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

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Running title: Aspergillus PCR for the prognosis of invasive aspergillosis

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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 5,146 serum samples. Fifty-one patients had proven/probable aspergillosis. We compared galactomannan, PCR, and mycological analysis of pulmonary samples in both neutropenic and non-neutropenic 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 galactomannan index yielded 78.4% sensitivity, 87.5% specificity, 27% positive predictive value and 98.6% negative predictive value. The inclusion of PCR in the EORTC/MSG mycological criteria permitted the reclassification of nine other cases from possible to probable aspergillosis and increased sensitivity to 71.7%. Combining the galactomannan index with serum PCR increased the detection rate of invasive aspergillosis with 88.2% sensitivity. PCR was systematically negative in 16 patients with non-invasive 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 galactomannan index, the initial fungal load as determined by PCR was highly predictive of 90-day mortality, with the rate of this latter being 15.8% for patients with less than 150 copies/mL vs 73.2% for patients at or above that cut-off (p<0.0001). Therefore, PCR appears to be a very powerful and interesting tool for the identification of patients with invasive aspergillosis who might benefit from more intense care.

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 mycological criteria, such as those defined jointly by the European Organisation 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 non-invasive mycological diagnostic methods currently available. Although these assays are of interest and widely used, they lack sensitivity, especially in non-neutropenic 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 fungal 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 due to a lack of standardisation, it has not yet been included in the EORTC/MSG mycological criteria. Very recently however, a panel of experts 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 non-neutropenic, 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 La Pitié-Salpêtrière hospital, in 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 serological 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 ICU and severe acute respiratory distress syndrome as host factors, as already reported [12, 13]. GM either in serum or in broncho-alveolar lavage (BAL) was used as mycological criterion for probable case. GM determination and PCR results were also available for some patients experiencing non-invasive 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’CTCGCAATGTATCACCTCTCGG3’ and 5’TCCTCGGTCCAGGCAGG3’ and the probe was 5’(6FAM)TGTCTTATAGCCGAGGGTGCAATGGG(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 5 serial ten-fold dilutions of the plasmid PGEMT Easy-Afu28S containing the target. The final PCR result was expressed in numbers of copies per mL 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 as a positive result.

Galactomannan determination. The GM index was determined by enzyme immuno-assay (BioRad) 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 and the free 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 (Figure 1). Clinical data were available for 941 patients (5,146 serum samples). A diagnosis of proven or probable IA was made respectively for 6 and 45 patients according to the extended EORTC/MSG criteria. Moreover, a non-invasive form of aspergillosis was diagnosed in 16 patients. Also, 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 others 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 (Table 1).

Among the 51 patients with proven/probable aspergillosis, 19 were female and 32 male. Their median age was 56 years (range 20-82). 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%), haematological malignancies (n=13; 25.5%), heart transplantation (n=9; 17.7%), liver transplantation (n=3; 5.9%), kidney transplantation (n=1; 2%) and 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%), oncological diseases (n=2; 3.8%), alcoholic liver cirrhosis (n=1; 2%), cardiogenic shock (n=1; 2%), multi-organ failure and long stay in intensive care unit (n=1; 2%). Overall three-month mortality was 49%. Neutropenic patients had a rate of mortality of 54.5% (12/22) vs 44.8% (13/29) for non-neutropenic patients (p=0.49 by Fischer exact test).
Performance of the galactomannan index, mycological examination of respiratory sample and PCR for the 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 (Figure 2). PCR was positive in 34 patients (including 11 with a positive GM in serum, 4 with a positive mycological analysis of respiratory samples, 18 with both positive GM in serum and positive mycological analysis of respiratory samples and 1 with an isolated positive GM in BAL), including five with a negative serum GM index. In one patient with a positive PCR, the diagnosis of probable aspergillosis was based only on a positive GM index in a bronchoalveolar lavage sample.

Sensitivities of the serum GM index, mycological 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 indices, 11 with positive PCR results, and 2 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 galactomannan 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 non-invasive forms of aspergillosis.

Among the 16 non-invasive aspergillosis patients, 10 were immunocompetent, 3 had metastatic malignancy, 2 had solid organ transplantation and one had alcoholic liver cirrhosis.
Clinical forms were simple aspergilloma (n=7), colonisation (n=4), chronic cavitary aspergillosis for (n=4) and chronic bronchitis (n=1). In addition to clinical condition, host history and radiological features, the patient diagnoses were further supported by mycological examination (n=11), the presence of anti-Aspergillus antibodies (n=1) or both (n=4). None of these patients had positive serum GM or PCR.

**Addition of PCR to the mycological criteria.**

Of the 9 patients treated for IA but classified as possible cases due to a lack of EORTC/MSG mycological criteria, 7 were male and 2 were female. Six patients were neutropenic (five haematological malignancies and one haematopoietic stem cell transplant) and 3 were not (one liver transplantation, one kidney transplantation and one severe acute respiratory distress syndrome). All 9 of these patients had positive PCR results.

Thus, considering PCR as a mycological criterion would have enabled the reclassification of 9 patients from possible to probable IA, increasing the number of patients with proven/probable IA to 60 (Table 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 galactomannan index would yield 27% PPV (95% CI, 19.8-34.2%) and 97.5% NPV (95% CI, 96.4-98.6%).

**Neutropenic versus non-neutropenic.**

Considering these 60 patients, PCR sensitivity tended to be better in neutropenic patients (82.1%) than in non-neutropenic patients (62.5%). The difference did not reach statistical
significance (p=0.09) but the power of the test (P=39\%) was insufficient to conclude. PCR specificity did not differ 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 non-neutropenic patients (91.2\%) (p<0.005).

Effect of antifungal therapy.

Among our 60 patients with IA, 49 have at least one sample before the targeted antifungal therapy initiation. All patients had a sample after treatment initiation except for 5 patients who died early. At the time of diagnosis, 24 patients received antifungal therapy: 12 had empirical therapy with caspofungin and 12 have more targeted anti-Aspergillus therapy with voriconazole or amphotericin B. Among the patients who received antifungal drugs, PCR and GM yield similar sensitivity of 70.8\% (17/24) and there was also no difference according to the antifungal drug that was used. 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 with antifungal therapy and patients without, there were also no statistically significant difference of sensitivity either for PCR, or for GM.

Variation of the fungal load after treatment initiation and prediction of outcome.

Real time PCR allows the quantification of fungal load with results expressed as the number of Aspergillus gene copies per mL 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 as fungal 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 with 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 by Fischer exact test). PCR results between day 14 and day 20 were also predictive of day 90 outcome (Table 3).

**Initial fungal load and prediction of outcome.**

We also assessed initial fungal loads as predictors of outcome. ROC curves for the evaluation of fungal load as a marker of 90-day mortality in invasive aspergillosis indicated that the 150 copies/mL cut-off offered the most efficient value (Figure 3a). Patients with PCR results strictly below 150 copies/mL had a higher probability of survival 90 days after the diagnosis (n=30/41; 73.2% survival), compared to those with PCR results equal to or above this cut-off (n=3/19; 15.8% survival; median survival 20 days) (Figure 3b). This result was highly statistically significant (p<0.0001 with 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 but the difference was not statistically significant (p=0.19 by Log-rank test) (Figure 3c).

**DISCUSSION.**

PCR was originally excluded from the 2008 EORTC/MSG definitions of invasive fungal diseases, but expert consensus now 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 inter-laboratory checks, and 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 stark 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 non-neutropenic 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 non-neutropenic patients and non-haematological 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 reported a relation between GM and outcome [19] and in 2012 Bergeron et al reported that patients with poor outcomes 45 days after the initiation of antifungal treatment had high baseline serum GM [20]. We however found only a non-statistically-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 study. We also
showed that PCR quantification is useful in follow-up to predict outcomes. After only one
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 7 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 clearly
demonstrated that PCR performed on plasma gives better results than PCR performed on
serum [21]. 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 others 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 and, strikingly, all were fumigatus. Going forward, the utility of
PCR in IA and the determination of cut-offs will have to be evaluated in other laboratory
specimens, such as cerebrospinal fluid and bronchoalveolar lavage.
CONCLUSION.

Aspergillus DNA detection in serum by PCR is a interesting tool for the diagnosis of invasive aspergillosis in both neutropenic and non-neutropenic patients and should be used concomitantly with the galactomannan index in at-risk populations. Moreover, PCR allows quantification of fungal load and 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. So, initial fungal load and variations in fungal 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.

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Part of the data was presented in French at the Congress of the French Society for Medical Mycology (SFMM: Société Française de Mycologie Médicale) held 21 May 2015. The presentation was awarded the Oral Communication Prize. A presentation of part of the data has been performed as an oral communication at the Trends in Medical Mycology international congress, held in October 2015 in Lisbon, Portugal.

TRANSPARENCY DECLARATION. The authors declare no conflicts of interest.

FUNDING. This work was supported by internal funding.

FIGURES CAPTION

Figure 1: flow chart describing the number of patients and samples included in the study.
1: extended EORTC/MSG criteria included host factors as published in 2008 plus several other host factors now recognised as leading to a risk of developing invasive aspergillosis, namely alcoholic liver cirrhosis, severe acute respiratory syndrome, long stay in intensive care unit, and solid organ cancer.

2: the study design did not include an exhaustive collection of possible cases; we present only the possible cases with positive PCR results that were considered as invasive aspergillosis by clinicians and treated as such.

Figure 2: Venn diagram showing data for patients with invasive aspergillosis (n=60). Diagram shows data for patients for whom PCR products and galactomannan in sera as well as mycological analysis of respiratory samples were available. Among the 10 patients with only positive PCR, one was positive for galactomannan in bronchoalveolar lavage samples.

Figure 3: serum Aspergillus PCR is highly predictive of 90-day mortality in invasive aspergillosis.

3a. ROC curve for the evaluation of PCR (square) or GM (triangle) as a marker of 90-day mortality in invasive aspergillosis. The 150 copies/mL cut-off offers the most efficient value and an area under the curve of 0.837. For GM index cut-off of 2 (the most efficient value) is related to a small area under the curve of 0.546.

3b. Patients with initial fungal loads below 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). 3c. Patients with initial GMs below 2 (n=28; 50% survival) appear to have more favourable outcomes than others.
(n=12; 25% survival) but the 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).

REFERENCES.


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<table>
<thead>
<tr>
<th>Characteristics of patients with proven/probable invasive aspergillosis</th>
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<sup>a</sup>: two patients in the “>150 copies/mL” group died before the start of antifungal therapy
Figure 1

Total
970 patients screened for PCR products and GM in serum

29 patients excluded (lacking data)

941 patients screened for both PCR products and GM in serum

865 patients without aspergillosis

60 patients with invasive aspergillosis

16 patients with non-invasive aspergillosis

Proven/probable cases based on extended EORTC/MSG criteria

Possible cases based on extended EORTC/MSG criteria

6 proven invasive aspergillosis

45 probable invasive aspergillosis

9 possible aspergillosis

9 chronic aspergillosis

7 aspergilloma
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Table 2: Performance of PCR to detect *Aspergillus fumigatus* in serum, determination of galactomannan index in serum and mycological 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 mycological criteria

a IA: invasive aspergillosis; criteria used for classification were those defined jointly by the EORTC/MSG consensus and published in 2008 with, additionally, the inclusion of PCR as a mycological criteria and minor modifications for host factors (e.g. inclusion of alcoholic liver cirrhosis)

b as calculated by the Chi-square test between neutropenic and non-neutropenic patients

c not available
Figure 2

- **PCR**: 10
- **Galactomannan index**: 11, 18, 7, 4
- **Mycology**: 4, 6

Counts: 10, 11, 18, 7, 4, 6
Figure 3: serum *Aspergillus* PCR is highly predictive of 90-day mortality in invasive aspergillosis.