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Aspergillus Polymerase Chain Reaction—An Update on Technical Recommendations, Clinical Applications, and Justification for Inclusion in the Second Revision of the EORTC/MSGERC Definitions of Invasive Fungal Disease

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Aspergillus polymerase chain reaction testing of blood and respiratory samples has recently been included in the second revision of the EORTC/MSGERC definitions for classifying invasive fungal disease. This is a result of considerable efforts to standardize methodology, the availability of commercial assays and external quality control programs, and additional clinical validation. This supporting article provides both clinical and technical justifications for its inclusion while also summarizing recent advances and likely future developments in the molecular diagnosis of invasive aspergillosis.

Keywords. *Aspergillus* PCR; EORTC/MSGERC definitions; technical aspects; clinical performance.

Aspergillus polymerase chain reaction (PCR) testing of blood and bronchoalveolar lavage fluid (BALF) has been recently accepted as a mycological criterion for probable invasive aspergillosis (IA) in consensus guidelines for research studies [1]. The basis for inclusion is the significant progress that has been made in the standardization of *Aspergillus* PCR methodology through the efforts of the European Aspergillus PCR Initiative (EAPCRI; now known as the Fungal PCR Initiative (FPCRI; [2] www.fpcri.eu), the availability of commercial assays, and increased confidence in performance as highlighted by a Cochrane review; various meta-analyses; and randomized, controlled trials that incorporate PCR technology [3–8].

When considering the suitability of any test for clinical use, the technical robustness and applicability, analytical and clinical performance, and clinical utility must be determined. All of these may be influenced by the reason for testing (screening vs diagnostic confirmation), which affects testing frequency, specimen choice, and subsequent result interpretation, where the emphasis will change dependent on the reason for testing [9]. While all parameters are important when considering a test for inclusion in the EORTC/MSGERC definitions, assay specificity is paramount, as accuracy in confirming a diagnosis is critical when enrolling patients into clinical trials of novel therapeutics or when assessing performance of new tests [1, 10, 11].

In this review, we summarize the evidence for inclusion of *Aspergillus* PCR into the recent EORTC/MSGERC definitions and describe recent advances, unmet clinical needs, and potential future developments.

TECHNICAL CONSIDERATIONS

Nucleic Acid Extraction

For years, the lack of commercial assays and limited methodological standardization prevented the incorporation of *Aspergillus* PCR into the EORTC/MSGERC definitions [10, 11]. The work of the EAPCRI/FPCRI demonstrated that the performance of molecular methods for the detection of *Aspergillus* was dependent on the nucleic acid (NA) extraction protocol to provide high-quality DNA of sufficient quantity with minimal inhibitory compounds [12]. The PCR amplification stage was not rate-limiting, providing consistent performance when testing comparable NA concentrations across methods. Following this, research commenced to develop optimal NA extraction protocols for whole blood (WB), serum, and plasma testing [12–14]. For all specimen types, sample volume (≥3 mL EDTA WB, ≥0.5 mL serum/plasma) and NA elution volume (<100 μL) were determined critical to success. The testing of WB to target organism-sourced DNA requires the processing of large volumes and manual procedures prior to automated extraction that increase processing time and limit the uptake of testing. The

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testing of serum/plasma for the detection of circulating DNA (DNAemia) was methodologically straightforward, using NA fully automated extraction platforms available in most molecular diagnostic laboratories [9]. NA extraction from serum likely provides enhanced specificity but compromises sensitivity compared with WB samples [3, 15]. However, the extraction from plasma was superior to that of serum, providing sensitivity comparable to testing WB but without the methodological complexity, which likely compromised specificity when testing WB [3, 14–16]. Given the documented presence of organisms in the respiratory tract and subsequent samples, mechanical disruption of the fungal cell is required to provide efficient NA extraction. However, the overall extraction process is less labor-intensive compared with WB extractions that require lysis of red and white blood cells prior to targeting the fungi [12]. Free fungal DNA will also be present in the respiratory tract through the actions of the host's immune response or antifungal therapy; this could be targeted exclusively using fully automated NA extraction platforms. By introducing BALF supernatant post-mechanical lysis of the BALF pellet, both organism-sourced and free DNA can be targeted in a single NA extraction procedure [9]. The FPCRI is currently finalizing NA extraction recommendations to standardize *Aspergillus* PCR testing of BALF and allow all potential DNA sources to be targeted, which will potentially improve performance. Given the viscous nature of some BALF, the sample may need to be liquefied before extraction to allow manipulation.

PCR Amplification

While PCR amplification has not been deemed critical to success when performing *Aspergillus* PCR, targeting a multicopy gene enhances the analytical sensitivity of PCR, and the ribosomal RNA gene cluster (18S/28S rRNA and the internal transcribed spacer (ITS) regions) has been frequently targeted [17]. As IA is most commonly caused by *Aspergillus fumigatus*, most assays are optimized to detect this organism. However, given that aspergillosis can occasionally be caused by other species, it is beneficial for assays to be pan-*Aspergillus.* However, a disadvantage is potential cross-detection of other genera (eg, *Penicillium* species) [9, 17]. Current *Aspergillus* PCR assays are better suited for the detection of *A. fumigatus*; however, the use of PCR assays that provide a genus level of detection and target the rRNA genes improves the detection of non-*A. fumigatus* species [17].

The use of real-time quantitative (qPCR) instruments minimizes the potential for contamination, provides rapid species-level identification, and generates a quantification cycle (Cq) that is proportional to the fungal burden, which is useful when interpreting the significance of a positive result. Typically, when testing blood samples, Cq values will be late (>35 cycles), entering the nonreproducible range of detection. Performing PCR amplification in duplicate to enhance detection of low NA concentrations and including an internal control are essential [8, 12]. Interpretation of PCR positives with late Cq values remains difficult. Determining the clinical significance of low burden is complicated, as it may be a consequence of testing specimens not directly associated with the infected site or a result of disease with little or no angioinvasion. Conversely, contamination can arise from both the clinical and laboratory settings and generate false-positive Cq values >35 cycles [9]. Negative controls should be used to monitor for procedural contamination. Commercial kits usually provide both positive and negative control material, and it is paramount that "in-house" methods follow suit.

While Cq is unique to each real-time PCR platform and the algorithm used for its determination, analysis across 29 protocols that tested blood samples spiked with varying burdens of *Aspergillus* genomic DNA identified a Cq threshold of 43 cycles as optimal, generating sensitivity and specificity of 86% and 95%, respectively, while lowering the thresholds to 34 cycles provided 100% specificity [13]. Setting a lower threshold of positivity (eg, an earlier Cq value) is a trade-off between sensitivity and specificity, and sensitivities <50% will compromise the positive likelihood ratio (<10), even if the specificity were 95%. Very high/late thresholds will optimize sensitivity but will produce more false positives, sacrificing specificity. This may be desirable in a clinical scenario where a clinician might prioritize avoidance of missing a true case over treating patients who may not be truly infected, since the consequence of not treating early IA can be devastating. However, the purpose of the EORTC/MSGERC guidelines is to restrict classification to cases that have a high degree of certainty for clinical trials, thus prioritizing high specificity. The recent Cochrane systematic review of the literature and meta-analysis on *Aspergillus* PCR testing of blood (29 studies, 34 datasets, 4718 patients, mean IA prevalence of 16.3%) that used the new EORTC/MSGERC criteria showed that pooled sensitivity/specificity for 2 consecutive positives was 60%/95%, corroborating the current inclusion criteria of requiring 2 positive PCR results [1, 3]. This trade-off between assay sensitivity and specificity emphasizes the limitations of trying to use the more restrictive criteria of the guidelines in the clinic.

BALF testing is invariably used to confirm suspected infection in a symptomatic high-risk patient. Thus, the pre-test probability is high and assay specificity is paramount. Meta-analyses have highlighted the high specificity (94%–95%) of *Aspergillus* PCR testing of BALF, and corresponding positive likelihood ratios (>12) confirm its suitability for confirming infection and its inclusion in the current definitions [5, 8, 18, 19]. Real-time PCR positivity is associated with a Cq value that is proportional to the fungal burden in the sample; this should allow thresholds that aid in the differentiation of infection from colonization/contamination in the respiratory sample to be implemented.

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The availability of commercial *Aspergillus* PCR assays provides quality assurance and technical consistency, including the provision of control samples that facilitates adoption by more laboratories outside of specialty mycology reference facilities. Surprisingly, commercial assays have not demonstrated superior performance over laboratory-developed methods [3]. The commercial assays do not recommend specific NA extraction methods. Combining commercial assays with the FPCRI methodological recommendations for NA extraction provides a fully standardized method that can easily be replicated across centers. This methodological consistency coupled with the availability of external quality control methods for *Aspergillus* PCR testing (Quality Control for Molecular Diagnostics) [20] provide a process that is very robust. The availability of an international *Aspergillus* DNA calibrator, which is currently being used to develop an international standard for *Aspergillus* PCR, allows tests that use multiple platforms to be referenced to a single control material [21]. Given all of this information, ideally, *Aspergillus* PCR testing should only be performed using real-time PCR platforms.

CLINICAL APPLICATION AND PERFORMANCE

Screening vs Diagnosis

Aspergillus PCR testing is principally used by clinicians to either confirm diagnosis in patients suspected to have IA or to screen individuals at risk for developing IA in order to facilitate early diagnosis. Screening strategies are best applied in patients at moderate to high risk of IA (eg, acute leukemia or transplant recipients) since the pre-test probability governs how well the test performs.

When *Aspergillus* PCR was used to screen blood samples, meta-analytical reviews generated sensitivity and specificity values of 84%–88% and 75%–76%, respectively [4, 22]. The recent Cochrane review of *Aspergillus* PCR testing of blood generated similar statistics (sensitivity, 79%; specificity, 80%) [3].

Anti-*aspergillus* prophylaxis significantly reduces the pre-test probability of IA and was associated with a significant reduction in specificity (79%–64%) coupled with a nonsignificant increase in sensitivity (75%–82%) [3, 9, 23]. While this may seem counterintuitive and contradictory to the influence of antifungal therapy on galactomannan-enzyme immunoassay (GM-EIA), it may be explained by the possibility that prophylaxis will prevent an initial infection from progressing to overt disease while the presence of *Aspergillus* DNA is maintained or even enhanced due to release of NAs by antifungals that target the cell wall or membrane [3]. *Aspergillus* PCR testing of BALF to confirm a breakthrough diagnosis in a patient on prophylaxis is feasible [24].

When confirming IA in patients with suspected disease, specimens from the infection site are more advantageous than blood samples. In a retrospective, multicenter evaluation that compared *Aspergillus* PCR testing of BALF with concurrently taken blood samples, PCR sensitivity was significantly greater in BALF (63%) vs blood (8%). Also, although 75% of samples were taken during antifungal therapy, this did not have a major impact on performance in BALF [24]. Studies that directly compare the performance of screening and diagnostic confirmatory PCR approaches are currently lacking. However, in 73% of cases of IA regularly screened using both PCR and GM-EIA, a positive screening result in blood was recorded on average 11 days prior to bronchoscopy to confirm the diagnosis. This was due to the logistical delays inherent in getting bronchoscopy performed promptly [3, 25].

The meta-analytical performance of *Aspergillus* PCR for the testing of BALF is comparable to that for GM-EIA with comparable sensitivities and specificities that range from 76.8 to 79.65 and 93.7 to 94.5, respectively [5, 19, 20].

The optimal use of *Aspergillus* PCR is likely to be in combination with antigen detection [26]. In a study that tested BALF, the combination of PCR with GM (I>1.0) generated 100% sensitivity and 98% specificity [27]. This approach was confirmed using the commercial Pathonostics AsperGenius assay, where PCR combined with GM (I>1.0) generated 96% sensitivity and 100% specificity [28]. These findings provide some clinical validation of a combined strategy of using commercial PCR and antigen assays. A meta-analysis of antigen/PCR testing of BALF generated sensitivity and specificity of 84% and 94%. While combination testing of BALF increased sensitivity by 5%–9%, the specificity remained sufficient to confirm IA (positive likelihood ratio, 14) [29]. Various randomized, controlled trials and prospective cohort studies have highlighted the benefit of combined antigen/PCR testing of blood for the management of IA [6, 7, 30, 31]. A meta-analysis confirmed that if both were consistently negative, the sensitivity (99%) would be sufficient to exclude IA, whereas the specificity when both assays were positive was 98% [26]. The improved specificity achieved through combination testing of both blood and BALF may instill confidence regarding the certainty of a case of probable IA when both tests are positive. Conversely, if both tests are consistently negative, disease can be confidently excluded, which is critical if antifungal stewardship strategies are to be used successfully.

While the kinetics of release of fungal biomarkers have been studied, data are limited, and the relationship between the release of the individual biomarkers and stages of disease is unclear [32–34]. Combination testing enhances the opportunity to detect the biomarkers that may vary differentially at various stages of the infection [35]. The recent The European Society for Clinical Microbiology and Infectious Diseases, the European Confederation of Medical Mycology and the European Respiratory Society (ESCMID/ECMM/ERS) guidelines for management of *Aspergillus* diseases moderately support the use of PCR to diagnose IA when testing blood, BALF, or cerebrospinal fluid (BII), and the strength of that recommendation is increased for combined GM-EIA and PCR

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testing of BALF [36]. The 2016 Infectious Diseases Society of America Aspergillosis Guidelines advise that PCR be performed on an individual basis and in conjunction with other tests and clinical context [37]. The development of real-time *Aspergillus* PCR assays has raised the possibility of using the assay as a prognostic marker during therapy. Unfortunately, the late Cq values that are regularly encountered when testing blood samples only permit a qualitative interpretation, with patients usually becoming negative promptly after starting treatment. While PCR positivity in BALF is regularly associated with earlier Cq values that could be monitored for response to therapy, the invasive nature of obtaining the sample prohibits prognostic evaluations.

Nonneutropenic Patients

Most of the data regarding evaluation of *Aspergillus* biomarker assays have been generated in neutropenic and Hematopoietic stem cell transplant (HSCT) patients. Evaluations of the performance of biomarker assays in other nonneutropenic patients are limited, but the need is growing, with apparent greater frequency of IA occurring in the intensive care setting, including in coronavirus disease 2019 (COVID-19) patients [38, 39]. The utility of high-frequency screening of blood samples in nonneutropenic patients may be limited by less angioinvasion of IA. IA in nonneutropenic patients may be restricted to the respiratory tract (eg, *Aspergillus* tracheobronchitis post-influenza infection), and symptoms are attributed to the inflammatory response rather than tissue infarction, which is more characteristic of neutropenic infections. For these reasons, the use of PCR assays for diagnostic confirmation is preferred at present. However, given the increasing incidence of IA in certain intensive care unit (ICU) cohorts (19% in post-influenza and 33% in post–COVID-19 patients), screening strategies are a high priority [40, 41]. Using a commercial *Aspergillus* PCR, sensitivity was 100% and specificity was 99%–100% in BALF from ICU patients [42, 43]. Chong and colleagues showed that the performance of the Pathonostics AsperGenius assay when testing BALF was identical for hematologic and critical care populations, generating a sensitivity and specificity of 80% and 91%, respectively, for the ICU population [44].

Pediatrics

The number of studies that have evaluated the performance of *Aspergillus* PCR in children is limited, even more so for neonates. Most data are derived from studies that evaluated performance in high-risk pediatric populations (eg, leukemia, transplant, and chronic granulomatous disease). Overall clinical performance is variable, but data analysis is complicated because some studies included possible IA with proven/probable IA, incorporated a pan-fungal PCR technology, or failed to use the EORTC/MSGERC definitions for case classification [45–51]. For studies where the data were retrievable, the overall pooled sensitivity was 82.3% (95% confidence interval [CI], 75.8–87.3) and pooled specificity was 72.8% (95% CI, 68.8–76.4), which are comparable to the results included in the recent Cochrane review of *Aspergillus* PCR and suggest that the diagnostic yield of *Aspergillus* PCR does not differ significantly between adult and pediatric studies [3, 46–48, 51, 53–57].

Nevertheless, given the limited number of studies, there are no recent recommendations for the diagnosis and management of IA in pediatric patients as they relate to PCR testing [57]. While it is likely that the performance of *Aspergillus* PCR will be similar in adults and children, it is important to remember that radiological imaging, which is critical to attaining a diagnosis of probable IA in the EORTC/MSGERC, is typically nonspecific in pediatrics, making validation of IA using data from adult studies difficult [57].

FURTHER DEVELOPMENTS AND FUTURE REQUIREMENTS

Since different *Aspergillus* species may have different antifungal susceptibility profiles it is desirable for molecular methods to be able to differentiate between species. Analysis of the analytical specificity of mainly laboratory-developed PCR methods demonstrated that the detection of species other than *A. fumigatus* (eg, *Aspergillus terreus*) was reduced. While the detection of non-fumigatus species (eg, *Aspergillus lentulus*) within the *Aspergillus fumigati* complex was possible, most assays did not differentiate species within this complex [17]. Recently, several commercial assays (eg, Fungiplex Aspergillus, Bruker UK Limited, Glasgow, UK, and AsperGenius, Pathonostics, Maastricht, Netherlands) have been designed to identify *A. terreus* separately from other species. The AsperGenius assay has also been used "off-label" to distinguish *A. lentulus* and *Aspergillus felis* from other members of the *A. fumigati* complex via melt-curve analysis, although testing was limited to DNA extracted from cultures [58]. More work is needed to address the unmet need for species identification.

PCR technology has the potential to identify potentially resistant organisms, overcoming limitations of classic susceptibility testing which is time consuming and may be hindered by the poor growth in culture [28]. The identification of single nucleotide polymorphisms (SNP) or tandem repeats associated with triazole resistance in *A. fumigatus* is now well documented and commercial assays (Mycogenie, Ademtech, Pessac, France; Pathonostics AsperGenius) that target the most frequently encountered mutations (TR_{34}/L98H and TR_{46}/Y121F/T289A) are now available [59, 60]. An alternative approach is to use molecular tests to identify persistent organisms during therapy [61]. Unfortunately, the practicality is limited due to rapid disappearance of the NA signal in blood and persistent NA in BALF may not correlate with viable organisms [62].

Initially, PCR sequencing was required to identify mutations. However, this approach is time-consuming and the development

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of real-time PCR that targets common mutations negates the need for gene sequencing and improves the time to result but limits the range of mutations that can be detected [62, 63]. Newer real-time PCR tests have been designed to detect multiple (n = 7, TR_{34}, TR_{46}, G54W, L98H, Y121F, and M220I) *cyp51A* mutations, but direct application to clinical samples has not yet been demonstrated [64]. A multicenter evaluation of the AsperGenius assay of BALF showed that the presence of mutations was significantly associated with treatment failure (75% vs 27%, $P = .01$) and increased 6-week mortality (50% vs 19%, $P = .07$) [65]. Nevertheless, the performance of these assays is variable [60, 66]. A comparison of the performance of the AsperGenius assay with direct PCR sequencing to identify mutations directly from samples, showed that PCR sequencing was only slightly better than real-time PCR [59]. Rapid pyrosequencing methods also have the capacity to detect an increasing number of mutations and have been applied directly to clinical specimens [67]. While direct sample testing to identify mutations has been applied to blood-based samples, the low circulating burden often limits successful amplification of the target genes [60, 68].

The application of next-generation sequencing (NGS) for the detection and identification of fungi directly from a clinical specimen likely represents the future of clinical mycological investigations, with the potential to identify to a species level (even within the mycobiome) and determine antifungal susceptibility and genotype organisms during outbreaks [69]. Currently, several limitations need to be overcome before it is suitable for routine use, including the identification of an optimal gene(s) to provide a sufficient degree of species differentiation (eg, ITS 1/2 regions only differentiate 75% of fungal species [70]), while maintaining the required analytical sensitivity (multicopy vs single-copy genes), optimization of the entire process from sampling through DNA extraction, PCR design, and overcoming the lack of required NGS bioinformatic tools and pipelines [69]. In a review of studies that used molecular approaches to study the complexity of the respiratory mycobiome, it was determined that *Candida* species were the dominant fungi, confirming the commensal nature of this yeast [71]. This highlights how NGS methods may need to be designed to avoid an overwhelming presence of a single commensal/colonizing species/genus of limited clinical importance, restricting the detection of less evident but clinically relevant fungi [72]. The application of digital droplet *Aspergillus* PCR represents an exciting development, potentially enhancing sensitivity and quantification [73].

CONCLUSIONS

Considerable evidence has been gathered about *Aspergillus* PCR assays to support both clinical utility and application to clinical research trials. With continued advances in molecular technology coupled with applications to address important unmet clinical needs, further developments in molecular technology will improve its use in clinical screening, diagnosis, and treatment selection.

Notes

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