**Title:** The impact of anti-mold prophylaxis on *Aspergillus* PCR blood testing for the diagnosis of invasive aspergillosis.

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**Running title:** Impact of antifungal prophylaxis on *Aspergillus* PCR performance

## SYNOPSIS

### Background

The performance of the galactomannan enzyme immunoassay (GM-EIA) is impaired in patients receiving mold-active antifungal therapy. The impact of mold-active antifungal therapy on *Aspergillus* PCR testing needs to be determined.

### Objectives

To determine the influence of anti-mold prophylaxis (AMP) on the performance of PCR blood testing to aid the diagnosis of proven/probable invasive aspergillosis (IA).

### Methods

As part of the systematic review and meta-analysis of 22 cohort studies investigating *Aspergillus* PCR blood testing in 2912 patients at risk of IA, subgroup analysis was performed to determine the impact of AMP on the accuracy of *Aspergillus* PCR. The incidence of IA was calculated in patients receiving and not receiving AMP. The impact of two different positivity thresholds, requiring either a single PCR positive test result or ≥2 consecutive PCR positive test results, on accuracy was evaluated. Meta-analytical pooling of sensitivity and specificity was performed by logistic mixed-model regression.

### Results

In total, 1661 (57%) patients received prophylaxis. The incidence of IA was 14.2%, significantly lower in the prophylaxis group (11-12%) compared to non- prophylaxis group (18-19%) ($P$<0.001). The use of AMP did not affect sensitivity, but significantly decreased specificity (Single PCR positive threshold: 26% reduction ($P$: 0.005; ≥2 consecutive PCR positive threshold: 12% reduction ($P:$ 0.019).

### Conclusions

Contrary to its influence on GM-EIA, AMP significantly decreases *Aspergillus* PCR specificity, without affecting sensitivity, possibly a consequence of AMP limiting the clinical

progression of IA and/or leading to false negative GM-EIA results, preventing the classification of probable IA using the EORTC/MSGERC definitions.

## INTRODUCTION

There is convincing evidence showing that both the sensitivity and specificity of the galactomannan enzyme immunoassay (GM-EIA) are impaired in patients receiving mold-active antifungal therapy (AFT) [1, 2]. Previous exposure to AFT also needs to be considered when interpreting *Aspergillus* PCR results, as animal studies and clinical trials both indicate that AFT may adversely affect test performance. [3, 4]. Recently, a systematic review and meta-analysis investigating *Aspergillus* PCR blood testing to aid the diagnosis of invasive aspergillosis (IA) in immunocompromised patients was performed [4]. Most patients had a haematological malignancy, had undergone hematopoietic stem cell transplantation (HSCT) or were solid organ transplant (SOT) recipients. The mean prevalence of proven or probable IA was 16.3 % (769/4718 patients) [4]. Pooled data showed that PCR has moderate diagnostic accuracy when used as a screening test for IA in high-risk patient groups. [4] The sensitivity and specificity of PCR for the diagnosis of IA varied according to the interpretative criteria used to define a test as positive. Considering a single positive test result as significant the sensitivity and specificity were 79.2% and 79.57%, respectively, changing to 59.6% and 95.1% when requiring two consecutive positive results. Diagnostic odd ratios (DORs), negative (NPV) and positive predictive values (PPV) were 14.8/28.8, 95%/92% and 42/70%, respectively for a single positive test, and two consecutive positive tests. [4]

As part of the systematic review and meta-analysis of cohort studies investigating *Aspergillus* PCR blood testing in patients at risk of IA, subgroup analysis was conducted and included an evaluation of the impact of anti-mold prophylaxis (AMP) on the diagnostic accuracy of *Aspergillus* PCR. This manuscript describes those findings.

## PATIENTS AND METHODS

### Meta-Analytical Review

The index tests included PCR testing of blood specimens (whole blood or serum/plasma) and subsequently methodological heterogeneity was evident (different DNA extraction methods and PCR methods (e.g. nested, PCR-ELISA, qPCR)). Depending on the original date of publication, proven/probable IA was defined using either the original (2002) or the revised (2008) EORTC/MSG consensus definitions of invasive fungal disease (IFD) [5, 6]. At the time of analysis there had been no studies using the recently published second revision of the EORTC/MSG consensus definitions, subsequently *Aspergillus* PCR was not a mycological criterion for defining IA. [7]. Systemic AMP was defined when patients received itraconazole, voriconazole, posaconazole, amphotericin B or caspofungin.

The cumulative incidence of IA was calculated in both patients receiving and not receiving AMP. The impact of two different positivity thresholds, requiring either a single PCR positive test result or ≥2 consecutive PCR positive test results, on diagnostic accuracy was evaluated, as the latter threshold is associated with increased specificity. A meta-analytical pooling of sensitivity and specificity was performed by logistic mixed-model regression, where the dependent variable was the positivity of the PCR test, and the covariates were "IA", AMP (yes/no), and itraconazole versus other AMP [8]. The final comparison included as the efficacy of itraconazole prophylaxis could be inferior to other AMP. As post-estimation results, DOR, positive likelihood ratio (LR +tive), and negative likelihood ratio (LR -tive) were obtained. PPV and NPV were calculated using the Bayes' rule as indicated by WHO, using sensitivity, specificity and prevalence data [9]. For this purpose, the considered incidence was the value calculated for each of the four individual groups, according to positivity threshold and prophylaxis. Logistic mixed-model regression analysis was used over conventional meta-analytical pooling (Supplementary Table 2) as it

preserves the randomization of each individual study, thereby limiting any confounding bias introduced through simple data pooling [8]. Calculations were performed with Stata v. 16.0 and MS Excel

## RESULTS

Of the 29 primary studies included in the primary meta-analysis [4], 12 used AMP across the entire population or in subsets of patients, 17 studies did not use AMP, although four of these studies used fluconazole for prophylaxis against certain *Candida* species. The sensitivity/specificity data for *Aspergillus* PCR associated with EORTC/MSGERC defined IA and the administration of antifungal prophylaxis was available from 22 primary studies (Supplementary table 1). Ten studies administered AMP to all patients (n=1438), 10 studies did not administer prophylaxis to any patients (n=1027) and two studies differentiated patients receiving (n=223), or not receiving prophylaxis (n=224). In total, 1661 patients received prophylaxis and 1251 did not receive prophylaxis.

The overall incidence of IA was 14.2% (95% CI: 13.0-15.5). The incidence of IA was significantly lower in the prophylaxis group compared to non- prophylaxis group: 11.9% (164/1373, 95% CI:10.3-13.8) vs 18.7% (216/1156, 95% CI: 16.5-21.0) in studies using a single PCR positivity threshold, and 11.4% (155/1356, 95% CI: 9.8-13.2) vs 18.0% (72/401, 95% CI: 14.5-22.0) in those requiring ≥2 consecutive positive test results; the differences were statistically significant ($P$<0.001), irrespective.

The use of AMP had no relevant effect on sensitivity, LR -tive, and NPV, but decreased specificity, LR +tive, and PPV (Table 1). When examining data under the criterion “single positive test result” (21 studies, 2529 patients, 1373 receiving prophylaxis and 1156 without prophylaxis) the use of AMP decreased specificity (from 0.86 to 0.60; $P$: 0.005), PPV (from 0.57 to 0.22) and DOR (from 25.7 to 7.60; $P$: 0.01) (Table 1). Requiring ≥2 consecutive positive results (12 studies, 1757 patients, 1356 receiving prophylaxis and

401 without prophylaxis), AMP use decreased specificity (from 0.98 to 0.86; *P*: 0.019), PPV (from 0.87 to 0.37) and DOR (from 98.1 to 11.8; *P*: 0.02), but again had no significant impact on sensitivity. Excluding studies with itraconazole prophylaxis did not significantly affect the effect performance (data not shown).

## DISCUSSIONS

Sensitivity and specificity data were determined in subgroups of patients receiving or not receiving AMP from 22 cohort studies reporting the diagnostic accuracy of PCR testing of blood for the diagnosis of IA in immunocompromised patients. As expected, the cumulative incidence of IA was significantly lower in patients receiving AMP. Prophylaxis significantly decreased the specificity of PCR, irrespective of the interpretative criteria used to define positivity. Conversely, AMP had no significant impact on sensitivity. Likewise, AMP decreased PPVs considerably, but had no relevant effect on NPVs. A decrease in DOR with both interpretative criteria was observed. One limitation of the study is the effect of AMP on the PCR performance of assays compliant with FPCRI methodological recommendations was not performed. Given most studies predated the availability of these recommendations, the number of compliant methods will be limited and subsequent additional analysis will be needed to determine if optimal methods minimized the effect of AMP.

Data from clinical trials and systematic review show that mold-active antifungals affect the accuracy of GM-EIA [1, 2, 10]. However, in these studies the effect of anti-mold drugs was heterogeneous, depending on the incidence of breakthrough infections, time of drug administration, and positivity threshold used for the GM-EIA test. In one study, AMP had only a minor effect on sensitivity and decreased specificity, but the pretest probability of IA was very low (1.9%) [2]. By contrast, other observations suggest that receipt of mold-active antifungal drugs decrease sensitivity, without any relevant effect on specificity [1,

10]. This can be explained by antifungal drugs limiting the detectable burden of GM antigen, through inhibition of growth and reducing the *Aspergillus* hyphal load able to shed the antigen, which is only released into the circulation during infection, when the fungus invades the endothelial compartment [11]. Indeed, GM is no longer recommended for routine blood screening in patients receiving mold-active AFT or prophylaxis [12, 13]. The effect of antifungal therapy on the sensitivity of PCR assays for IA has long been debated. There is some evidence from animal models and clinical trials that a mold-active antifungals limit PCR detection, but this effect is not consistent across studies [3,14-17]. Variation in the antifungal administered, the incidence of IA, and the study population will influence the pretest probability of IA and potential assay performance. Our findings, based on a considerable number of trials and patients, did not show a significant reduction in the sensitivity of *Aspergillus* PCR testing of blood from patients receiving AMP. Contrary, AMP reduced the proportion of EORTC/MSGERC defined proven/probable cases of IA, and lowered specificity (i.e. increased PCR false positivity) [5, 6]. It is possible that active AMP reduces the clinical progression of IA, limiting the manifestations typically associated with IA that are essential when classifying probable IA using the EORTC/MSGERC definitions. Furthermore, given AMP has been associated with reduced GM-EIA sensitivity, the use of AMP could result in false negative GM-EIA results preventing cases of possible IA becoming probable IA and compromising PCR specificity. Conversely, the shedding of genomic material into the circulation still occurs during early infection, with the release of DNA (DNAemia) potentially being enhanced by antifungal therapy disrupting the fungal cell membrane or wall and detection of this target could define probable IA cases that would be otherwise missed using GM-EIA. Recently, *Aspergillus* PCR has been included in the updated EORTC/MSG definitions for IFD, as it provides a robust diagnostic test for screening and confirming the diagnosis of *Aspergillus* infection. [7] While the use of AMP may limit the diagnostic specificity of a single PCR

positive test, the specificity for multiple PCR positive tests (as required in the EORTC/MSGERC definitions) remains excellent, suitable for confirming a diagnosis of IA. Given the reasons above and that clinical/radiologic manifestations typical of overt IFD are required to achieve a classification of probable IA, the presence of *Aspergillus* PCR positivity in this setting will likely continue to provide sufficient mycological specificity. From a clinical perspective *Aspergillus* PCR of blood is best used to exclude IA, based on an adequate sensitivity, which from this study appears to be unaffected by the use of AMP.

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**Conflicts of Interest**

**PLW**: Performed diagnostic evaluations and received meeting sponsorship from Bruker, Dynamiker, and Launch Diagnostics; Speakers fees, expert advice fees and meeting sponsorship from Gilead; and speaker and expert advice fees from F2G and speaker fees MSD and Pfizer. Is a founding member of the European *Aspergillus* PCR Initiative.
**JL**: Is a founding member of the European *Aspergillus* PCR Initiative and is head of the FPCRI
**DB**: received research grants from Gilead Sciences and Pfizer, served on the speakers' bureau of Gilead Sciences, Merck Sharp & Dohme/Merck and Pfizer and received travel grants from Merck Sharp & Dohme/Merck and Pfizer.

**AW**: is supported by the MRC Centre for Medical Mycology (grant MR/N006364/2) at the University of Exeter, and has received research support from Gilead and served at the speaker's bureau of Gilead.

**TRR**: Served on the advisory board and at the speaker's bureau of Pfizer Healthcare Ireland, Gilead Sciences, and Menarini Pharma.

**JPD**: Has provided consultancy for F2G and Gilead and served at the speaker's bureau of Gilead and Pfizer. Is a founding member of the European *Aspergillus* PCR Initiative.

**RAB**: Is a founding member, treasurer and steering committee member of the Fungal PCR Initiative.

**MC, CM, COM, LK, JM, WJH, BW, DL, BJ and CC**: No conflicts declared

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**Table 1.** Effect of anti-mold prophylaxis on Aspergillosis incidence and diagnostic test estimates as determined by logistic mixed-model regression. PPV and NPV values were calculated using the Bayes' rule.

| Parameter | Positivity Threshold: 1 positive PCR test | | Positivity threshold: ≥2 positive PCR tests | |
|---|---|---|---|---|
| | Prophylaxis | | Prophylaxis | |
| | Yes | No | Yes | No |
| **Incidence (n/N, %)** | 164/1373, 11.94 | 216/1156, 18.68[a] | 155/1356, 11.43 | 72/401, 17.96[a] |
| **Sensitivity (95% CI)** | 0.83 (0.72, 0.91) | 0.81 (0.70, 0.88) | 0.67 (0.51, 0.79) | 0.70 (0.50, 0.84) |
| **Specificity (95% CI)** | 0.60 (0.43, 0.75) | 0.86 (0.75, 0.92)[b] | 0.86 (0.71, 0.93) | 0.98 (0.91, 0.99)[c] |
| **DOR (95% CI)** | 7.60 (3.77, 15.31) | 25.69 (13.32, 49.54)[d] | 11.80 (4.39, 31.69) | 98.06 (20.79, 462.60)[e] |
| **LR +tive (95% CI)** | 2.10 (1.30, 2.90) | 5.72 (2.64, 8.79) | 4.62 (1.21, 8.02) | 30.29 (NE, 72.30) |
| **LR -tive (95% CI)** | 0.28 (0.14, 0.41) | 0.22 (0.12, 0.32) | 0.39 (0.23, 0.56) | 0.31 (0.13, 0.49) |
| **PPV (95% CI)** | 0.22 (0.15, 0.28) | 0.57 (0.38, 0.67) | 0.37 (0.13, 0.51) | 0.87 (NE, 0.94) |
| **NPV (95% CI)** | 0.96 (0.95, 0.98) | 0.95 (0.93, 0.97) | 0.95 (0.93, 0.97) | 0.94 (0.90, 0.97) |

**Footnote:** PPV, positive predictive value; NPV, negative predictive value; DOR, Diagnostic odds ratio; LR +tive, Likelihood ratio positive; LR -tive, Likelihood ratio negative; NE, no estimate available. 95% CI, 95% Confidence interval.

[a] Difference in incidence of IA with and without prophylaxis was significant ($P<0.001$). [b] The specificity was significantly lower under prophylaxis (P = 0.005). [c] The specificity was significantly lower under prophylaxis (P = 0.019). [d] The DOR was significantly lower under prophylaxis (P = 0.013) [e] The DOR was significantly lower under prophylaxis (P = 0.022).