specific recommendations for IA diagnosis. Overall, this study demonstrated the worth of molecular detection of *Aspergillus* in ICU patients BAL for the diagnosis of IA, particularly in non-hematological patients and those without prior exposure to antifungals. Such major information associated to other published data in non-hematological patients should be taken into account, independently to valuable hesitations and debates in the specific context of hematology. Checking the susceptibility profile by *in vitro* testing from cultures remains essential for triazole resistance monitoring.

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TABLES

Table 1. Characteristics of patients with invasive aspergillosis (N=61)

	EORTC/MSG classification	
Clinical features	Proven/probable (N=38) n (%)	Possible (N=23) n (%)
Age (years, [range])	62 [37-81]	52 [11-86]
Gender; Sex ratio	30/8; 3.75	15/8 ; 1.8
Hospitalization ward		
Intensive Care Unit	20 (52.6)	7 (30.4)
Haematology unit	11 (28.9)	11 (47.8)
Infectious disease unit	2 (5.3)	2 (8.7)
Others	5 (13.2)	3 (13.0)
Underlying condition		
Hematological malignancies	20 (52.6)	21 (91.3)
Acute myeloid leukemia	6 (30.0)	10 (47.6)
Acute lymphoid leukemia	0	1 (4.8)
Lymphoma	6 (30.0)	3 (14.3)
Myelodysplastic syndrome	1 (5.0)	2 (9.5)
Multiple myeloma	2 (10.0)	0
Others	5 (25.0)	5 (23.8)
Allogeneic HSC transplantation [a]	3 (7.9)	11 (47.8)
No hematological malignancies	18 (47.4)	2 (8.7)
Solid tumor	3 (22.2)	0
Solid organ transplantation [b]	7 (38.9)	0
Others	8 (44.4)	2 (100)
Neutropenia <0,5G/L	17 (44.7)	13 (56.5)
Immunosuppressant therapy[c]	33 (86.8)	19 (82.6)
Antifungal therapy before bronchoscopy	7 (18.4)	13 (56.5)
Prophylaxis	3 (7.9)	8 (34.8)
Empirical or curative[d]	4 (10.5)	5 (21.7)
12-week overall mortality		
Dead	11[e] (30.6)	7 (30.4)

HSC : Hematopoietic stem cell.

[a] 14/41 patients with hematological malignancies
[b] Liver n=5 ; kidney n=1 ; heart n=1
[c] Corticosteroid or rejection prophylaxis or cytotoxic chemotherapy
[d] Mold-active antifungal agent administered since at least 7 days before BAL performing
[e] 2 patients lost to follow-up

Table 2. Performances of PCR assays in broncho-alveolar lavage samples compared to galactomannan detection and culture for the diagnosis of invasive aspergillosis (N=387)

Method	Positive results n (%)		
	Patients with invasive aspergillosis (n=61)		Patients without invasive aspergillosis (n=326)
	Proven or probable IA n=38	Possible IA n=23	
Alone			
Af mito PCR	23 (60.5)	6 (26.1)	10 (3.1)[b]
28S PCR	27 (71.1)	9 (39.1)	16 (4.9)[c]
MycoGenie® PCR	28 (73.7)	9 (39.1)	na
AsperGenius® *Af* PCR	23 (60.5)	6 (26.1)	na
AsperGenius® *Asp sp* PCR	26 (68.4)	6 (26.1)	na
Combined			
Af mito PCR and EORTC/MSG criteria[a]	38 (100)	6 (26.1)	29 (8.9)
28S PCR and EORTC/MSG criteria[a]	38 (100)	9 (39.1)	33 (10.1)
Mycogenie® PCR and EORTC/MSG criteria[a]	38 (100)	9 (39.1)	na
AsperGenius® Af PCR and EORTC/MSG criteria[a]	37 (97.4)	6 (26.1)	na
AsperGenius® Asp PCR and EORTC/MSG criteria[a]	37 (97.4)	6 (26.1)	na
EORTC/MSG criteria[a]	35 (92.1)	na	27 (8.3)

[a] BAL GM + BAL culture + serum GM

[b] Aspergilloma (n=2), chronic necrotising pulmonary aspergillosis (CNPA) (n=2), pulmonary tumor (n=2), bronchiectasis (n=1), chronic obstructive pulmonary disease (COPD) (n=1), normal pulmonary functions (n=2)

[c] Aspergilloma (n=2), CNPA (n=2), pulmonary tumor (n=2), COPD (n=2), bronchiectasis (n=1), severe asthma (n=1), normal pulmonary functions (n=6)

na : not applicable; Af-mito PCR: targeting a sequence of an *A. fumigatus* mitochondrial gene; 28S PCR: targeting a sequence of a 28S rRNA gene of *A. fumigatus*; BAL GM ELISA: sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of galactomannan (GM) in broncho-alveolar fluid (BAL); AsperGenius® *Af* PCR: sub-assay of the AsperGenius® multiplex assay detecting 28S rRNA of the *A. fumigatus* complex; AsperGenius® *Asp sp*: sub-assay of the AsperGenius® multiplex assay detecting 28S rRNA of *A. terreus* and *Aspergillus* spp.; MycoGenie®: quadruplex real-time PCR assay which targets *A. fumigatus* (*28SrRNA* gene), TR34, L98H, and an internal control to monitor sample inhibition.

A result was considered as positive when a signal was detected at Ct<45.

Table 3. PCR results according to broncho-alveolar (BAL) sample culture results

Culture results	Positive *Aspergillus* PCR assay results				
	Af mito PCR n/N (%)	28S PCR n/N (%)	Mycogenie® PCR[a] n/N(%)	Aspergenius® *Af* PCR[a] n/N(%)	Aspergenius® *Asp sp* PCR[a] n/N(%)
A. fumigatus	19/22 (86.4)[g]	20/22 (90.9)[h]	15/16 (93.8)	15/16 (93.8)	15/16 (93.8)
Non *fumigatus Aspergillus* species[b]	0/4 (0)	2[c]/4 (50.0)	1[d] /2 (50.0)	0/2 (0)	1[d] /2 (50.0)
Others moulds[e]	1[f]/6 (16.7)	1[f]/6 (16.7)	1[f] /2 (50.0)	1[f] /2 (50.0)	1[f] /2 (50.0)
Negative	19/355 (5.3)	29/355 (8.2)	20/41 (48.8)	13/41 (31.7)	15/41 (36.6)

[a] Tested only on BAL samples from IA cases (n=61)
[b] *A. flavus* (n=1), *A. glaucus* (n=1), *A. ochraceus* (n=1), *A. calidoustus* (n=1). *A. flavus* and *A. calidoustus* were grown from IA BAL samples.
[c] *A.flavus, A. ochraceus*
[d] *A. flavus*
[e] *Penicillium sp*. (n=4), *Scedosporium sp.* (n=1), *S. apiospermum* (n=1)
[f] *S. apiospermum*
[g] 15/16 proven or probable IA with positive culture and 4/6 excluded IA with positive culture
[h] 15/16 proven or probable IA with positive culture and 5/6 excluded IA with positive culture
Af-mito PCR: targeting a sequence of an *A. fumigatus* mitochondrial gene; 28S PCR: targeting a sequence of a 28S rRNA gene of *A. fumigatus*; MycoGenie®: quadruplex real-time PCR assay which targets *A. fumigatus* (*28SrRNA* gene), TR34, L98H, and an internal control to monitor sample inhibition; AsperGenius® *Af* PCR: sub-assay of the AsperGenius® multiplex assay detecting 28S rRNA of the *A. fumigatus* complex; AsperGenius® Asp sp: sub-assay of the AsperGenius® multiplex assay detecting 28S rRNA of *A. terreus* and *Aspergillus* spp..

Table 4. Detection of *cyp51A* mutation using Mycogenie® and AsperGenius® assays

PCR assay		Number of positive samples (%)				
		A.fumigatus PCR	TR34	L98H	Y121F	T289A
AsperGenius®	Target amplification	29/61 (47.5)	18/29 (62.1)	18/29 (62.1)	19/29 (65.5)	18/29 (62.1)
	Detection of mutated allele[a]	na	0/18	0/18	0/19	0/18
Mycogenie®	Detection of mutated allele	37/61	0/37	0/37	na	na

na : not applicable

[a] Tm value of melting peaks in "mutant" range, according to manufacturer's instructions

MycoGenie®: quadruplex real-time PCR assay which targets *A. fumigatus* (*28SrRNA* gene), TR34, L98H, and an internal control to monitor sample inhibition; AsperGenius® *:* multiplex assay detecting 28S rRNA of the *A. fumigatus* complex, *A. terreus* and *Aspergillus* spp..

Table 5. Diagnostic performances of PCR assays, galactomannan (GM) detection and Culture of broncho-alveolar lavage (BAL) samples, according to the underlying disease in patients with IA

Method	Hematological disease n=41	No hematological disease n=20	p-value	Neutropenia <0.5G/L n=30	No neutropenia n=31	p-value
	Sensitivity n(%)	Sensitivity n(%)		Sensitivity n(%)	Sensitivity n(%)	
Alone						
Af mito PCR	15 (36.6)	14 (70.0)	0.028*	9 (30.0)	20 (64.5)	0.010*
28S PCR	21 (51.2)	15 (75.0)	0.100	15 (50.0)	21 (67.7)	0.198
MycoGenie® PCR	22 (53.7)	15 (75.0)	0.163	16 (53.3)	21 (67.7)	0.300
AsperGenius® *Af* PCR	17 (41.5)	12 (60.0)	0.275	12 (40.0)	17 (54.8)	0.309
AsperGenius® *Asp sp* PCR	18 (43.9)	14 (70.0)	0.063	14 (46.7)	18 (58.1)	0.446
Combined						
Af mito PCR and EORTC/MSG criteria[a]	24 (58.5)	20 (100)	<0.001***	19 (63.3)	25 (80.6)	0.161
28S PCR and EORTC/MSG criteria[a]	27 (65.9)	20 (100)	0.002**	21 (70.0)	26 (83.9)	0.235
Mycogenie® PCR and EORTC/MSG criteria[a]	27 (65.9)	20 (100)	0.002**	21 (70.0)	26 (83.9)	0.235
AsperGenius® Af PCR and EORTC/MSG criteria[a]	24 (58.5)	19 (95.0)	0.003**	20 (66.7)	23 (74.2)	0.582
AsperGenius® Asp PCR and EORTC/MSG criteria[a]	24 (58.5)	19 (95.0)	0.003**	20 (66.7)	23 (74.2)	0.582
EORTC/MSG criteria[a]	18 (43.9)	17 (85.0)	0.003**	16 (53.3)	19 (61.3)	0.609

*p<0.05 ; **p<0.01 ; ***p<0.001

[a] BAL GM + BAL culture + serum GM

Af-mito PCR: targeting a sequence of an *A. fumigatus* mitochondrial gene; 28S PCR: targeting a sequence of a 28S rRNA gene of *A. fumigatus*; BAL GM ELISA: sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of galactomannan (GM) in broncho-alveolar fluid (BAL); AsperGenius® *Af* PCR: sub-assay of the AsperGenius® multiplex assay detecting 28S rRNA of the *A. fumigatus* complex; AsperGenius® Asp sp: sub-assay of the AsperGenius® multiplex assay detecting 28S rRNA of *A. terreus* and *Aspergillus* spp.; MycoGenie®: quadruplex real-time PCR assay which targets *A. fumigatus* (*28SrRNA* gene), TR34, L98H, and an internal control to monitor sample inhibition.

Table 6. Diagnostic performances of PCR assays compared to galactomannan (GM) detection and Culture of broncho-alveolar lavage (BAL) samples according to the antifungal exposure prior to BAL sampling

Method	Prophylaxis n=11	No prophylaxis n=50	p-value	Previous treatment[b] (n=20)	No previous treatment[b] (n=41)	p-value
	Sensitivity n(%)	Sensitivity n(%)		Sensitivity n(%)	Sensitivity n(%)	
Alone						
Af mito PCR	4 (36.4)	25 (50.0)	0.514	7 (35.0)	22 (53.7)	0.187
28S PCR	5 (45.4)	31 (62.0)	0.333	8 (40.0)	28 (68.3)	0.052
MycoGenie® PCR	5 (45.4)	32 (62.0)	0.315	8 (40.0)	29 (70.7)	0.028*
AsperGenius® *Af* PCR	4 (36.4)	25 (50.0)	0.514	7 (35.0)	22 (53.7)	0.187
AsperGenius® *Asp sp* PCR	4 (36.4)	28 (56.0)	0.323	7 (35.0)	25 (61.0)	0.100
Combined						
Af mito PCR and EORTC/MSG criteria[a]	5 (45.4)	39 (78.0)	0.058	9 (45.0)	35 (85.4)	0.002**
28S PCR and EORTC/MSG criteria[a]	6 (54.5)	41 (82.0)	0.106	10 (50.0)	37 (90.2)	0.001***
Mycogenie® PCR and EORTC/MSG criteria[a]	6 (54.5)	41 (82.0)	0.106	10 (50.0)	37 (90.2)	0.001***
AsperGenius® Af PCR and EORTC/MSG criteria[a]	5 (45.4)	38 (76.0)	0.067	9 (45.0)	34 (82.9)	0.006**
AsperGenius® Asp PCR and EORTC/MSG criteria[a]	5 (45.4)	38 (76.0)	0.067	9 (45.0)	34 (82.9)	0.006**
EORTC/MSG criteria[a]	3 (27.3)	32 (62.0)	0.042*	6 (30.0)	29 (70.7)	0.005**

[a] BAL GM + BAL culture + serum GM

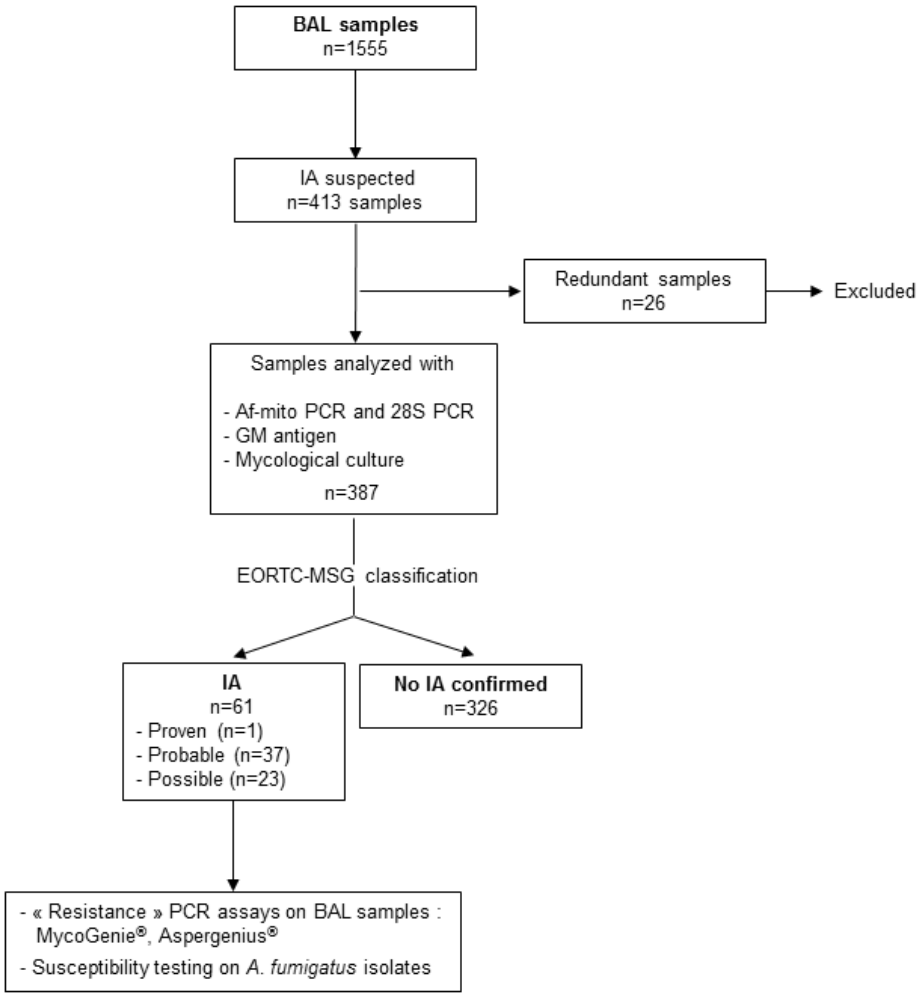
[b]prophylactic or curative (empirical or targeted) antifungal therapy

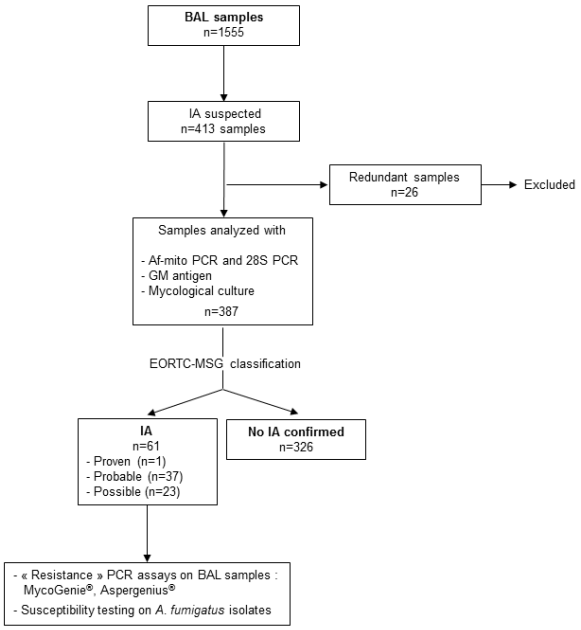
*p<0.05 ; **p<0.01 ; ***p<0.001

Af-mito PCR: targeting a sequence of an *A. fumigatus* mitochondrial gene; 28S PCR: targeting a sequence of a 28S rRNA gene of *A. fumigatus*; BAL GM ELISA: sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of galactomannan (GM) in broncho-alveolar fluid (BAL); AsperGenius® *Af* PCR: sub-assay of the AsperGenius® multiplex assay detecting 28S rRNA of the *A. fumigatus* complex; AsperGenius® Asp sp: sub-assay of the AsperGenius® multiplex assay detecting 28S rRNA of *A. terreus* and *Aspergillus* spp.; MycoGenie®: quadruplex real-time PCR assay which targets *A. fumigatus* (*28SrRNA* gene), TR34, L98H, and an internal control to monitor sample inhibition.

FIGURES

Fig.1 : Flow-chart of the study population





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