

Real-time PCR on the first galactomannan-positive serum sample for diagnosing invasive aspergillosis in liver transplant recipients

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Abstract: Invasive aspergillosis (IA) is a life-threatening complication of liver transplantation. Detection of circulating galactomannan (GM) in serum samples is a method to improve the microbiological diagnosis in patients at risk for IA. However, the assay is hampered by false-positive results. The search for circulating *Aspergillus* DNA in the first GM-positive sample could improve the specificity of the test. Among 484 liver transplant recipients followed in a single center over 4 years, 25 patients had at least 1 GM-positive serum sample. The threshold of GM positivity was a ratio ≥ 1 . These 25 patients were classified by the clinicians as probable IA ($n = 11$), possible IA ($n = 2$), and no IA ($n = 12$) using the EORTC/MSG criteria with blinding to the polymerase chain reaction (PCR) results. After 1 mL aliquots of the first GM-positive serum sample were thawed, 2 independent DNA extractions were performed using the MagNA Pure Compact apparatus. Real-time amplification targeted at *Aspergillus fumigatus* mitochondrial DNA was performed on 10 μ L of the final eluate in duplicate in the 2 independent DNA extractions using a LightCycler instrument. A sample was considered positive when the crossing point was ≤ 43 cycles in at least 2 out of the 4 replicates. Among the 13 probable or possible IA, 8 patients were PCR positive. The other 12 patients who had no IA were all PCR negative. Our data suggest that a concomitant real-time PCR performed on the first GM-positive sample improves the specificity of the first GM-positive assay result.

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Key words: invasive aspergillosis; galactomannan; real-time PCR; liver transplantation

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Received 12 September 2007, revised 7 February 2008, accepted for publication 17 March 2008

DOI: 10.1111/j.1399-3062.2008.00323.x

Transpl Infect Dis 2008; 10: 333–338

Invasive aspergillosis (IA) occurs in 1–8% of liver transplant recipients and is associated with a high mortality rate, ranging from 60% to 80% (1). This poor prognosis may be due at least in part to a late diagnosis, which in turn results in delayed antifungal specific therapy. Indeed, the diagnosis of IA remains challenging. Imaging is not specific and is difficult to analyze after abdominal surgery. Mycology cultures are positive in <1.5% of cases (1). Therefore, additional biological tests are under investigation to improve the diagnosis (2). Among these tests, galactomannan (GM) detection is the most studied, and several teams have implemented

serial screenings of GM detection in their patients at risk for IA (3, 4). GM is a polysaccharide cell-wall component whose release varies during fungal growth (5). Detection is achieved using a sandwich ELISA commercially available (Platelia Aspergillus, Bio-Rad, Marnes-la-Vallée, France), which has been approved by the US Food and Drug Administration for use with serum samples. The performance of the assay has been shown to be reliable enough to be included in the microbiological criteria for probable and possible IA in patients with hematological malignancy (6). However, a recent meta-analysis found only a moderate accuracy of the test for diagnosis of IA in solid organ transplant (7). Additionally, GM contamination of some antibiotic batches of piperacillin-tazobactam (8–11) or amoxicillin-clavulanic acid (12–14) has increased the possibility of irrelevant false-positive results. Therefore,

This study was presented in part at the 16th Congress of International Society for Human and Animal Mycology, June 25–29, 2006, Paris, France.

to confirm a first positive result, a second test with another sample is required. This procedure could improve the predictive positive value but might delay specific therapy.

In contrast to GM, the results of polymerase chain reaction (PCR) assays are not included in the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) consensus defining IA (6). This omission is mainly the consequence of divergent results obtained with non-standardized PCR assays. The reliability of the PCR results is much improved using real-time PCR, mainly because the risk of false-positives due to cross-contamination is dramatically decreased (15). Moreover, the quantitative results are more amenable to comparison between laboratories. This new technology is replacing the so-called classical PCR (16–19).

We wondered whether detecting circulating *Aspergillus* DNA in the first positive GM sample by real-time PCR could help in diagnosing IA in liver transplant patients. A real-time PCR performed on the same serum sample, as soon as the result of GM is known, could shorten the time response and help in therapeutic decision-making. Therefore, we retrospectively evaluated the first GM-positive serum sample, collected as part of our routine practice, for *Aspergillus* DNA by real-time PCR.

Patients and methods

Between January 2000 and December 2004, 484 patients underwent a liver transplantation at the Paul Brousse Hospital (Villejuif, France). The transplanted patients were routinely screened for GM once a week during the first 3 weeks post transplantation and thereafter occasionally if fever or respiratory symptoms occurred. When received at the laboratory, the serum was split into 2 parts, an aliquot was used for the GM assay and the other one immediately stored at -20°C and subsequently used for PCR testing. The GM assay was performed according to the manufacturer's instructions. Only samples with a ratio ≥ 1 were considered positive, as common practice in 2000 (20, 21), and were stored. The local ethical committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale) confirmed that no ethical approval from the patients was required, as this observational study did not modify current diagnostic or therapeutic strategies.

The first positive GM serum sample was retrospectively analyzed by real-time PCR with a LightCycler instrument (Roche Diagnostics, Meylan, France) as previously described (17). After thawing, 2 independent DNA extractions were performed for each serum sample from 1 mL using total nucleic acid isolation kit – large volume on a

MagNA Pure Compact Instrument (Roche Diagnostics) and eluted in 50 μL . PCR was performed in duplicate with 10 μL of the elution volume. The DNA target was the mitochondrial DNA from *Aspergillus fumigatus* and *Aspergillus flavus* (17). The primers and probes used were also able to amplify and detect *Aspergillus terreus* and *Aspergillus niger* DNA. Fluorescence curves were analyzed using the LightCycler software, version 3.5. Quantitative results were expressed by determination of the threshold of detection, or crossing point (C_p), which marked the cycle at which fluorescence of the sample became significantly different from the baseline signal. Thus, the higher the C_p value was, the smaller was the amount of DNA in the sample.

Performing 2 DNA extractions and duplicate PCR reactions, we obtained 4 C_p values for each serum sample. Sample was considered positive when C_p was ≤ 43 cycles for at least 2 values. The 43rd cycle was retained as the last one giving a complete amplification curve that can be analyzed. One positive control, using *A. fumigatus* DNA, and 2 negative controls were added to each run. The negative controls were a GM-negative serum sample submitted to the extraction and amplification protocols and a sterile water sample submitted only to the amplification protocol. Strict measures were taken to prevent DNA contamination. Contamination with previously amplified products was prevented by the systematic use of uracil-*N*-glycosylase.

The 25 patients were classified according to the EORTC/MSG criteria as probable, possible, and non-IA (6) by clinicians who were blinded to PCR results. This classification was performed based upon analysis of the entire medical file and was not the diagnosis at the time of the first GM-positive result.

The Epi Info (version 7.0) was used for all the analyses. Sensitivity and specificity were calculated in reference to the diagnosis of IA. Fisher's exact test was used to evaluate differences in categorical data. A Mantel Haenszel test was used to evaluate the role of the piperacillin-tazobactam known to be responsible for false GM EIA-positive results.

Results

Out of the 484 patients who underwent liver transplantation between January 2000 and December 2004, 25 had at least 1 GM-positive result. Table 1 summarizes the clinical and microbiological data from these 25 patients and their outcome. There were 21 males and 4 females with a mean age of 49 years (range 13–69 years, median 48 years). The classification of these 25 patients according to the consensual MSG/EORTC criteria was as follows: 11 patients had

EORTC/MSG classification of invasive aspergillosis, (IA), real-time PCR, and first galactomannan ELISA (GM EIA) results, possible causes of false-positive GM EIA results (piperacillin-tazobactam treatment and renal dialysis), and outcome at 3 months of the studied patients

Sex, age (years)	Underlying disease (number of LT performed)	EORTC/MSG classification	PCR results (cut-off value)	GM EIA ratio	Piperacillin-tazobactam	Dialysis	Outcome (day post GM EIA-positive result)
F, 38	PBC (3)	Probable	+ (38.1)	3.1	No	Yes	Deceased (D1)
M, 62	HCC, HBV (2)	Probable	+ (39.9)	1.1	No	Yes	Deceased (D77)
M, 58	AC (1)	Probable	+ (41.7)	2.2	Yes	No	Deceased (D11)
M, 45	AC (1), HCV	Probable	+ (37.4)	3.6	No	No	Deceased (D16)
M, 67	AC (1)	Probable	Negative	1.7	No	No	Deceased (D27)
M, 66	NASH, HCC	Probable	Negative	3.7	Yes	Yes	Deceased (D9)
M, 62	HCV (2)	Probable	+ (41.2)	1.7	No	No	Deceased (D16)
M, 42	HIV, HCV (1)	Probable	+ (40.7)	3.9	No	No	Deceased (D4)
M, 63	HCV, AC (1)	Probable	Negative	2.2	No	Yes	Deceased (D9)
F, 69	HCC, PBC (1)	Probable	Negative	1.7	Yes	Yes	Deceased (D21)
M, 43	HBV	Probable	Negative	1.5	Yes	Yes	Deceased (D70)
M, 18	FH (2)	Possible	+ (41.3)	6.3	No	No	Alive
F, 38	PBC (1)	Possible	+ (39.5)	1.1	Yes	Yes	Deceased (D33)
F, 64	PBC (2)	Non-IA	Negative	3.5	Yes	Yes	Deceased (D6)
M, 37	FAP (1)	Non-IA	Negative	1.0	Yes	No	Alive
M, 69	AC (1)	Non-IA	Negative	1.3	Yes	Yes	Deceased (D3)
M, 39	HCV, AC (2)	Non-IA	Negative	1.3	Yes	Yes	Deceased (D41)
M, 48	AC (1)	Non-IA	Negative	1.9	No	No	Alive
M, 47	AC (1)	Non-IA	Negative	1.7	No	No	Deceased (D45)
M, 67	AC (1)	Non-IA	Negative	2.3	Yes	No	Alive
M, 24	FH (1)	Non-IA	Negative	5.8	No	No	Alive
M, 63	HCC, HCV (1)	Non-IA	Negative	1.9	No	No	Alive
M, 13	Biliary atresia (1)	Non-IA	Negative	1.5	No	No	Alive
M, 67	HCC, HCV (1)	Non-IA	Negative	1.8	Yes	No	Deceased (D24)
M, 15	Wilson disease (1)	Non-IA	Negative	2.9	Yes	No	Alive

LT, liver transplantation; PBC, primary biliary cirrhosis; HCC, hepatocellular carcinoma; HBV, hepatitis B virus infection; AC, alcoholic cirrhosis; NASH, non-alcoholic steatohepatitis; HCV, hepatitis C virus infection; HIV, human immunodeficiency virus infection; FH, fulminant hepatitis; FAP, familial amyloid polyneuropathy; PCR, polymerase chain reaction; EORTC, European Organization for Research and Treatment of Cancer; MSG, Mycoses Study Group; ELISA, enzyme-linked immunosorbent assay.

Table 1

probable IA, 2 patients had possible IA, and 12 patients had no AI (Table 1). The immunosuppressive regimen consisted of low-dose prednisone and tacrolimus, with or without mycophenolate mofetil. Identified risk factors for IA were primary non-function or dysfunction of the graft ($n = 10$), retransplantation ($n = 4$), acute renal failure requiring hemodialysis ($n = 7$), CMV coinfection ($n = 2$), and fulminant hepatic failure ($n = 1$). For the 13 patients with probable or possible IA, the median time from transplantation to the diagnosis of probable IA was 150 days (range 8–3240 days). Twelve patients had pulmonary lesions and 1 patient

had only central nervous system involvement. The mycological criterion for defining IA was isolation of *A. fumigatus* from the respiratory tract for 3 patients, typical hyphae observed using wet mounting with negative culture for 2 other patients, and more than 2 positive GM results for the remaining 8 patients. All the patients with probable or possible IA but one died in the first 3 months after their first positive GM (range 1–77 days, median 16 days). No autopsy was performed.

Of the 25 patients with GM-positive tests, 12 (48%) were receiving piperacillin-tazobactam and therefore suspected

of false-positive results and 5 of them were finally classified as probable or possible IA (Table 1). No patient was given amoxicillin-clavulanic acid. Three patients had chronic rejection, which has been reported to give false-positive GM results (22). Dialysis has also been reported to increase the false-positive GM results (4). Ten of the 25 patients required dialysis, 7 patients in the IA group and the 3 other patients in the group without IA.

No positive PCR result was observed in the 12 non-IA patients (Table 1) and in the negative controls. Among the 13 patients with IA, 8 patients were PCR-positive with 6 probable and 2 in the possible IA group ($P < 0.001$). The C_p of the positive control at 28.9 fg/ μ L was 32.7 ± 0.5 cycles. As the C_p of the patient results was always > 37 cycles, the circulating DNA was always < 28.9 fg/ μ L. Four patients had 4 positive replicates, 1 patient had 3 positive replicates, and 3 patients had 2 positive replicates. No quantitative correlation was observed between GM titers and the PCR results.

When considering the final classification according to the MSG/EORTC criteria as the reference, the sensitivity, specificity, positive, and negative predictive values of the real-time PCR performed on the first GM-positive sample were 62% (8/13), 100% (12/12), 100% (8/8), and 71% (12/17), respectively. The difference between PCR-positive and PCR-negative patients was significant ($P \leq 0.01$ by Fisher's exact test). The relative risk to have a probable or possible IA when the GM test was positive was 3.4 times (1.6–7.1) higher when the PCR result on the same serum sample was also positive. When the analysis was adjusted according to receiving or not receiving piperacillin-tazobactam, the difference between PCR-positive and PCR-negative patients remained significant ($P \leq 0.001$ by Mantel Haenszel test), and the relative risk to have a probable or possible IA was 3.4 (1.4–7.9) times higher when the PCR result was positive.

Discussion

Our study suggests that real-time PCR performed on the first GM-positive serum samples anticipates the final classification of IA in liver transplant recipients. The relative risk to have a final diagnosis of IA is 3.4 times higher than when the PCR test is negative. The study is not a prospective comparison of GM EIA and real-time PCR, and the aim was not to propose a serial screening using both the tests. To know whether previous or subsequent samples were also PCR positive or not, to support a diagnosis of IA as already suggested (19, 23), was not possible, as the GM-negative samples were not available. The aim of our study was to know the potential value of an additional test

performed on the first GM-positive sample without waiting for supplementary serum samples.

When considering the first GM result alone, our result underlines the high rate of false-positive results (48%), as either no additional GM-positive samples or positive microbiological culture was obtained, or no radiological sign developed after the first GM-positive result, excluding the diagnosis of probable or possible IA according to the EORTC/MSG definitions. Several reasons could have contributed to the poor performance of the first GM-positive result in our patients. Ten patients required renal dialysis, reported to increase the rate of false-positive results (4). Twelve patients received piperacillin-tazobactam, the antibiotic most known for possible GM contamination (8–11). Five of them further developed an IA and 7 did not, showing the difficulty in interpreting a first GM result when the patient is given piperacillin-tazobactam. As a consequence, according to the EORTC/MSG classification, a single GM-positive result could not be considered as a microbiological criterion for the definition of IA. This underlines the necessity to obtain additional serum samples (5), which can be problematic for outpatients. For the patients receiving piperacillin-tazobactam, one can also recover the piperacillin-tazobactam batch number to check for the presence of GM. However, the relative risk of having an IA when the PCR test is positive remains, whether or not piperacillin-tazobactam was administered.

In a recent meta-analysis, Pfeiffer et al. (7) have underlined the poorer performance of the GM in solid organ transplant recipients compared with patients with hematological malignancy. The sensitivity was 41% and 58% and the specificity 86% and 96% for solid organ transplant recipients and for patients with hematological malignancy, respectively (7). One of the reasons is that the pretest probability of IA greatly influences the predictive values of any test. In liver transplant recipients, the prevalence of IA remains low, between 1% and 8% (1). In the present study, the prevalence was 2.7% (13/484), although some patients with isolation of *Aspergillus* sp. from respiratory specimens were not included because of their negative GM results. However, additional tests are not needed for the diagnosis of possible or probable IA when molds are isolated from respiratory specimens (6). The use of antifungal prophylaxis and the protected ICU environment with HEPA filters in our ICU could have contributed to the low prevalence of IA.

Given the difficulties in interpreting a first GM-positive result in liver transplant recipients at high risk for IA, the search for another marker such as circulating DNA could be of high interest. The positive PCR results were all observed in probable and possible IA. Interestingly, none of the 12 patients with a final diagnosis of no IA was PCR-positive. As for patients with hematological malignancies (24), the PCR-positive patients were more likely to die (7/8

patients) than the PCR-negative patients (10/17 patients), but the main finding was the 70% (17/25 patients) mortality rate among the GM-positive patients, whatever be the PCR results. The poor prognosis of patients with positive GM results has already been underlined and can be multifactorial (4). In the present series, the PCR results could have helped in giving antifungal drugs in 1 patient who died without any mold-active antifungal agent because the cerebral signs were attributed to a possible toxoplasmosis. On the other hand, at this time, the withdrawal of antifungal drugs in GM + /PCR – patients cannot be recommended because PCR-negative patients were classified as having probable IA and it is always very difficult to exclude the diagnosis of IA.

Intriguingly, the amount of DNA on the real-time PCR test was always low (17, 24). A role of the storage condition of the serum is always possible. However, there is no clear evidence that storage at -20°C for several years can alter the amounts of DNA (25). Uncontrolled cycles of freezing and thawing can occur at -20°C more often than at -80°C but this has probably little consequence when amplification involves very short DNA sequences as in the present real-time PCR assay. The restricted specificity of the primers and probes for *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger* could have missed an IA with another *Aspergillus* species, but this hypothesis seems unlikely to us. When a positive culture was obtained for the patients studied, only *A. fumigatus* was identified.

The low amount of DNA raises several problems in interpreting real-time PCR results. First, quantification of very small DNA amounts limits the reproducibility of real-time PCR results. Second, PCR results cannot be consistently positive at these very low concentrations, below 10 copies of target DNA. Thus, one replicate can be positive and the other one can be negative, depending on whether the target DNA is present or not in the volume tested. Therefore, we think that several replicates are mandatory to avoid false-negative results. Third, the small DNA amounts prevent any analysis of correlation between DNA and GM quantification. A larger DNA burden could indicate a more advanced disease, as recently suggested with the use of nucleic acid sequence-based amplification (26). Despite the difficulties in analyzing the quantitative results, the main advantage of real-time PCR technology is the low risk of false-positive results due to contamination from the environment (15). In the present study, we used commercial DNA extraction kits and all of our negative controls were negative.

In conclusion, our study suggests that performing real-time PCR, as soon as the first serum sample is GM positive, predicts the final diagnosis and classification of IA. This can help when false-positive GM results are suspected, for example in patients given potentially GM-contaminated

antibiotics. Therefore, the finding of positive GM and PCR results can initiate more invasive search for the fungi, such as bronchoalveolar lavage. Our results also underline the difficulties in interpreting the meaning of biological tests using definitions adapted for hematological patients, and in the absence of definite standards, as autopsies are rarely performed. Prospective and multicentric studies might be promoted to better define the performance of GM and PCR tests but the low prevalence of IA suggests that such studies may be difficult to lead.

Acknowledgements:

The authors thank the staff of the liver transplantation department for taking care of the patients, and the technicians of the mycology laboratory.

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