

# *Aspergillus* polymerase chain reaction (PCR) diagnosis

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In recent years novel molecular methods, notably polymerase chain reaction (PCR) assays, have been developed to improve the diagnosis of life-threatening invasive aspergillosis in patients at high risk, especially patients with malignant hematological disease. Early diagnosis and treatment are essential for adequate therapeutic management. Management, however, often remains difficult since most of the diagnostic tools used clinically at present either lack specificity or acceptable sensitivity. The clinical value, advantages and remaining problems of PCR approaches to detect the emerging fungal pathogen are reviewed.

**Keywords** *Aspergillus* spp., detection, hematology, molecular methods, PCR

## Introduction

There is an increasing incidence of life-threatening systemic fungal infections in immunocompromised patients, especially invasive infections with *Aspergillus* spp. [1]. Those who are immunocompromised as a result of HIV infection or organ transplantation, patients from intensive care units, and premature infants can be affected, but at highest risk for these severe infections are those with prolonged neutropenia after intensified chemotherapy. These include patients with acute leukemia during induction therapy or after transplantation of allogeneic hematologic stem cells [1–5]. Invasive aspergillosis has a high mortality rate and, in surviving patients, leads to considerable complications often limiting further anti-neoplastic therapies [4,6–10].

The reliability of current diagnostic tools in early detection of fungal infections in neutropenic patients is limited. Conventional microbiological, serologic or imaging techniques are often insufficient to ensure both the early diagnosis as well as monitoring the course of invasive infections caused by *Aspergillus* spp. [4,8,11]. Computed tomography of the lung is clinically accepted as a relevant suggestion of invasive pulmonary aspergillosis (IPA), but not as a proof [12,13]. A novel serological enzyme linked immunosor-

bent assay (ELISA) based on the detection of fungus specific galactomannan in blood samples showed – in some studies – high sensitivity and specificity rates, but other studies have not had such good results, and additional appropriate imaging techniques are recommended to improve the diagnosis [14–16]; reactivity with antibiotics may be an additional problem [17].

The clinical presentation of invasive fungal disease is non-specific and variable [4,11]. As a result, many cases are diagnosed late or even only at autopsy. Because of the low specificity and sensitivity rates of the available tools, definite diagnosis of invasive mycosis has until now been proven only by positive histopathology or culture results [2,4,8,9,18–20]. Culture results from blood and bronchoalveolar lavage (BAL) show low yields but, especially in mould infections, are susceptible to contamination; tissue biopsy specimens are rarely obtained in these neutropenic, often critically ill and thrombocytopenic patients [11,21,22].

In the usual clinical situation, the results of all early diagnostic tools are so insensitive that antifungal treatment is generally commenced based on empirical considerations [4,8,23]. Early diagnosis and early appropriate antifungal treatment is important in reducing mortality and morbidity; research at present is therefore focused on the development of reliable novel diagnostic modalities with both high sensitivity and specificity rates [24] in order to detect fungal pathogens earlier and reduce high mortality rates in this group of patients.

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## Molecular approaches to diagnose invasive aspergillosis

Owing to the disappointing performance of serologic assays, research has – since the early 1990s – been focused on molecular methods to detect the fungal pathogen early and with high sensitivity. In addition, molecular diagnostics of *Aspergillus* spp. attempt to cover a wide range of diagnostic problems that result from the difficulty in detecting and characterizing the pathogen. Problems that urgently need solving include the detection of the fungus from blood or BAL samples of high risk patients at an early stage of the disease, identification of the pathogen *in situ*, in histological slides, and its identification for epidemiologic, ecologic and typing questions [8,9,25–32].

As with other difficult-to-detect pathogenic organisms (e.g., *Borrelia burgdorferi* [33], *Cytomegalovirus* [34], *HIV* [35]), polymerase chain reaction (PCR) has led to the development of more sensitive and rapid detection assays [36] particularly since parts of the fungal genome, especially multicopy gene targets, were identified and sequenced [37–41]. Early studies to evaluate PCR-mediated detection of fungi showed significantly improved sensitivity compared with other diagnostic tools at that time, but were performed with different assays and different objectives – partly to optimize culture assays [42,43], partly for typing in epidemiologic studies – and often in nonclinical settings [44,45].

Epidemiologic studies, based on identification and typing of the pathogen from culture or environment samples, have resulted in more and more assays using different PCR techniques or other molecular approaches being published; however, a molecular ‘gold standard’ assay has yet to be determined [24,46–49].

For clinical use, in order to detect the pathogen in clinical samples, an increasing number of groups have succeeded in defining targeting gene sequences, practicable primers and effective DNA extraction methods. They have also overcome technical difficulties such as differentiation and specification of the amplicons, for which different approaches have been described (e.g., species-specific oligonucleotide probes [50] and PCR ELISA [51]).

The first PCR assays detected quantities down to 1–20 pg of genomic DNA from *Aspergillus fumigatus in vitro* [52]. The sensitivity could be increased up to 100 fg by combination with Southern Blot hybridization [40]. Melchers *et al.* [41] described a PCR assay that detects DNA from *Aspergillus* spp. in immunocompromised patients using primers based on sequences coding for the multicopy 18S rRNA gene; the targets for most

novel PCR assays are located in this gene, particularly since amplification of several hundred copies of this gene improves the sensitivity of the assay. Verweij *et al.* [53] compared the results of a then novel *Aspergillus* galactomannan antigen ELISA with those of a PCR assay; the ELISA showed fewer false positive results from BAL samples. Kappe *et al.* [54,55] designed universal fungus-specific primer systems and showed their specificity experimentally in infected human tissue samples. With another PCR assay, Makimura *et al.* [38,39] obtained PCR-products from BAL in immunocompromised and neutropenic patients, but no amplicons from immunocompetent individuals. Bretagne *et al.* [56] demonstrated the high risk of BAL-specimen contamination by *Aspergillus* conidia which resulted in approximately 23% false positive results. Yamakami *et al.* [57] first described a two-step PCR technique for the detection of *Aspergillus* spp. from blood with improved specificity and sensitivity rates compared to a conventional antigen assay.

Einsele *et al.* [50] developed a PCR technique targeting a multi-copy ribosomal gene sequence, followed by a genus- and species-specific hybridization step detecting fungal pathogens (including *Aspergillus* spp.) in blood samples, with a threshold of approximately 1 CFU/ml blood (*in vitro*). Prospectively using this PCR assay to screen BAL specimens from bone marrow transplantation patients, Einsele *et al.* [58] found a significant correlation between positive PCR results and the development of invasive aspergillosis; this indicates that previous contamination of the airways with *Aspergillus* spp. could be an additional risk factor for the development of invasive aspergillosis. Three further studies based on this assay have since been published. Hebart *et al.* [59] described sensitivity and specificity rates of 100% and 65%, screening 84 recipients of an allogeneic stem cell transplant and thus identifying patients at high risk for invasive aspergillosis. In prospective screening of patients with acute leukemia or those receiving high-dose therapy and hematologic stem cell transplantation, PCR was found to be the earliest indicator of invasive fungal infection, preceding clinical evidence by approximately five days [60]. Serial monitoring of blood samples from 121 patients with hematological disorders showed a sensitivity and a specificity rate of 75% and 96%, respectively [61]. Another study described a similar novel panfungal PCR to detect fungal pathogens in human blood specimens with similar preliminary results [62].

Jones *et al.* [51] developed an *Aspergillus* PCR-ELISA and showed high sensitivity and specificity rates in BAL samples from small number of patients with

definite or probable invasive pulmonary aspergillosis. Bretagne *et al.* [63] retrospectively compared the results of another PCR assay with antigen assay findings from blood samples of 41 selected hematologic patients, and found no convincing advantages for the PCR assay. Yamakami *et al.* [57] evaluated their two-step PCR assay for detection of *Aspergillus* spp. testing blood samples of 30 patients with various forms of pulmonary aspergillosis, and observed that the sensitivity of the assay depends on the extent of fungemia. Kawamura *et al.* [64,65] compared the results of another two-step PCR assay with two antigen assays and again demonstrated the superiority of the PCR assay to the serological tools for diagnosis of invasive aspergillosis. A possible new diagnostic area was shown by Kami *et al.* [66], who tested cerebrospinal fluid using PCR and combined these results with the findings of an imaging technique (MRI), thus detecting early central nervous system aspergillosis (in a single patient). Skladny *et al.* [67], using a novel two-step PCR assay without subsequent hybridization, showed a detection threshold up to 1–5 CFU per ml of blood *in vitro*, and both high sensitivity and specificity rates. Based on these *in vitro* results and in order to assess the clinical implications and applicability of the assay, clinical samples obtained from a large number of febrile neutropenic patients with malignant hematologic diseases at high risk for invasive fungal disease were tested in clinical trials. Using this assay, 67 BAL samples and 907 blood samples from 67 and 218 febrile neutropenic patients, respectively, were examined prospectively [68]. For BAL samples, the PCR assay had a specificity of 92.6% and a sensitivity of 100%; for blood samples, the test sensitivity was 91.7%, the specificity 81.3%. The contamination rate was low, confirmed in another study using BAL samples [69]. Similar results, testing a smaller number of patients, were described by Williamson *et al.* [70] using another nested PCR assay to examine 175 blood samples from 37 bone marrow recipients. Here, sensitivity of PCR in diagnosing patients was 100% and specificity was 79% using the criterion of a single positive result. If two positive results were required, these values were 81% and 100%, respectively. In this study, positive PCR results predated the institution of antifungal therapy in two-thirds of patients.

With the aim of detecting infection early and monitoring antifungal treatment, the screening of blood samples is obviously clinically more applicable as samples can be obtained repeatedly and non-invasively. However, the testing of BAL samples has been shown to be a valuable, more sensitive diagnostic tool to detect IPA early, with both higher

sensitivity and specificity rates than blood samples; *Aspergillus* infections being primarily airborne pulmonary infections with secondary hematogenous spread.

In recent years, numerous additional novel assays have been published in order to improve or to optimize procedures and techniques and to overcome or reduce methodological drawbacks, confirming the increased interest in the emerging clinical problem of detecting invasive aspergillosis sensitively and specifically. On the other hand, clinical studies with established assays were performed to assess the clinical significance of the PCR technology [69,71–93]. Some of the newer assays are successfully using real-time PCR technology, either the LightCycler™ technology or the TaqMan technology, both combining rapid *in vitro* amplification of DNA with immediate detection of the amplicon. Thus, rapid quantification of the DNA amount of the pathogen can be achieved as well as detection of the pathogen; mostly from blood samples but also from BAL samples. The sensitive and specific quantification of the fungal burden seems to be of clinical relevance, since the assessment of the individual fungal burden may possibly allow therapeutic monitoring.

All sensitive PCR assays have distinct limitations. Since *Aspergillus* conidia are ubiquitous, contamination problems can occur [56,94] leading to false positive results. Strict precautions against contamination, such as the use of sterile benches for processing samples, are recommended to minimize contamination rates. Sampling errors are another limiting factor [62,68,93], as is the impossibility of distinguishing – in clinical samples, especially in BAL samples – between colonization and infection [62,69]. Questions regarding the kinetics of the PCR findings, according to the kinetics of fungemia, remain unanswered, even in animal models of invasive aspergillosis [95–97].

Since published comparative and interlaboratory studies of different PCR assays are lacking – most assays are ‘in house assays’ – the comparability even of methodologically similar assays is unclear, and, up to now, no standardized assays are commercially available. A ‘gold standard’ *Aspergillus* PCR assay has yet to be defined [24], and the significance of different DNA extraction methods, different primer sequences, PCR conditions and other factors is unclear. One crucial step of *Aspergillus* PCR diagnosis is the extraction of fungal DNA, which contributes significantly to the quality of the PCR assay. Despite these problems, at least three methodologically different PCR assays, published by Einsele *et al.* [50], Skladny *et al.* [67] from our group and the assay from the MD Anderson Cancer Center Group [77,78] have shown in large clinical trials

that the PCR technique represents a highly sensitive diagnostic tool for the detection of small amounts of *Aspergillus* spp. in diverse specimens. These results point to a high value of the test in clinical use, primarily in the clinically most important attempts to improve early diagnosis of invasive aspergillosis.

Despite the advances that have been made, some drawbacks and questions should be reconsidered. Since our knowledge about *Aspergillus* fungemia and/or 'DNAemia' is preliminary, the timing of drawing blood samples remains unclear; sensitivity rates decline

significantly under antifungal treatment [93,96]. We do not know anything about the reduction of mortality or morbidity rates by using PCR diagnostics, and no cost-benefit analysis exists up to now. Nevertheless, in these authors' opinion, high sensitive and specific PCR assays to detect *Aspergillus* spp. are an important part of the clinical diagnostic mosaic that includes host factors, microbiological and serologic results and findings from imaging techniques. Further clinical validation in prospective multicenter studies, aiming to include PCR assays into clinical management

**Table 1** *Aspergillus* PCR assays from blood and bronchoalveolar lavage samples for clinical purposes.

Author	Reference	Patients/controls	Number of samples	Sensitivity (%)	Specificity (%)
PCR from BAL samples					
Tang <i>et al.</i> (1993)	37	23/28	51	100	94
Bretagne <i>et al.</i> (1995)	56	28/9	37	n.p.	n.p.
Jones <i>et al.</i> (1998)	51	12/-	12	100	100
Einsele <i>et al.</i> (1997)	58	134/-	261	63	98
Hayette <i>et al.</i> (2001)	87	74/103	177	100	96
Buchheidt <i>et al.</i> (2001)	68	67/33	100	100	93
Buchheidt <i>et al.</i> (2002)	69	141/35	197	93.9	94
Raad <i>et al.</i> (2002)	78	249/199	249	64-80	93
Meletiadiis <i>et al.</i> (2003)	79	42	42	n.p.	n.p.
Kawazu <i>et al.</i> (2003)	80	1	1	n.p.	n.p.
Spiess <i>et al.</i> (2003)	82	11/20	32	n.p.	n.p.
Sanguinetti <i>et al.</i> (2003)	88	20/24	44	n.p.	n.p.
Rantakokko <i>et al.</i> (2003)	89	66/33	99	45-73	93-100
Lass-Flörl <i>et al.</i> (2004)	98	36	21	66-100	n.p.
PCR from blood samples (>5 pts.)					
Einsele <i>et al.</i> (1997)	50	172/35	601	100	98
Bretagne <i>et al.</i> (1998)	63	41	281	n.p.	n.p.
Yamakami <i>et al.</i> (1998)	57	30	30	n.p.	n.p.
van Burik <i>et al.</i> (1998)	62	24	«multiple»	n.p.	n.p.
Kawamura <i>et al.</i> (1999)	64	44	44	n.p.	n.p.
Skladny <i>et al.</i> (1999)	67	93/47	250	100	89
Williamson <i>et al.</i> (2000)	70	37/-	175	100	79
Hebart <i>et al.</i> (2000)	59	84/-	1193	100	65
Hebart <i>et al.</i> (2000)	60	92/-	333	100	73
Löffler <i>et al.</i> (2000)	72	9	9	n.p.	n.p.
Lass-Flörl <i>et al.</i> (2001)	61	121/-	619	75	96
Buchheidt <i>et al.</i> (2001)	68	218/60	907	92	81
Kami <i>et al.</i> (2001)	66	33/89	323	79	92
Bialek <i>et al.</i> (2002)	84	17/-	71	67	88
Costa <i>et al.</i> (2002)	76	14/20	210	n.p.	n.p.
Ferns <i>et al.</i> (2002)	85	17/-	94	n.p.	n.p.
Raad <i>et al.</i> (2002)	77	54/36	54	57-100	100
Pham <i>et al.</i> (2003)	86	71/35	559	n.p.	n.p.
Spiess <i>et al.</i> (2003)	82	9/50	64	n.p.	n.p.
Imhof <i>et al.</i> (2003)	90	6/-	9	n.p.	n.p.
Pryce <i>et al.</i> (2003)	91	8/45	?	n.p.	n.p.
Challier <i>et al.</i> (2004)	92	41	207	50-100	n.p.
Buchheidt <i>et al.</i> (2004)	93	165	1522	64	64-92
Kawazu <i>et al.</i> (2004)	81	96	1251	55	93
Lass-Flörl <i>et al.</i> (2004)	98	205	36	44-40	n.p.

n.p. = not performed.

strategies, is mandatory to improve the prognosis of patients with life-threatening infections caused by *Aspergillus* spp.

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