

Diagnosis of invasive aspergillosis using bronchoalveolar lavage in haematology patients: influence of bronchoalveolar lavage human DNA content on real-time PCR performance

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Abstract In order to improve invasive pulmonary aspergillosis (IPA) diagnosis, a real-time polymerase chain reaction (PCR) assay detecting *Aspergillus* spp. was developed. Its detection limit reached 2–20 conidia. The retrospective evaluation on 64 bronchoalveolar lavage (BAL) fluids from 57 patients at risk for IPA, including 20 probable and five proven IPA patients, revealed a 88% or 38% sensitivity in direct examination (DE)/culture-positive or culture-negative BAL, respectively, whereas galactomannan (GM) sensitivity reached 88% or 58%, respectively. Influence on the *Aspergillus*-PCR yield of BAL fluid volume, cellular count and DNA content

(evaluated by human β -globin quantification) was assessed. Significantly higher β -globin levels were detected in *Aspergillus* PCR-positive (median 5,112 pg/ μ l) than negative (median 1,332 pg/ μ l) BAL fluids, suggesting that the β -globin level could reflect BAL yields and DNA extraction. Using β -globin for the interpretation of fungal PCR could improve the negative predictive value of this test.

Introduction

Invasive pulmonary aspergillosis (IPA) is a life-threatening infection, occurring mostly in immunocompromised patients. *Aspergillus fumigatus* is the most common species to cause aspergillosis, but *A. flavus*, *A. terreus* and *A. niger* are also fairly common forms of *Aspergillus* infection [1]. Diagnosis is often difficult, based on host factors, clinical and radiological findings, and mycological criteria. The European Organization for Research and Treatment of Cancer (EORTC) and the Mycosis Study Group (MSG) have defined three levels for invasive fungal infections, “possible,” “probable” and “proven,” giving a rational basis for the evaluation of treatments and radiological or biological diagnosis tools [2].

Currently, microbiological tools have difficulty in providing a sensitive and early diagnosis of IPA [3, 4], whereas these two criteria are essential for adapted antifungal therapy and improved prognosis. Only positive direct examination (DE) and culture of pulmonary samples obtained by sterile procedures (lung or transbronchial lung biopsies) can confirm the IPA diagnosis and allow identification at the species level, leading to appropriate antifungal therapy. However, these samples, which require invasive procedures for sampling, lack sensitivity, espe-

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cially if the patient received empirical antifungal treatment [5]. Galactomannan (GM) detection in serum by enzyme-linked immunosorbent assay (ELISA) has a variable sensitivity (29–100%), depending on the host group (immunocompromised or not, probability of IPA in patients included, antifungal treatment) [3, 6, 7]. The specificity of GM generally reaches over 90%, but false-positives can occur, specifically when patients received antibiotics such as piperacillin/tazobactam [6, 8]. Cross-reactivity with other fungal species have also been reported [6, 9]. In bronchoalveolar lavage (BAL), GM detection could lead to an earlier diagnosis of IPA in patients with haematological malignancy, yielding an increased sensitivity when compared to serum, e.g. 85–100% versus 47% [10].

In order to improve IPA diagnosis, molecular methods have been recently developed [11–16]. They can either be specific to one fungal species (mainly *A. fumigatus*) or target several species (panfungal polymerase chain reaction [PCR]), detecting fungal DNA in serum/blood, biopsies or BAL samples. In addition to being helpful in IPA diagnosis, DNA amplification from biological samples allow species identification when culture remains negative [11–16]. In BAL, which is commonly used for the diagnosis of opportunistic infections in immunosuppressed patients with pulmonary infiltrates, *Aspergillus* DNA detection yields high sensitivity (67–100%) and specificity (80–100%) [15–22], and has been shown to be clinically relevant for the diagnosis of IPA [23]. However, the features of the BAL fluid could influence fungal detection, as in the diagnosis of alveolar haemorrhage, or interstitial lung diseases, for which the influence of BAL quality on diagnosis has been reported [24], and, consequently, criteria for the selection of suitable samples representative of the lower respiratory tract were defined for the diagnosis of these diseases [25].

With the aim of exploring how a low-quality BAL could result in a decreased sensitivity/specificity of diagnosis tests, we retrospectively evaluated the detection of *Aspergillus* spp. by real-time PCR on BAL from patients with haematological malignancy. We compared PCR to other available IPA diagnosis tools (DE, culture and GM detection) and determined how PCR can contribute to improve IPA diagnosis sensitivity when BAL quality using cytological analysis and the evaluation of human DNA content by β -globin gene molecular quantification are assessed.

Materials and methods

Patients

Sixty-four BAL from 57 patients referred between December 2000 and February 2004 in the Hematology Department of

Lille University Hospital were retrospectively analysed. All of these 57 patients were at risk for IPA, i.e. neutropaenic, with persistent fever under broad-spectrum antibiotics treatment, graft-versus-host disease (GVHD), immunosuppressive agent or corticosteroid treatment. According to the criteria of the EORTC/MSG [2], 25 of 57 patients (yielding 32 BAL for analysis) were classified by a physician as probable ($n=20$) or proven ($n=5$) IPA, and the 32 remaining patients (32 BAL) were considered as “no IPA” patients (Table 1).

BAL sampling and conventional analysis

In our institution, BAL is performed using a 150–200-ml volume of sterile saline. The reaspirated volume is noted and the sample divided into two parts. One part is sent to the Pathology Department, where the cytological analysis is performed. Cytological data (cellularity and formula) were collected when available. The second part of the BAL is sent to the Microbiology Department for bacteriological, virological and mycological analysis. For mycological conventional and molecular analysis, BAL samples were then subdivided into two fractions.

The conventional analysis included a centrifugation step. A pellet was used for DE after staining with toluidine blue O and May-Grünwald-Giemsa and fungal cultures on Sabouraud agar medium. The BAL supernatant was used to perform the *Aspergillus* GM antigen test (Platelia *Aspergillus* EIA, BioRad®), using index ≥ 1 as positive [10, 26]. BAL GM was used as an EORTC/MSG criteria to classify IPA patients [2].

BAL molecular analysis

The molecular analysis included a centrifugation step and a DNA extraction from the pellet as previously described [27]. Briefly, after centrifugation, the supernatant was discarded and DNA was extracted from 200 μ l of BAL pellet using the QIAamp DNA Mini Kit (Qiagen SA, Courtaboeuf, France), following the manufacturer’s instructions, and eluted in 200 μ l of sterile water. DNA extracts were stored at -20°C until analysis.

The detection of *Aspergillus* spp. by real-time PCR was performed with a 5- μ l DNA extract on a LightCycler instrument (Roche Molecular Biochemicals, Meylan, France), as previously described [11, 28]. Fluorescence curves were analysed with LightCycler software version 3.5. A standard curve was generated by running eight positive controls containing *A. fumigatus* IPP 22–7994 genomic DNA (from 1 fg/ μ l to 10 ng/ μ l). Two samples (one sterile water submitted to extraction and one PCR mix control) were included as negative controls. Two DNA amplifications were initially performed on each BAL sample. An amplification was considered to be positive

when the C_p value was ≤ 43 [29]. When both replicates were positive, the sample was considered to be positive. When only one of the replicates was positive, DNA amplification was performed again twice: 2/4 or 3/4 PCR-positive results yielded a PCR-positive sample.

PCR inhibitor control was performed using 50 picograms (pg) of *A. fumigatus* IPP 22–7994 added to separate PCR mixtures containing 5 μ l of each BAL fluid. Such quantities of exogenous *Aspergillus* DNA was supposed to minimise the interference with endogenous fungal DNA when PCR BAL was positive. The amplification kinetics were compared to the kinetics of the 50-pg standard (C_p values of each BAL sample versus the mean of 50-pg standard replicate C_p values). A difference of over three PCR cycles (1 log) was considered as significant inhibition.

In order to evaluate human DNA content in the BAL samples, a quantitative human β -globin PCR was performed using the Control Kit DNA (Roche Molecular Biochemicals, Meylan, France), according to the manufacturer's instructions; the results were expressed as pg of human DNA per μ l.

Performance of the combined extraction–amplification method

The analytical sensitivity of the combined extraction–amplification method was evaluated using serial dilutions of *A. fumigatus* conidia (10^4 to 10^1 conidia/ml) in pooled serums from patients not at risk for IPA (absence of *Aspergillus* antigen [GM] or antibodies had been checked). For each dilution, DNA extraction was performed twice in two independent experiments, from 200 μ l of serum with conidia (corresponding to 2,000, 200, 20 and 2 conidia), using the QIAamp DNA Mini Kit (Qiagen SA, Courtaboeuf, France), as described above. One extraction control (200 μ l of serum without conidia) was included in each series. DNA extracts were then analysed in duplicate using the real-time PCR method described above.

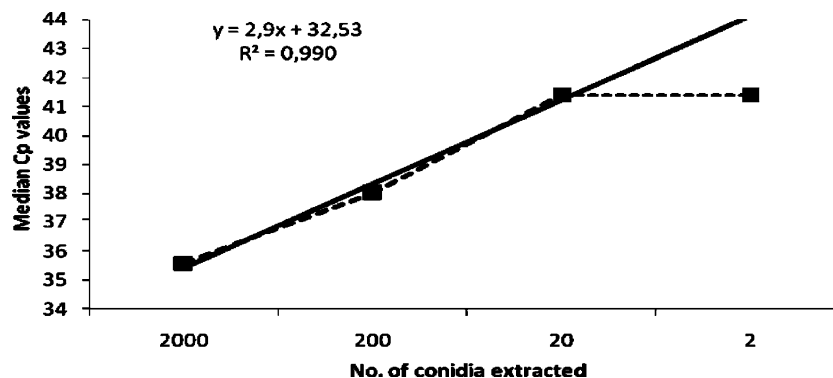


Fig. 1 Combined extraction–amplification method for the determination of the detection limit. Median C_p values were calculated for each *Aspergillus fumigatus* conidia dilution (10^4 , 10^3 , 10^2 and 10^1 /ml) and

Assessment of BAL quality

As BAL composition (alveolar macrophages, ciliated cells, mucus, total cellularity etc.) reflects BAL quality [25], we analysed BAL using the following indicators: (i) volume recovered for analysis, which could be associated with the sample quality [30]; (ii) total cellularity and detailed cell count, which reveal potential contamination by proximal airways or terminal bronchioles [25]; and (iii) human β -globin gene quantification by real-time PCR, which could reflect high cellularity of the BAL and confirm a good quality of DNA extraction, required to perform an efficient PCR reaction. For cell count analysis, alveolar macrophages under 80%, lymphocytes over 15% and neutrophils or bronchial cells over 10% were considered as abnormalities in the BAL formula according to the published data [25, 30].

Statistical methods

The Wilcoxon test was used to compare the duration of the antifungal treatment, BAL volume recovered, total cellularity and the β -globin level medians between IPA patient subgroups classified according to their positive or negative result for DE/culture, GM and PCR. A P -value equal to or less than 0.05 was considered to be statistically significant.

Results

Analytical sensitivity of the DNA extraction and amplification methods

First, we quantified the threshold for fungal DNA detection by real-time PCR using serial dilutions of *A. fumigatus* genomic DNA (from 1 fg/ μ l to 10 ng/ μ l), which reached 1–10 fg/ μ l, according to previous data [11]. Secondly, the ability and reproducibility of the QIAamp DNA Mini Kit extraction method to extract fungal DNA was evaluated

were related to the corresponding number of conidia extracted in 200 μ l of serum (2,000, 200, 20 and 2 conidia, respectively)

Table 1 Characteristics of patients and bronchoalveolar lavage (BAL) results

Patient no.	Sex, age	Underlying disease ^a	BAL	Time sample was taken, when IPA was suspected (days after diagnosis)	Fungal culture (positive direct examination [DEI])	GM detection	Molecular data		Total cellularity (10^2 /ml)	Volume (ml)	Nature of the antifungal treatment ^c (duration, in days)	IPA diagnosis (EORTC criteria)
							<i>Aspergillus</i> PCR (mean Cp)	β -globin (pg/ μ l)				
1	M, 42	MM/BMT		0	<i>A. fumigatus</i>	+	+	(26.02)	135,400	NA	VCZ (5)	Probable
2	M, 47	NHL		0	<i>A. fumigatus</i>	+	+	(34.17)	88,900	3030	NA	Probable
3	F, 61	AML		2	<i>A. fumigatus</i>	+	+	(39.98)	134	30	No	Probable
				15	-	-	-	-	1,370	NA	ABL, VCZ (13)	
4	M, 71	MM		1	<i>A. fumigatus</i> / <i>C. albicans</i>	+	+	(35.68)	3,885	170	NA	Probable
5	F, 40	AML		0	<i>A. fumigatus</i>	+	-	-	3,114	57	AB, ABL (3)	Probable
6	M, 66	CLL		13	<i>A. fumigatus</i>	-	+	(40.50)	6,347	580	ABL (4)	Probable
7	M, 56	CLL/BMT		67	- (+ mycelium)	+	+	(31.98)	107,200	70	VCZ, CSF, ABL (67)	Proven
8	M, 42	AML		1	-	+	-	-	102	NA	ABL (1)	Proven
				30	- (+ mycelium)	+	+	(38.78)	76,800	NA	ABL (30)	
9	F, 52	AML		0	-	+	+	(35.89)	2,010	NA	AB, ABL (12)	Proven [#]
10	M, 51	AML		1	-	+	+	(42.91)	15,690	220	ABL (3)	Probable
11	F, 28	CML/BMT		1	-	+	+	(36.2)	342	136	ABL, VCZ, CSF (19)	Probable
12	M, 36	ALL		2	-	+	+	(37.48)	717	150	ABL, VCZ, CSF (32)	Probable
13	M, 74	AML		7	-	+	-	-	141	NA	ABL (4)	Probable
14	M, 69	AML		0	-	+	-	-	1,394	17	ABL (1)	Probable
15	F, 80	AML		9	-	+	-	-	13,200	NA	ABL, CSF (13)	Probable
16	F, 40	AML/BMT		10	-	+	-	-	1,366	200	AB, ABL (5)	Probable
17	F, 58	ALL		2	-	+	-	-	74	70	AB, ABL (14)	Probable
18	M, 58	ALL		3	-	+	+	-	225	60	AB, ABL, CSF (31)	Probable
19	F, 43	AML		0	-	+	-	-	6	NA	AB (11)	Probable
20	M, 39	AML		1	-	+	-	-	8,180	70	No	Proven [#]
				15	-	-	-	(38.83)	866	110	No	
				28	-	+	+	(37.23)	86,300	700	ABL (6)	
21	F, 20	AML		1	-	-	-	-	42	90	ABL (2)	Probable
				10	-	-	-	-	113	200	ABL (3)	
				30	-	+	+	(39.55)	947	60	ABL (5)	
22	M, 29	AML		0	-	-	-	(40.08)	13,500	254	AB (4)	Probable
				14	-	-	-	(39.01)	2,170	NA	AB, ABL (18)	
23	M, 55	MDS		7	-	-	-	-	14,300	212	AB, ABL (34)	Proven [#]
24	M, 39	AML		64	-	-	-	-	5,955	230	ABL, VCZ, CSF (61)	Probable
25	M, 70	RC		1	-	-	-	-	1,299	50	No	Probable
26	M, 39	HL/APBSCT		NA ^b	-	-	-	-	38,385	650	NA	No IPA
27	M, 35	HL		NA	-	-	-	-	18,885	154	NA	No IPA

28	M, 23	ALL/APBSCT	NA	-	-	3,252	152	52.5	NA	No IPA
29	M, 73	VD	NA	-	-	1,935	93	43	NA	No IPA
30	F, 78	AML	NA	-	-	752	90	21	NA	No IPA
31	F, 45	AML/MDS	NA	-	-	99	17.5	40	NA	No IPA
32	M, 54	AML/APBSCT	NA	-	-	1,215	NA	69	NA	No IPA
33	M, 42	AML	NA	-	-	5,873	194	48	NA	No IPA
34	M, 38	ALL/BMT	NA	-	-	5,471	72	7.5	NA	No IPA
35	M, 31	ALL/BMT	NA	-	-	68	72	70	NA	No IPA
36	M, 33	ET/BMT	NA	-	-	2,802	NA	NA	NA	No IPA
37	M, 26	IMA/BMT	NA	-	-	30	54	20	NA	No IPA
38	F, 54	ALL	NA	-	-	5	10	33	NA	No IPA
39	M, 52	MDS/BMT	NA	-	-	28,935	54	5	NA	No IPA
40	M, 35	MDS/BMT	NA	-	-	2,727	192	50	NA	No IPA
41	M, 43	ALL/BMT	NA	-	-	3,065	72	58	NA	No IPA
42	M, 70	MDS	NA	-	-	84	65	68	NA	No IPA
43	M, 28	AML/APBSCT	NA	-	-	3,183	435	92	NA	No IPA
44	F, 55	AML	NA	-	-	260	7	9	NA	No IPA
45	M, 53	RAEB/BMT	NA	-	-	399	36	52	NA	No IPA
46	F, 73	AML	NA	-	-	3,623	NA	NA	NA	No IPA
47	M, 52	NHL/BMT	NA	-	-	653	140	30	NA	No IPA
48	M, 51	NHL	NA	-	-	15,086	515	65	NA	No IPA
49	M, 56	NHL	NA	-	-	4,283	396	106	NA	No IPA
50	M, 56	MDS/BMT	NA	-	-	4,175	NA	NA	NA	No IPA
51	M, 57	NHL	NA	-	-	1,224	346	81	NA	No IPA
52	F, 80	NHL	NA	-	-	186	149	51	NA	No IPA
53	M, 28	AML/BMT	NA	-	-	26,865	697	74	NA	No IPA
54	M, 42	MM/BMT	NA	-	-	7,484	83	40	NA	No IPA
55	M, 41	CLL/BMT	NA	-	-	19,160	100	20	NA	No IPA
56	F, 64	NHL	NA	-	-	11,960	220	55	NA	No IPA
57	M, 29	IMA/BMT	NA	-	-	9,750	23	55	NA	No IPA

^a ALL, acute lymphoblastic leukaemia; AML, acute myelogenous leukaemia; APBSCT, autologous peripheral blood stem cell transplantation; BMT, bone marrow transplantation; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; ET, essential thrombocythaemia; IMA, Idiopathic macrocytic anaemia; HL, Hodgkin lymphoma; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; RAEB, refractory anaemia with excess of blasts; RC, refractory cytopaenia; VD, Vaquez' disease

^b NA, not available

^c VCZ, voriconazole; CSF, caspofungin; AB, amphotericin B; ABL, liposomal amphotericin B

[#] Histologically confirmed IPA (lobectomy)

Table 2 Results of *Aspergillus* tests in proven or probable invasive pulmonary aspergillosis (IPA) and no-IPA patients

	Positive			Negative		
	DE/culture	GM	PCR	DE/culture	GM	PCR
Proven or probable IPA	8	21	16	24	11	16
Combined positive tests						
GM	7 (88%)	NC*	NC	14 (58%)	NC	NC
PCR	7 (88%)	12 (57%)	NC	9 (38%)	4 (36%)	NC
No IPA	0	0	0	32	32	32

*Not calculated

using serial dilutions of *A. fumigatus* conidia. The median Cp values of the 10^4 , 10^3 and 10^2 conidia/ml dilutions ($n=4$) reached 35.6, 38.0 and 41.4, respectively, yielding a linear increase in the median Cp as a function of the number of conidia added for extraction ($R^2=0.990$, Fig. 1). The 10^1 conidia/ml dilution (corresponding to the DNA extraction of two conidia, Fig. 1) exhibited positive DNA amplification in 2 out of 4 replicates (median Cp value of 41.4), suggesting that our combined DNA extraction–amplification method reached a 10^1 – 10^2 conidia/ml (2–20 conidia, Fig. 1) overall sensitivity, and was similar to the most sensitive methods [31, 32].

Contribution of real-time PCR to IPA diagnosis

The positive results of each mycological test (DE/culture, GM and PCR) are summarised in Table 2, with the

performance of either PCR or GM when they are combined with other tests. The sensitivity of PCR was higher when other tests were also positive: 88% versus 38% in DE/culture-positive BAL, and 57% versus 36% in GM-positive BAL. The sensitivity of GM was similar to that of PCR in DE/culture-positive BAL (88%), but higher in DE/culture-negative BAL (58% versus 38%, Table 2). Both PCR and GM were independently negative in a BAL with positive culture (patients nos. 5 and 6, Table 1, respectively). Within the 26 BAL with negative culture, the detection of *Aspergillus* spp. DNA by PCR in 11 BAL (patients nos. 7–12, 20–22, Table 1) was the only test supporting the *Aspergillus* infection (DE and GM are not specific to *Aspergillus* spp.). Within the ten BAL from seven patients with proven or probable IPA that were negative for DE/culture and GM (patients nos. 3, 20–25, Table 1), three BAL yielded a positive PCR result (patients nos. 20 and 22, Table 1). For these patients, PCR was the only test supporting the diagnosis of IPA in BAL. None out of the 32 BAL from patients without suspected IPA showed a PCR-positive result. A variation under 1 PCR cycle between BAL sample Cp values when 50 pg is added and the mean of 50-pg standard replicate Cp values (25.05 ± 0.22 Cp) confirmed the absence of PCR inhibitors.

Influence of antifungal treatment on the performance of *Aspergillus* tests

Because of host clinical risks, most patients received antifungals before BAL was performed. The number of molecules used and the total duration of the treatment, which ranged from 0 to 67 days, are indicated in Table 1. The treatment duration medians between negative and

Table 3 Analysis of the influence of antifungal treatment duration and BAL quality (volume of fluid recovered for analysis, cellularity, β -globin level and BAL formula) on the performance of DE/culture, GM detection and *Aspergillus* DNA detection

	Antifungal treatment duration (days)		Volume of fluid recovered for analysis (ml)	
	Number of BAL	Median (range)	Number of BAL	Median (range)
No IPA	NA ^a	NA	29	50 (5–106)
Proven or probable IPA	30	5 (0–67)	23	45 (7–110)
DE/culture				
positive	6	4.5	6	47.5
negative	24	5.5	17	29.5
GM				
positive	19	6	14	36
negative	11	4	9	66
PCR				
positive	14	5.5	12	41
negative	16	4.5	11	58
Total	NA	NA	52	49 (5–100)

^aNA, not available

*AM, alveolar macrophages; L, lymphoplasmocytes; N, neutrophils; BC, bronchial cells

[‡]Significant difference between positive and negative subgroups ($P \leq 0.05$)

positive subgroups for each test (DE/culture, GM detection, PCR) were compared and yielded no significant difference for any of the three tests (Table 3, $P>0.05$).

Alveolar micro-environment: evaluation of BAL quality and influence on *Aspergillus* detection

The volume recovered for analysis, total cellularity, detailed cell count (when available) and human β -globin gene quantification by real-time PCR were used as indicators of BAL quality (Table 1). The range variations and medians were compared when patients are classified according to their IPA status (proven or probable IPA versus no IPA, Table 3). BAL abnormalities of cell analysis were more frequent in patients without IPA, especially due to an increase in lymphoplasmocyte or neutrophil counts (Table 3), and probably related to the underlying haematological disease or to a non-fungal infection (e.g. bacterial). In terms of BAL quality, there was no significant difference between the proven or probable IPA and no IPA patient groups ($P>0.05$, Table 3), even if large variations of volume recovered, total cellularity and β -globin individual values were observed.

In patients with proven or probable IPA, the medians of the volume recovered, total cellularity and β -globin levels between negative and positive subgroups for each test (DE/culture, GM detection, PCR) were compared (Table 3). We observed a significantly higher β -globin level in DE/culture- and PCR-positive subgroups ($P\leq 0.05$, Table 3), reaching 41,573 pg/ μ l (1,368 pg/ μ l in DE/culture-negative samples) and 5,116 pg/ μ l (1,332 pg/ μ l in PCR-negative samples), respectively. All BAL with β -globin levels over 15,690 pg/ μ l were *Aspergillus* PCR-positive (six BAL, patients nos. 1, 2, 7, 8, 10 and 20, Table 1). Few samples with low β -globin levels and which were fungal PCR-positive have also been observed (e.g. patients nos. 3, 11 or

12, Table 1). The volume recovered and cellularity were not significantly different in the positive and negative subgroups for any of the three tests ($P>0.05$, Table 3).

Discussion

Contribution of real-time PCR to IPA diagnosis

The retrospective evaluation of a real-time PCR assay on BAL in patients with haematological malignancy at risk for IPA ($n=57$; 64 BAL) confirmed previous analytical sensitivity [11], which reached 1–10 fg/ μ l, similarly to the most accurate real-time PCR or nested PCR assays targeting the classical rRNA genes [13–15, 33, 34] or other mitochondrial genes [16]. Our combined DNA extraction–amplification method was able to detect 2–20 conidia. Our detection limit was similar to the detection limit of 1–10 conidia reported when *A. fumigatus* DNA extraction methods were used [31, 32] and higher than a recently reported 100-conidia sensitivity in BAL samples [35].

We found no false-positive PCR in patients without IPA documented. This excellent specificity agrees with the most recent PCR studies on BAL [16, 21, 26] and could result from the development of real-time PCR assays that have decreased the risk of contamination [36]. Furthermore, performing PCR in BAL from patients at risk for IPA, and considering a positive PCR result only in patients with clinical findings suggesting IPA, could avoid false-positive results in colonised patients [23].

PCR and GM both reached a 88% sensitivity in BAL positive for DE/culture, which confirmed the recent studies reporting a 75–96% and 67–78% sensitivity, respectively [20, 22, 26]. The absence of DNA amplification or GM detection whereas DE/culture is positive in two patients (patients nos. 5 and 6, Table 1) confirmed previous data: false-negatives

Cellularity (10^3 /ml)		β -globin level (pg/ μ l)		Abnormalities in BAL formula				
Number of BAL	Median (range)	Number of BAL	Median (range)	Number of patients	AM<80%*	L>15%*	N>10%*	BC>10%*
28	93 (7–697)	32	2,082 (5–25,590)	29	17 (59%)	14 (48%)	14 (48%)	0 (0%)
23	136 (17–3,030)	32	1,702 (6–134,400)	22	11 (50%)	6 (27%)	6 (27%)	2 (9%)
6	120	8	41,573 [§]					
17	136	24	1,368 [§]					
14	103	21	1,394					
9	200	11	2,170					
12	160	16	5,116 [§]					
11	70	16	1,332 [§]					
51	110 (7–3,030)	64	2,764 (5–135,400)	51	28 (55%)	20 (39%)	20 (39%)	2 (4%)

can occur but are rare [20]. Since no inhibition has been found, it could result from either the absence of fungal material in the part of BAL used for analysis or from a low yield of DNA extraction. In terms of quantification, culture and DNA detection seem not to be correlated, since the absence or very low fungal DNA quantities have been observed, whereas culture was positive (e.g. patients nos. 5 and 6, Table 1). The absence of GM detection whereas DE/culture is positive could also result from the low quantities which we failed to detect because of a too high cut-off value (≥ 1). Although no cut-off value has been validated in the BAL, and using lower cut-off values (e.g. 0.5 or 0.7) could have improved the performance of the assay [26], it could not be assessed here, since only qualitative results of GM were available.

In DE/culture-negative BAL, the PCR and GM sensitivity were lower (38% and 58%) and confirmed previous studies which reported a 33–36% and 41–67% sensitivity, respectively [20, 22, 26]. Positive control amplification in all samples excluded the presence of PCR inhibitors, which was consistent with previously reported increased DNA quality provided by using commercial kits [31]. The sensitivity could also have been decreased by potential damage on DNA samples (stored at -20°C and retrospectively collected) or by non-*Aspergillus* infections that could yield positive DE or GM (cross-reactivity with other species), whereas PCR detection remained negative.

The comparison of PCR sensitivity according to GM results yielded a lower sensitivity in GM-negative (36%) than in GM-positive (57%) BAL. Although other studies usually used GM as a criterion for the selection of patients and rarely included patients at risk of IPA with GM-negative BAL samples, our results seemed to be consistent with the recent study which included two GM-negative BALs and reported a PCR sensitivity of 50%, whereas the sensitivity of PCR in GM-positive samples reached 60% [20]. In addition, PCR was the only positive test in 3 out of 4 GM-negative-PCR-positive BAL. In these samples, PCR improved BAL sensitivity and confirmed that this highly specific test could contribute to IPA diagnosis.

Influence of antifungal treatment on the performance of *Aspergillus* tests

The duration of the antifungal therapy was not significantly related to the results of DE/culture, GM or PCR in our study. However, the antifungal treatment most patients received could have contributed to decrease *Aspergillus* tests' sensitivity, as previously reported in experimental models of IPA [37, 38]. The absence of statistical significance could result from clinical bias, such as the low number of patients or antifungal resistance of the strain responsible for IPA.

Alveolar micro-environment: evaluation of BAL quality and influence on *Aspergillus* detection

Among the criteria analysed, β -globin seems to be the most discriminating, significantly related to an increased *Aspergillus* detection by DE/culture or PCR, suggesting that a high cellularity (reflected by a high β -globin level) could increase conidia, hypha and DNA detection. On the other hand, GM detection seems to be independent from β -globin levels, even if false-positive GM results have been described in BAL [39] and cannot be totally excluded in our study. The absence of the influence of β -globin on GM detection in BAL supernatants could also result from its release in the alveolar micro-environment during growth [7], reducing the impact of cell-recovering efficiency during bronchoscopy on its detection. This could make the GM test fairly independent of BAL quality and explain its high sensitivity compared to DE/culture and PCR, which is more dependent on BAL quality.

Detection in the cell pellet of conidia, hyphae (by DE/culture) and fungal DNA (by PCR) results from their presumed localisation in phagocytes. As suggested by previous studies using cell pellets to detect DNA [15–20, 29] and by a recent study comparing the detection of fungal DNA in BAL pellets and supernatants [33], the major localisation of fungal DNA is probably conidia and hypha, in BAL pellets, rather than free in BAL supernatants, released by live and dead fungal material. However, using BAL supernatants could contribute to IPA diagnosis and should be further evaluated in prospective studies that might contribute to improve our understanding of the physiopathological mechanisms of *Aspergillus* infection.

Even if cellularity followed the same trend as β -globin quantification (higher in PCR-positive and in GM-negative subgroups), there was no significant difference between subgroups when volume and cellularity were analysed (Table 3). Therefore, only β -globin quantification was found to be a significant relevant marker of BAL overall quality when we evaluated the IPA diagnosis tools. The accuracy of β -globin quantification could result from both biological and technical criteria, including cellularity, centrifugation of BAL, efficiency of DNA extraction, PCR method and the absence of inhibitors. Contrarily to cellularity, β -globin levels could be less influenced by cell lysis during the handling of the BAL fluids, whereas nuclear DNA remains in the pellet and seems more reliable to control the yield of the BAL.

The significant differences observed in β -globin subgroups could be useful for the interpretation of both DE/culture and PCR results. Given that a high level of β -globin is associated with positive PCR results in IPA patients, negative PCR with high β -globin levels could more likely reflect the true absence of *Aspergillus* DNA in the sample,

rather than a lack of sensitivity. Similarly, when human DNA content is low, a negative *Aspergillus* PCR result should be interpreted carefully, since false-negatives could occur more frequently, as suggested by a recent study [33]. In BAL with high β -globin levels, the inhibition of the detection of *Aspergillus* DNA present in small quantities by human background DNA should also be taken into account (e.g. patients nos. 15 or 23, Table 1) [40].

Conclusion

Polymerase chain reaction (PCR) on bronchoalveolar lavage (BAL) fluids could contribute to the diagnosis of invasive pulmonary aspergillosis (IPA). Despite recent developments of real-time PCR assays resulting in a high analytical sensitivity of fungal DNA detection, the sensitivity of PCR is still lower than galactomannan (GM) detection in the BAL. Taking into account the DNA content of BAL fluids, using human β -globin DNA quantification for the interpretation of PCR results could improve the value of this test. A minimal β -globin value yielding a 100% NPV could be determined in further prospective studies, and might contribute to validate a consensus approach of fungal PCR in IPA diagnosis.

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