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ORIGINAL ARTICLE

Aspergillus PCR in serum for the diagnosis, follow-up and prognosis of invasive aspergillosis in neutropenic and nonneutropenic patients

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Abstract

We evaluated the usefulness of a serum *Aspergillus* PCR assay for the diagnosis and prognosis of invasive aspergillosis in a study involving 941 patients for a total of 5146 serum samples. Fifty-one patients had proven/probable aspergillosis. We compared galactomannan (GM), PCR and mycologic analysis of pulmonary samples in both neutropenic and nonneutropenic patients. PCR performed in serum yielded 66.7% sensitivity, 98.7% specificity, 75.6% positive predictive value and 98.0% negative predictive value, while the GM index yielded 78.4% sensitivity, 87.5% specificity, 27% positive predictive value and 98.6% negative predictive value. The inclusion of PCR in the European Organization for Research and Treatment of Cancer (EORTC) and the Mycosis Study Group (MSG) mycologic criteria permitted the reclassification of nine other cases from possible to probable aspergillosis and increased the sensitivity to 71.7%. Combining the GM index with serum PCR increased the detection rate of invasive aspergillosis with 88.2% sensitivity. PCR was systematically negative in 16 patients with noninvasive forms of aspergillosis (namely aspergilloma and chronic aspergillosis). Remaining PCR positive after a period of 14 to 20 days of treatment was related to poor outcome at 30 and 90 days. Our results also indicate that, unlike the determination of the GM index, the initial fungus load as determined by PCR was highly predictive of 90-day mortality, with the rate of the latter being 15.8% for patients with <150 copies/mL vs. 73.2% for patients at or above that cutoff ($p < 0.0001$). Therefore, PCR appears to be a powerful and interesting tool for the identification of patients with invasive aspergillosis who might benefit from more intense care.

Keywords: *Aspergillus fumigatus*, fungal infection, galactomannan, haematology, solid organ transplant
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Introduction

Invasive aspergillosis (IA) is a major threat for immunocompromised hosts. Early diagnosis and initiation of appropriate antifungal therapy are essential to improve the prognosis of the disease [1,2]. However, the diagnosis of IA remains difficult. Currently it is frequently based on a set of host, clinical and mycologic criteria, such as those defined jointly by the European Organization for Research and Treatment of Cancer (EORTC) and the Mycosis Study Group (MSG) [3]. In the EORTC/MSG criteria, galactomannan (GM) or β-D-glucan assays are the only noninvasive mycologic diagnostic methods currently available.

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Although these assays are of interest and widely used, they lack sensitivity, especially in nonneutropenic patients [4]. They also may be influenced by the use of certain medical devices [5] or antibiotics [6,7], leading to a large number of false-positive results. GM detection has also been reported in invasive fungal diseases not due to *Aspergillus* [8–10]. Moreover, β-D-glucan is present in many fungus species and is therefore not specific to aspergillosis. Other tools have been developed to improve diagnosis, including in particular real-time PCR in blood samples to detect circulating *Aspergillus* DNA. Several studies have shown the potential interest of this PCR approach, but because of a lack of standardization, it has not yet been included in the EORTC/MSG mycologic criteria. Recently however, a panel of experts has argued for its inclusion in those criteria [11].

For the present study, we aimed at evaluating the performance of an in-house *A. fumigatus* real-time PCR assay using 1 mL volume of serum for the diagnosis of IA in at-risk patients, both neutropenic and nonneutropenic, and comparing the PCR results with those of the GM assay to determine their 30- and 90-day prognostic contributions.

Materials and Methods

Design

A retrospective single-centre analysis was performed between February 2012 and October 2014 in Hôpital de La Pitié-Salpêtrière, Paris, France.

Patients

All patients who are at risk of IA are routinely subjected to monitoring with a serologic assay for the detection of GM antigen and serologic PCR for the detection of *A. fumigatus* DNA. For the present study, we focused on patients with proven/probable IA according to EORTC/MSG criteria [3], extended to include the additional criteria of alcoholic liver cirrhosis, a long stay in the intensive care unit and severe acute respiratory distress syndrome as host factors, as already reported [12,13]. GM either in serum or in bronchoalveolar lavage (BAL) was used as mycologic criterion for probable case. GM determination and PCR results were also available for some patients with noninvasive aspergillosis.

PCR

We used a real-time PCR that targets a previously described 67 bp segment of a 28S ribosomal RNA coding DNA [14,15], for which the primer sequences were 5′-CTCGCA ATGTATCACCTCTCGG3′ and 5′TCCTCGGTCCAGGCAG G-3′ and the probe was 5′-(6FAM)TGTCTTATAGCCGAGGG TGCAATGGG(TAMRA)-3′. DNA extraction was performed on 1 mL of serum with the MagNA Pure Compact large volume kit on a MagNA Pure device (Roche). Elution volume was 50 µL. Amplification was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems). Quantification was achieved using five serial tenfold dilutions of the plasmid PGEMT Easy-Afu28S containing the target. The final PCR result was expressed in numbers of copies per millilitre of serum. We used an internal control in our assay for all wells (TaqMan exogenous Internal Positive Control) as well as an extraction control (albumin gene) for each sample. All PCRs were performed in duplicate. A single positive well was considered to be a positive result.

GM determination

The GM index was determined by enzyme immuno-assay (Bio-Rad) according to the manufacturer's recommendations. A result was considered positive after two determinations, performed on two different assays but on the same sample, showing both an index above 0.5 for serum and 1 for BAL.

Statistical analysis

Tests were performed using GraphPad Prism 5 software and the online site BiostaTGV (http://marne.u707.jussieu.fr/biostatgv/).

Results

Patients

Over the study period, GM assay and *A. fumigatus* PCR were performed in 970 patients (Fig. 1). Clinical data were available for 941 patients (5146 serum samples). A diagnosis of proven or probable IA was made, respectively, for six and 45 patients according to the extended EORTC/MSG criteria. Moreover, a noninvasive form of aspergillosis was diagnosed in 16 patients. Although the study was not focused on an exhaustive identification of all possible cases, we did register diagnoses of IA based on host factors and clinical evaluation for nine other patients, classifying them as possible IA according to the EORTC/MSG criteria. These patients had positive serum PCR.

Characteristics and outcomes of patients with aspergillosis

Among the 51 patients with proven or probable aspergillosis, 19 were female and 32 male. Their median age was 56 years (range 20–82 years). Twenty-two patients (43%) were neutropenic (absolute neutrophil count <500/µL) at the time of diagnosis. Underlying conditions were haematopoietic stem cell transplant (n = 17, 33.3%), haematologic malignancies (n = 13, 25.5%), heart transplantation (n = 9, 17.7%), liver transplantation (n = 3, 5.9%), kidney transplantation (n = 1, 2%) and

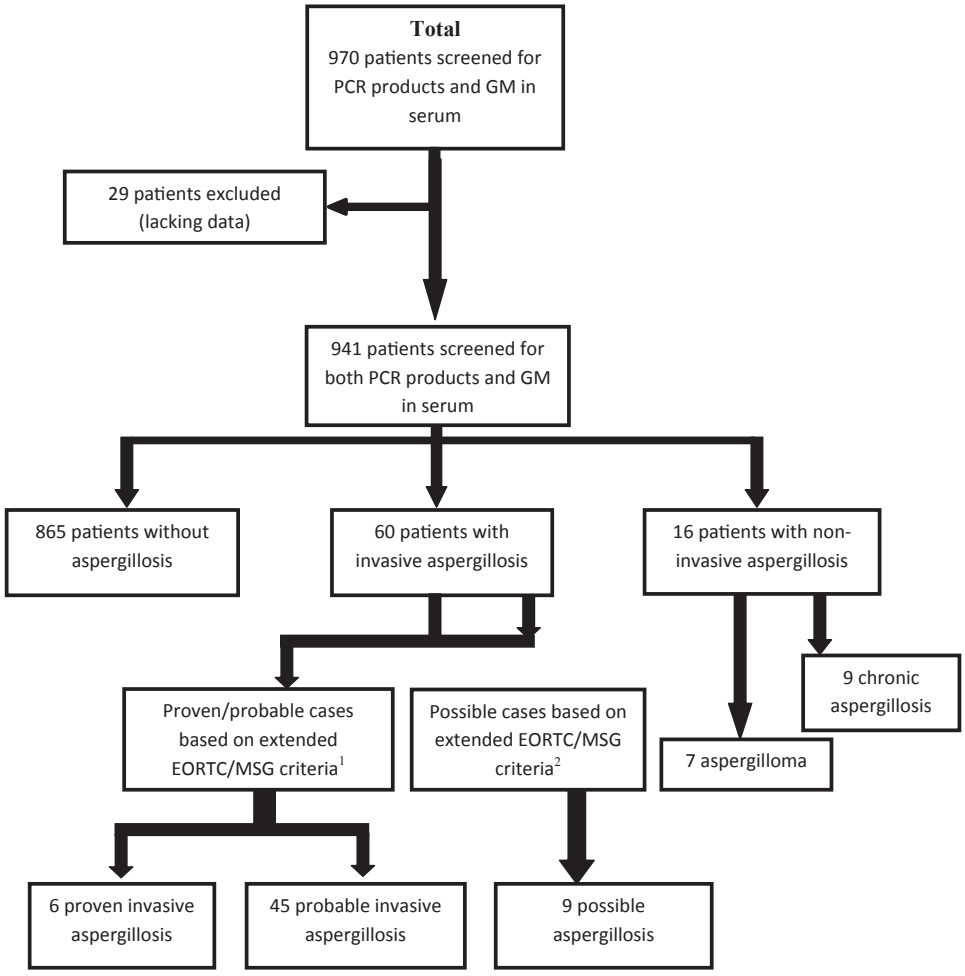


FIG. 1. Flow chart showing number of patients and samples. [1]Extended EORTC/MSG criteria included host factors as published in 2008 plus several other host factors now recognized as leading to risk of developing IA, namely alcoholic liver cirrhosis, severe acute respiratory syndrome, long stay in intensive care unit and solid organ cancer. [2]Study design did not include exhaustive collection of possible cases; we present only possible cases with positive PCR results that were considered as IA by clinicians and treated as such. EORTC/MSG, European Organization for Research and Treatment of Cancer/Mycosis Study Group; IA, invasive aspergillosis.

liver/kidney transplantation (n = 1, 2%). Others risk factors were present in seven patients as follows: severe acute respiratory distress syndrome (n = 2, 3.8%), oncologic diseases (n = 2, 3.8%), alcoholic liver cirrhosis (n = 1, 2%), cardiogenic shock (n = 1, 2%), multiorgan failure and long stay in intensive care unit (n = 1, 2%) (Table 1). Overall 3-month mortality was 49%. Neutropenic patients had a rate of mortality of 54.5% (12/22) vs. 44.8% (13/29) for nonneutropenic patients (p 0.49, Fisher's exact test).

Performance of GM index, mycologic examination of respiratory sample and PCR for diagnosis of proven/probable aspergillosis

A diagnosis of proven/probable IA (according to the extended EORTC/MSG criteria; n = 51) was made for 40 patients by a positive GM index (Fig. 2). PCR was positive in 34 patients (including 11 with a positive GM in serum, four with a positive mycologic analysis of respiratory samples, 18 with both positive GM in serum and positive mycologic analysis of respiratory samples and one with an isolated positive GM in BAL), including five with a negative serum GM index. In one patient with a positive PCR result, the diagnosis of probable aspergillosis was based only on a positive GM index in a BAL sample. Sensitivities of the serum GM index, mycologic examination and serum PCR were 78.4% (95% confidence interval (CI) 67.1–89.7), 62.7% (95% CI 49.4–76) and 66.7% (95% CI 53.8–79.6) respectively. Of note, sensitivity of GM and PCR in case of proven aspergillosis was similar (83.3%; 5/6 patients). Among the patients for whom the diagnosis of any clinical form of aspergillosis was excluded by clinicians, there were 108 with positive GM

TABLE 1. Characteristics of patients with proven/probable invasive aspergillosis according to EORTC/MSG criteria and *Aspergillus* PCR level

Characteristic	EORTC/MSG criteria		According to *Aspergillus* PCR level		
	Without PCR	With PCR	<150 copies	≥150 copies	p
No. of patients	51	60	41	19	
Haematologic malignancy	13 (25.5%)	18 (30%)	10 (24.4%)	5 (26.3%)	0.87
Haematopoietic stem cell transplant	17 (33.3%)	18 (30%)	10 (24.4%)	6 (31.6%)	0.78
Antifungal therapy[a]					0.6
Azole based	36 (70.5%)	42 (70%)	31 (75.6%)	11 (57.9%)	
Non–azole based	13 (25.5%)	16 (26.7%)	10 (24.4%)	6 (31.6%)	
SOT recipient	14 (27.4%)	16 (26.7%)	9 (22%)	5 (26.3%)	0.96
Other	7 (13.7%)	8 (13.3%)	5 (12.2%)	3 (15.8%)	0.7
Interval between time of sample and start of targeted antifungal therapy (mean days)	0.65	0.67	0.8	0.37	0.7
3-month mortality	25 (49%)	27 (45%)	11 (26.8%)	16 (84.2%)	<0.0001

EORTC/MSG, European Organization for Research and Treatment of Cancer/Mycosis Study Group; SOT, solid organ transplant.
[a]Two patients in the >150 copies/mL group died before start of antifungal therapy.

indices, 11 with positive PCR results, and two with positive GM indices and positive PCR results. Specificities of GM and PCR were 87.5% (95% CI 85.3–89.7) and 98.7% (95% CI 97.9–99.5) respectively. PCR yielded 75.6% positive predictive value (PPV) (95% CI 63.1–88.1) and 98.0% negative predictive value (NPV) (95% CI 97.1–98.9), while the GM index yielded 27% PPV (95% CI 19.8–34.2) and 98.6% NPV (95% CI 97.8–99.4). No differences were observed between PCR and GM as concerns the precocity of diagnosis (data not shown).

PCR results for noninvasive forms of aspergillosis

Among the 16 noninvasive aspergillosis patients, ten were immunocompetent, three had metastatic malignancy, two had solid organ transplants and one had alcoholic liver cirrhosis. Clinical forms were simple aspergilloma (n = 7), colonization (n = 4), chronic cavitary aspergillosis (n = 4) and chronic bronchitis (n = 1). In addition to clinical condition, host history and radiologic features, patient diagnoses were further supported by mycologic examination (n = 11), presence of anti-*Aspergillus* antibodies (n = 1) or both (n = 4). None of these patients had positive serum GM or PCR.

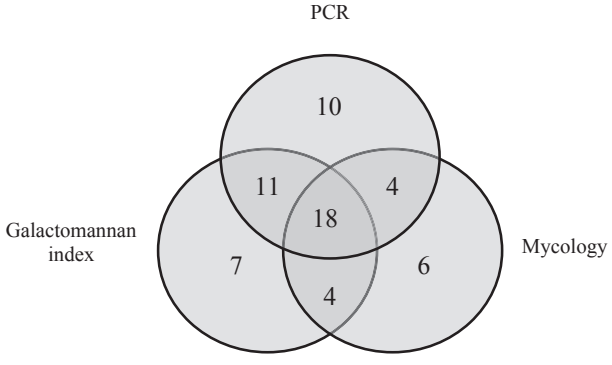


FIG. 2. Venn diagram showing data for patients with invasive aspergillosis (n = 60). Diagram shows data for patients for whom PCR products and GM in sera as well as mycologic analysis of respiratory samples were available. Among ten patients with only positive PCR, one was positive for GM in bronchoalveolar lavage samples. GM, galactomannan.

Addition of PCR to the mycologic criteria

Of the nine patients treated for IA but classified as possible cases due to a lack of EORTC/MSG mycologic criteria, seven were male and two were female. Six patients were neutropenic (five haematologic malignancies and one haematopoietic stem cell transplant) and three were not (one liver transplantation, one kidney transplantation and one severe acute respiratory distress syndrome). All nine of these patients had positive PCR results.

Thus, considering PCR as a mycologic criterion would have enabled the reclassification of nine patients from possible to probable IA, increasing the number of patients with proven/probable IA to 60 (Tables 1 and 2). In this scenario, GM determination and PCR would have been positive in 40 and 43 of the 60 patients respectively, conferring sensitivities of 66.7% (95% CI 54.8–78.6) and 71.7% (95% CI 60.3–83.1) and specificities of 87.7% (95% CI 85.5–89.9) and 98.8% (95% CI 98.1–99.5) for GM and PCR respectively. PCR would yield 79.6% PPV (95% CI 68.9–90.3) and 98.0% NPV (95% CI 97.1–98.9), while the GM index would yield 27% PPV (95% CI 19.8–34.2) and 97.5% NPV (95% CI 96.4–98.6).

Neutropenic versus nonneutropenic

Considering these 60 patients, PCR sensitivity tended to be better in neutropenic patients (82.1%) than in nonneutropenic patients (62.5%). The difference did not reach statistical significance (p 0.09), but the power of the test (P = 39%) was insufficient to draw conclusions. PCR specificity did not differ

TABLE 2. Performance of PCR to detect *Aspergillus fumigatus* in serum, determination of galactomannan index in serum and mycologic examination of respiratory samples for the diagnosis of invasive aspergillosis in 60 patients treated for proven/probable invasive aspergillosis according to extended EORTC/MSG criteria with the addition of PCR in the mycologic criteria

Method	Neutrophil status	Group	No. of samples with result		Sensitivity	Specificity	Positive predictive value	Negative predictive value	p[b]	
			Positive	Negative						
Galactomannan	All patients	IA[a]	40	20	66.7	87.7	27	97.5		
		Non-IA	108	773						
	Neutropenic	IA	19	9	67.8	83.9	22	97.5	0.93	Compared to nonneutropenic
		Non-IA	67	350					<0.005	Compared to nonneutropenic
	Nonneutropenic	IA	21	11	65.6	91.2	33.9	97.5		
		Non-IA	41	423						
Mycologic examination	All patients	IA	32	14	69.6	NA	NA	NA		
	Neutropenic		8	9	47					
	Nonneutropenic		24	5	82.7					
PCR	All patients	IA	43	17	71.7	98.8	79.6	98		
		Non-IA	11	870						
	Neutropenic	IA	23	5	82.1	98.1	74.2	98.8	0.09	Compared to nonneutropenic
		Non-IA	8	409					0.09	Compared to nonneutropenic
	Nonneutropenic	IA	20	12	62.5	99.4	87	97.5		
		Non-IA	3	461						

EORTC/MSG, European Organization for Research and Treatment of Cancer/Mycosis Study Group; IA, invasive aspergillosis; NA, not available.
[a]Criteria used for classification of IA were those defined jointly by the EORTC/MSG consensus and published in 2008 with additionally inclusion of PCR as a mycologic criteria and minor modifications for host factors (e.g. inclusion of alcoholic liver cirrhosis).
[b]As calculated by chi-square test between neutropenic and nonneutropenic patients.

between the two groups. As for the GM index, sensitivity did not differ between the two groups, but specificity was significantly lower in neutropenic patients (83.9%) compared to nonneutropenic patients (91.2%) (p <0.005).

Effect of antifungal therapy

Among our 60 patients with IA, 49 had at least one sample taken before initiation of targeted antifungal therapy. All patients had a sample taken after treatment initiation except for five patients who died early. At the time of diagnosis, 24 patients received antifungal therapy: 12 received empirical therapy with caspofungin and 12 received more targeted anti-*Aspergillus* therapy with voriconazole or amphotericin B. Among the patients who received antifungal drugs, PCR and GM yielded similar sensitivity of 70.8% (17/24), and there was also no difference according to the antifungal drug provided. Among the 36 patients without antifungal therapy, sensitivity was 72.2% (26/36) for PCR and 63.9% for GM (23/36). The difference is not statistically significant (p 0.6). When comparing patients who did and did not receive antifungal therapy, there was no statistically significant difference of sensitivity for PCR or GM.

Variation of fungus load after treatment initiation and prediction of outcome

Real-time PCR allows the quantification of fungus load, with results expressed as number of *Aspergillus* gene copies per millilitre of serum. GM results are presented as an index which can be interpreted quantitatively. Considering this, we assessed variations (decreases or increases) in PCR and the GM index to determine their ability to predict treatment efficacy and 30- and 90-day mortality.

Concerning the response to targeted antifungal therapy, we observed no early variations in PCR results because fungus loads did not decrease quickly after the initiation of treatment, even when outcomes were favourable. Moreover, an increase or decrease in the number of copies in the first week was not related to day 30 or day 90 outcome. However, patients who become PCR negative (and one who had cleared more than 99% of the initial load) between day 14 and day 20 after treatment initiation were all alive at day 30 (n = 9) while those who remained PCR positive during this period had poor outcomes, with 80% (4/5) mortality at day 30 (Table 3). This result was highly significant (p <0.005, Fisher's exact test). PCR results between days 14 and 20 were also predictive of day 90 outcome (Table 3).

TABLE 3. Results of *Aspergillus* PCR after 14 days of antifungal therapy related to day 30 and day 90 outcome

Aspergillus PCR result in serum sampled between days 14 and 20	Outcome at day 30			Outcome at day 90		
	Alive	Dead	p	Alive	Dead	p
Positive	1	4	<0.005	0	5	<0.05
Negative[a]	9	0		7	2	

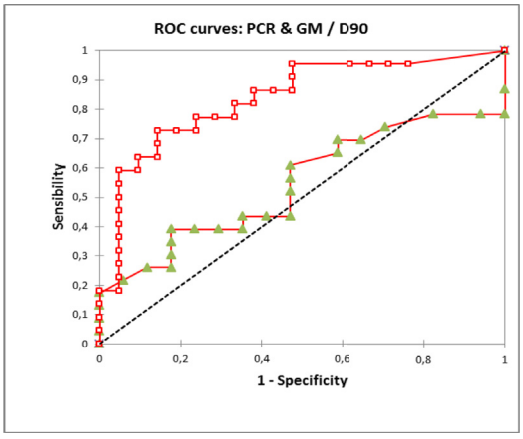
All patients had positive PCR result at time of diagnosis and had at least one serum sample taken between 14 and 20 days after initiation of targeted antifungal treatment and tested for PCR; p value was determined by Fisher's exact test.
[a]One patient who cleared more than 99% of the initial load was considered negative.

Initial fungus load and prediction of outcome

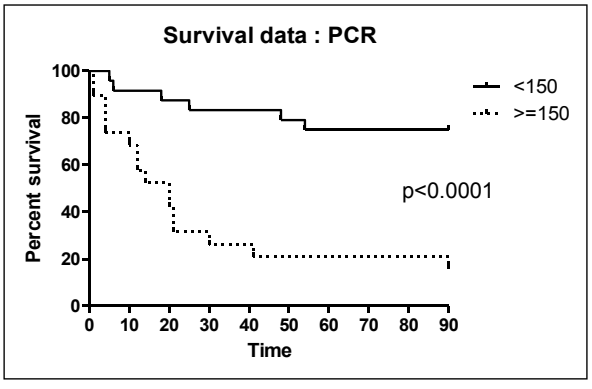
We also assessed initial fungus loads as predictors of outcome. Receiver operating characteristic curves for the evaluation of fungus load as a marker of 90-day mortality in IA indicated that the 150 copies/mL cutoff offered the most efficient value (Fig. 3a). Patients with PCR results strictly below 150 copies/mL had a higher probability of survival 90 days after diagnosis (n = 30/41, 73.2% survival) compared to those with PCR results at or above this cutoff (n = 3/19, 15.8% survival, median survival 20 days) (Fig. 3b). This result was highly statistically significant (p <0.0001, log rank test). The test had a hazard ratio of 0.14 (95% CI of ratio 0.05 to 0.34). Similar results were obtained when all patients were considered, i.e. including the 17 patients with negative PCR in the <150 copies/mL group. There were no differences between these two groups in terms of age, sex ratio, underlying diseases or antifungal therapy (Table 1). There were also no differences concerning the interval between sampling and the start of targeted antifungal therapy. In comparison, among patients with initial positive GM, patients with GMs below 2 (the most efficient value) appear to have more favourable outcomes than others, although the difference was not statistically significant (p 0.19, log rank test) (Fig. 3c).

Discussion

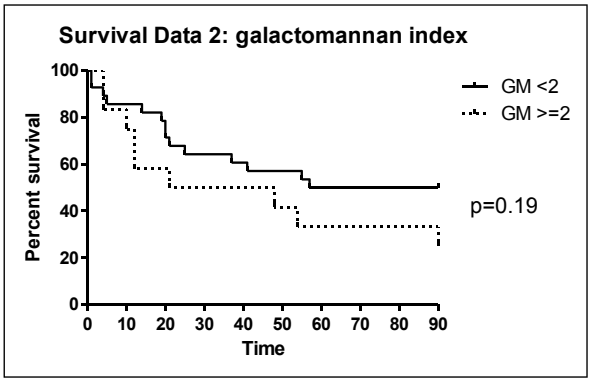
PCR was originally excluded from the 2008 EORTC/MSG definitions of invasive fungal diseases, but expert consensus now



(a)



(b)



(c)

FIG. 3. Serum *Aspergillus* PCR is highly predictive of 90-day mortality in IA. (a) ROC curve for evaluation of PCR (square) or GM (triangle) as marker of 90-day mortality in IA. Cutoff of 150 copies/mL offers most efficient value and area under curve of 0.837. For GM index cutoff of 2 (most efficient value) is related to small area under curve of 0.546. (b) Patients with initial fungus loads <150 copies/mL (n = 41, 73.2% survival) have more favourable outcomes than other patients (n = 19, 15.8% survival); p <0.0001 by log rank (Mantel-Cox) test, hazard ratio 0.14 (95% CI of ratio 0.05 to 0.34). (c) Patients with initial GMs below 2 (n = 28, 50% survival) appear to have more favourable outcomes than others (n = 12; 25% survival), but difference is not statistically significant (p 0.19 by log rank (Mantel-Cox) test; hazard ratio 0.5, 95% CI of ratio 0.20 to 1.29). CI, confidence interval; GM, galactomannan; IA, invasive aspergillosis; ROC, receiver operating characteristic.

considers that this method is mature enough for its inclusion [11]. Meta-analyses have indeed demonstrated that PCR offers sensitivity ranging from 77 to 88% and specificity from 75 to 94.1%, rates similar to those attained with GM assays [16–18]. Moreover, PCR permits the use of quality control and interlaboratory checks, and it provides a more robust quantification assessment than does the determination of the GM index.

The results of the present study add to this evidence, with PCR sensitivity reaching 71.7% and specificity 98.7%. In our study, PCR appeared to offer an interesting PPV, i.e. 79.6% for all patients, in contrast to the GM index, where a high-false positive rate, particularly in neutropenic patients, led to a poor overall PPV of 27%. PCR and GM both offered interesting negative predictive values above 97%. PCR was more sensitive in neutropenic patients (82.1%) than it was in nonneutropenic patients (62.5%), but the difference was not significant (p 0.09). Increasing the number of patients could lead to a significant result. Nevertheless, our study shows that PCR is also useful in nonneutropenic patients and nonhaematologic populations such as solid organ transplant recipients. As previously reported, our result indicate that performing both GM determination and PCR on the same sample increases the sensitivity [18].

In 2010 Koo *et al.* [19] reported a relation between GM and outcome, and in 2012 Bergeron *et al.* [20] reported that patients with poor outcomes 45 days after the initiation of antifungal treatment had high baseline serum GM. We, however, found only a nonstatistically significant trend for the GM index in the determination of outcomes. Nevertheless, Bergeron *et al.* also found no association between outcome and *Aspergillus* DNA detection, while our work supports that the initial level of *Aspergillus* DNA is highly predictive of the 90-day mortality rate. As concerns our results more specifically, we found that a PCR threshold of 150 copies/mL could discriminate patients with low (below the threshold) or high (above) probabilities of 90-day mortality. PCR thus has a much greater discriminative capacity than does the determination of the GM index. This result could be useful for identifying patients who may benefit from more intensive care and designing further clinical studies. We also showed that PCR quantification is useful in follow-up to predict outcomes. After only 1 week of therapy, an increase or decrease in the number of copies is not relevant for the assessment of treatment efficacy or outcome. However, PCR results after 2 weeks of treatment did appear to have relevance. In our study, patients who had a negative PCR (to which we add one patient who had cleared more than 99% of the initial load) between days 14 and 20 were all alive at day 30, and seven of them were still alive at day 90 vs. none of the five patients who remained PCR positive.

In a work initiated by the European *Aspergillus* PCR Initiative, White *et al.* [21] clearly demonstrated that PCR performed on plasma gives better results than PCR performed on serum. In consideration of this evidence, we are now changing our methods. The PCR that we used in the study was specific to the *fumigatus* species, and thus it would not have amplified the genomes of other species such as *flavus, niger* or *nidulans*. This may explain at least in part the relative lack of sensitivity for PCR in our study, as non-*fumigatus* species may account for more than 25% of disease [22]. We did, however, retrieve the causative species in 32 of our cases; strikingly, all were *fumigatus*. Going forward, the utility of PCR in IA and the determination of cutoffs will have to be evaluated in other laboratory specimens, such as cerebrospinal fluid and BAL.

Conclusion

Aspergillus DNA detection in serum by PCR is a interesting tool for the diagnosis of IA in both neutropenic and nonneutropenic patients and should be used concomitantly with the GM index in at-risk populations. Moreover, PCR allows quantification of fungus load; our results indicate that a threshold of 150 copies/mL is very powerful to discriminate patients with low (below the threshold) or high (above) probabilities of 90-day mortality. Therefore, initial fungus load and variations in fungus load during treatment as determined by PCR are robust predictive markers of mortality that may be used to identify patients who might benefit from closer and more attentive care.

Transparency Declaration

All authors report no conflicts of interest relevant to this article.

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