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Aspergillus galactomannan detection in comparison to a real-time PCR assay in serum samples from a high-risk group of patients

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Abstract

Invasive aspergillosis (IA) is a severe infection with a 70% mortality rate. Aspergillus fumigatus is responsible for over 90% of those infections.

The diagnosis of invasive aspergillosis is based on clinical sample culture and detection of fungal hyphae in histopathological examination. Additional tests may include the detection of the galactomannan antigen and of fungal genetic material in serum and bronchoalveolar washings. The present study was to assess the use of these two rapid tests in the diagnosis of invasive aspergillosis: serological one – to detect the galactomannan antigen (ELISA assay), and real-time PCR, and to establish a possible correlation between these two methods.

Key words: *Aspergillus, real-time PCR, galactomannan, aspergillosis, Mycassay™.*

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Introduction

Invasive aspergillosis (IA) has one of the highest mortality rates in patients under immunosuppressive treatment. Mainly post bone marrow or solid organ transplant patients treated for blood cancer are at high risk. Early detection is crucial in adequate therapy implementation [1].

Invasive aspergillosis diagnosis is a challenge as the infection presents a low radiographic sensitivity and no specific clinical signs. According to 2008 EORTC/MSG guidelines, an abnormal computed tomography (CT) scan is required to detect or rule out invasive infections. In the case of a normal CT scan, microbiological tests and host risk factors are not enough to diagnose invasive infections [2]. Abnormal X-rays do not make it possible to identify specific pathogens, and a biopsy is most often not a viable option in patients undergoing chemotherapy. Among infectious biological markers, testing for galactomannan in serum is the only sufficiently explored technique that can be used. The use of these markers in tissues other than peripheral blood increases galactomannan detection, especially when bronchoalveolar lavage (BAL) can be performed. Classical microbiological diagnostics, including pathogen culture and microscopic analysis of respiratory tract samples, cannot unambiguously confirm or rule out infections [1, 3]. Non-culture methods, such as detecting fungal cell wall components – galactomannan antigens and 1,3-β-D-glucan circulating in serum, and BAL, are useful in the diagnosis of invasive aspergillosis, but also limited by false positive or negative results related to many factors [3]. False positive results might be a consequence of the use of B-lactam antibiotic (piperacillin with tazobactam), cyclophosphamide, immunoglobulin, plasma-like products, or hydrating fluid containing galactomannan. Cases of cross reactions in patients infected with other fungal species (*Penicillium* sp., *Paecilomyces* sp., or *Fusarium*) or with *Bifidobacterium* sp. were also reported [1, 4-6]. Despite their limitations, commercial serological tests detecting cell wall components of yeast-like fungi, are widely used in diagnostics [7].

Progress in molecular biology made it possible to start diagnosing IA with PCR assay [7]. These tests identify the pathogen type or species in a fairly short time, without having to perform invasive procedures on patients (biopsy) [3]. Despite a vast diversity and availability of commercial tests, their use in IA diagnosis is limited because they are not standardized [1, 8]. Badiee *et al*. assessed, while

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comparing classical diagnostic techniques with PCR, the sensitivity, specificity, positive and negative PCR results at 86.6%, 82%, 96.5%, and 52%, respectively [9]. Real-time PCR is a modification of standard PCR. This assay delivers faster results than classical PCR. The high sensitivity and specificity of real-time PCR in diagnosing invasive aspergillosis was reported in other studies [10-21]. MycAssay™ is the only available commercial test detecting *Aspergillus* DNA in serum or BAL (Myconostica, currently Lab21 Company, Cambridge, UK). This test was designed to detect genomic DNA of 18 *Aspergillus* species, including *A. fumigatus, A. flavus, A. terreus*, and *A. niger*, using molecular beacon probes detecting 18S rRNA genes [8, 22].

Study objective

The study was to compare the clinical usefulness of the commercially available MycAssay™ in patients at higher risk of pulmonary aspergillosis with positive or negative galactomannan antigen levels.

Material and methods

Twenty patients suspected of IA, including three patients after their first kidney transplant, five patients preparing for a subsequent kidney transplant, and 11 patients post liver transplant, were included in the study. Serum samples, collected from patients during routine diagnostics, were first tested for the galactomannan antigen with the ELISA assay. Later on, the samples, kept at –20°C, were tested with MycAssay™ to establish a possible correlation between the two methods. Forty five serum samples from 20 patients (12 females and 8 males aged beetwen 23 and 90), suspected of IA and treated at the Medical University of Warsaw, Institute of Transplantology, were tested for galactomannan. None of the patients was following a treatment described by the producer as potentially inhibiting PCR. Serum samples to be tested were retrospectively selected according to their availability, IA clinical symptoms in patients, and clinical sample culture results.

GM PLATELIA *Aspergillus*

Sandwich ELISA to detect galactomannan in serum was performed following the producer's guidelines [23] (Platelia Aspergillus protocol: Bio-Rad, Marnes-la-Coquette, France). Optical density (OD) was measured spectrophotometrically with Bio-Rad Model PR5100 ELISA microplate reader (Bio-Rad, Marnes-la-Coquette, France). Results were interpreted based on the index calculated from the measured OD, using a 450 nm wavelength. Indexes ≥ 0.5 were considered positive.

The isolation of total genomic DNA was performed with the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). *Aspergillus fumigatus* ATCC 204305 was the reference strain used to control DNA isolation. MycAssay™, real-time PCR assay (Myconostica, currently Lab21, Cambridge, UK), was used, according to the producer's guidelines, to test for *Aspergillus* spp. specific gene in serum. Purified DNA was assayed with Real-Time PCR for targeted *Aspergillus* 18S rRNA gene. For every analysed sample, 10 µl of purified DNA were used together with reaction mixtures, totalling to a final volume of 25 µl. Negative and positive control reactions were also conducted. The MycAssay™ protocol includes the following reaction controls: internal amplification, and negative and positive controls. Furthermore, the DNA previously isolated from *Aspergillus fumigatus* ATCC 204305 served as a positive control. Samples with a Cp < 38 were considered positive. The crossing point (Cp) was the cycle number at which the real-time PCR test became positive. Samples with a Cp ≥ 38 or with zero crossing points were considered negative. White *et al.* study, sponsored by Myconostica, on serum samples from 18 healthy individuals was used as control group [24].

Results

Serum samples from patients suspected of IA, including positive and negative galactomannan antigen levels, were tested. The results of serological and genetic tests, mycological cultures, CT scans and X-rays were all taken into account. Study results are presented in Tables 1 and 2. Table 1 presents the results of eight serum samples from seven patients suspected of IA.

MycAssay™ confirmed the infection in four patients (around 50%) among those whose serum tested positive for galactomannan.

MycAssay™ did not detect any DNA of *Aspergillus* spp. in patient no. 2 despite a positive result for circulating antigens. Their transaminase level was normal.

Also for patients no. 6 and 7 with a positive glactomannan antigen no *Aspergillus* DNA was detected. No yeast-like *Aspergillus* fungi were cultured from patient no. 6, however *Aspergillus fumigatus* was cultured from BAL samples of patient no. 7. Both patients had elevated transaminase levels.

Table 2 presents the results of 37 serum samples from 13 patients suspected of IA with negative galactomannan antigen levels.

In four (no. 9, 10, 17, and 20) out of 13 patients, MycAssay™ detected *Aspergillus* DNA in all cultured serum samples. All patients had normal transaminase levels.

Patients no. 11, 12, 13, and 14 were suspected of pulmonary aspergillosis basing on their X-rays. MycAssay™ tested positive in one of the two serum samples in all of the above-mentioned patients. They had normal transaminase levels.

Patient no. 8 had proven pulmonary aspergillosis based on CT scan and X-ray results and an elevated transaminase

Table 1. Serum test in solid organ transplant recipients with positive galactomannan antigen levels

Patient no.	Sex/age	Transplanted organ	Stage after transplantation	Galactomannan antigen levels (ELISA method)		DNA detection by real-time PCR (MycAssay™)	Additional information			Clinical events
				(OD)	date of serum collection		culture	CT/X-RAY	transaminase level (U/0.5 ml)	
1	F/71	KTx	late	1.5	05.10.2012	+	none	no changes	< 22.2	
2	M/63	KTx	late	8.884	03.08.2011	–	none	no changes	< 22.2	
				0.51	20.08.2011	–	none	no changes	< 22.2	
3	F/67	OLTx	late	5.9	08.10.2012	+	none	no changes	> 22.2	
4	F/58	OLTx	intermediate	0.7	17.01.2012	+	none	no changes	> 22.2	TPN, fever of unknown aetiology
5	M/67	OLTx	late	0.8	07.01.2012	+	none	no changes	> 22.2	
6	F/60	OLTx	intermediate	0.69	28.12.2011	–	none	no changes	> 22.2	colitis, gastrointestinal bleeding
7	F/53	OLTx	late	0.5	18.11.2011	–	*A. fumigatus* culture – BAL	no changes	> 22.2	liver cirrhosis, HCV, HCC (hepatocellular carcinoma)

KTx – kidney transplantation; OLTx – orthotropic liver transplantation; OD – optical density; BAL – bronchoalveolar lavage

level. MycAssay™ detected *Aspergillus* DNA only in one of the three collected serum samples.

Patients no. 16 and 17 had elevated transaminase levels but no changes were visible in X-ray imaging. In these patients MycAssay™ delivered amibiguous results – some samples from the same patients gave positive and others – negative results.

The real-time PCR results of patients no. 15 and 19, suspected of aspergillosis based on their CT and X-ray results and having elevated transaminase levels, were amibiguous. *Aspergillus* DNA was detected in three out of 9 serum samples in patient no. 15. However, for patient no. 19 only one sample out of three tested positive.

Discussion

Invasive fungal infections are a serious complication, especially in solid organ transplant patients. In this group, fungal infections represent about 5% of all infections [25] and present a high mortality rate.

They are also extremely hard to detect. They do not present any specific clinical signs, lesions (especially in *Aspergillus* infections) are often encapsulated, which makes it more difficult to culture aetiological agents. There are no standardised techniques that would allow a rapid and early detection of invasive infections, which is also problematic. Galactomannan detection in blood serum and bronchoalveolar washings are one of the useful tests in diagnosing *Aspergillus* infections. Platelia Aspergillus (Bio-Rad, Marnes-la-Coquette, France) is the galactomannan antigen detection assay used *in vitro*.

Early diagnosis of *Aspergillus* spp. infections remains a great challenge, and with the development of molecular techniques, scientists focus on finding reliable solutions to detect *Aspergillus* spp. DNA in clinical samples [21]. The have recently introduced an assay, detecting *Aspergillus* spp. DNA with real-time PCR assay, which is a supplement to classical mycological diagnosis. This technique is very sensitive to and specific in aetiological agent detection (sensitivity and specificity of 94% and 77%, respectively, with positive and negative predictive results at 91% and 83%) [22]. The study was to assess the clinical effectiveness of MycAssay™ in patients at a higher risk of pulmonary aspergillosis with positive or negative galactomannan antigen levels.

Despite the high sensitivity of the assay, the elements that might have a negative impact on its results are worth noting. The level of transaminases in patients, which at 22.2 U/0.5 ml in serum might cause *Aspergillus* DNA to degrade, is crucial and might result in a false negative result. In the group with positive galactomannan levels, false negative results, a consequence of high aminotransferase levels, could be obtained in patients no. 6 and 7; whereas in the group with negative galactomannan levels, in patients no. 8, 15, 16, 18, and 19. It is also critical that serum

Table 2. Serum test in solid organ transplant recipients with negative galactomannan antigen levels

Patient no.	Sex/age	Transplanted organ	Stage after transplantation	Galactomannan antigen levels (ELISA method)		DNA detection by real-time PCR (MycAssay™)	Additional information			Clinical events
				(OD)	date of serum collection		culture	CT/X-RAY	transaminase level (U/0.5 ml)	
8	M/80	KTx	late	negative	10.06.2012	+	none	proven aspergillosis in X-ray and CT	> 22.2	COPD, pneumonia of mixed aetiology (CMV, *Klebsiella oxytoca* ESBL+, *Aspergillus* spp.)
				negative	09.07.2012	–	none		> 22.2	
				negative	30.07.2012	–	none		> 22.2	
9	M/49	KTx	late	negative	27.06.2012	+	none	aspergillosis suspected based on X-ray	< 22.2	pulmonary aspergillosis
				negative	10.08.2012	+	none		< 22.2	
10	F/60	KTx	before another transplantation	negative	10.01.2011	+	none	aspergillosis suspected based on X-ray	< 22.2	acute graft rejection
				negative	14.04.2011	+	none		< 22.2	
				negative	01.12.2012	+	none		< 22.2	
11	M/66	KTx	before another transplantation	negative	21.11.2011	–	none	aspergillosis suspected based on X-ray	< 22.2	acute graft rejection, dialysis
				negative	05.12.2011	+	none		< 22.2	
12	M/90	KTx	before another transplantation	negative	15.10.2012	+	none	aspergillosis suspected based on X-ray	< 22.2	acute graft rejection, pneumonia of fungal aetiology
				negative	10.11.2012	–	none		< 22.2	
13	F/78	KTx	before another transplantation	negative	28.06.2012	+	none	aspergillosis suspected based on X-ray	< 22.2	acute graft rejection, end-stage renal disease
				negative	20.07.2012	–	none		< 22.2	
14	F/30	KTx	before another transplantation	negative	14.06.2012	+	none	aspergillosis suspected based on X-ray	< 22.2	acute graft rejection, hydronephrosis
				negative	04.07.2012	–	none		< 22.2	

Table 2. Cont.

Patient no.	Sex/age	Transplanted organ	Stage after transplantation	Galactomannan antigen levels (ELISA method)		DNA detection by real-time PCR (MycAssay™)	Additional information			Clinical events
				(OD)	date of serum collection		culture	CT/X-RAY	transaminase level (U/0.5 ml)	
15	F/25	OLTx	late	negative	10.10.2011	–	none	aspergillosis suspected based on X-ray and CT scan	> 22.2	sepsis, cirrhosis of the liver, oral candidiasis
				negative	21.10.2011	+	none		> 22.2	
				negative	26.10.2011	–	none		> 22.2	
				negative	31.10.2011	–	none		> 22.2	
				negative	04.11.2011	+	none		> 22.2	
				negative	11.11.2011	–	none		> 22.2	
				negative	16.11.2011	–	none		> 22.2	
				negative	26.12.2011	–	none		> 22.2	
				negative	29.05.2012	+	none		> 22.2	
16	F/49	OLTx	late	negative	25.07.2012	+	none	no changes in X-ray	> 22.2	acute respiratory tract infection, diabetes, chronic graft rejection, oral candidiasis
				negative	15.08.2012	+	none		> 22.2	
				negative	30.08.2012	–	none		> 22.2	
17	M/40	OLTx	late	negative	12.07.2012	+	none	no changes in X-ray	< 22.2	liver abscess
				negative	01.08.2012	+	none		< 22.2	
18	F/62	OLTx	late	negative	26.06.2012	+	none	no changes in X-ray	> 22.2	
				negative	17.07.2012	–	none		> 22.2	
19	F/23	OLTx	late	negative	15.09.2012	+	none	no changes in X-ray; changes in the right lung in CT scan	> 22.2	
				negative	01.10.2012	–	none		> 22.2	
				negative	17.10.2012	–	none		> 22.2	
20	M/27	OLTx	late	negative	09.06.2011	+	*A. fumigatus* – tibia bone tissues	no changes in X-ray/CT scan	< 22.2	
				negative	20.06.2011	+	*A. fumigatus* and *A. flavus* – wound swab		< 22.2	
				negative	01.07.2011	+	none		< 22.2	

KTx – kidney transplantation; OLTx – orthotropic liver transplantation; IA – invasive aspergillosis; BAL – bronchoalveolar lavage; CT – computed tomography

be stored appropriately before testing. The present study was a retrospective serum assessment, therefore sample storage could have affected DNA degradation and lead to false negative results. The retrospective study was carried out in 2012. Too long or inappropriate sample storage might result in DNA degradation. Morton *et al*. pointed out that *Aspergillus* DNA was stable in blood serum only for a relatively short period of time < 144 h [26]. This could result in false results. Clinical factors (*Aspergillus* colonisation) and factors related to clinical sample preparation (*Aspergillus* airway contamination, PCR product contamination with another sample, and cross-reactivity between starters, probes, and the genetic material of other fungal species) could result in false positive results. White *et al*. study, sponsored by the MycAssay™ producer, was carried out on a group of generally healthy individuals. One out of 18 samples tested positive and therefore the study was repeated at an independent centre. The repeated study did not confirm the positive result. The serum sample contamination was stated as the reason behind the falsely positive result [24].

MycAssay™ *Aspergillus* PCR presents major cross-reactivity with most *Penicillium* spp. species, which rarely cause opportunistic infections in humans. On the other hand, galactomannan might even more often cross-react with *Penicillium* spp. or *Paecilomyces* spp. antigens.

Torelli *et al*. reported some patients who were colonised by *Aspergillus* but presented a negative galactomannan result in BAL samples assayed with ELISA. They assessed the specificity of this technique at 92% vs. 50% for *Aspergillus* DNA detection. They also pointed out that galactomannan needed to be detected earlier, and DNA detection should only confirm IA and improve the specificity of the results [1]. The same could have occurred in the present patients no. 9, 10, 17, and especially 20, out of whose samples *A. fumigatus* was cultured twice. White *et al*. stated that for *Aspergillus* PCR, it was technically better to sample serum instead of full blood. Both tests, galactomannan and PCR, could be performed on a single serum sample, enabling result comparison, which was beneficial for patients [24]. MycAssay™ might be compared to the commercial PCR assay and to galactomannan tests, previously introduced to IA diagnostics. It is necessary to conduct prospective tests on MycAssay™ effectiveness in IA diagnostics. Such a study would increase the clinical significance of the assay and limit DNA degradation.

Only the clinical picture, CT scan, clinical sample culture, and confirmation with one of the following methods – ELISA (serological) or MycAssay™ (genetic) should be decisive. It seems that negative results of serological and genetic tests, together with negative results of other tests, should rule out invasive aspergillosis. A positive MycAssay™ result in more than one serum sample and with infection symptoms, such as fever of unknown origin, abnormal CT, absence of clinical sample cultures, should be treated as potential IA. Such patients should be closely monitored and tested for invasive aspergillosis (e.g. patients no. 9, 10, 16, 17, and 20).

The present study revealed that both galactomannan antigen testing and the real-time PCR assay (MycAssay™) were useful, however, the absence of any correlation between the two methods could neither confirm nor rule out IA. Such an assay should be used for secondary testing when invasive aspergillosis was suspected, galactomannan antigen detected, or *Aspergillus* spp. cultured.

The authors declare no conflict of interest.

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