Clinical Infectious Diseases
EDITORIAL COMMENTARY

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Polymerase Chain Reaction of Plasma and Bronchoalveolar Lavage Fluid for Diagnosing Invasive Aspergillosis

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(See the Major Article by Mah et al. on pages 1282–90.)

In this editorial commentary, we examine 2 papers reporting the diagnosis of invasive aspergillosis (IA) using polymerase chain reaction (PCR) on plasma samples and bronchoalveolar lavage (BAL) fluid. Mah et al [1] reported the results of a single-center case-control study describing the improved accuracy of *Aspergillus* PCR targeting cell free DNA (DNAemia) in plasma samples compared with galactomannan enzyme-linked immunosorbent assay (GM) testing. By contrast, Huygens et al [2] conducted a prospective multicenter study to determine the clinical impact of PCR testing for the diagnosis of IA and detection of azole resistance when testing BAL fluid from hematology patients. Both studies used the revised and updated consensus definitions of invasive fungal disease (IFD) published by the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC/MSGERC) [2, 3].

In the study of Mah et al, when testing 43 cases of proven/probable IA, as designated by the 2020 EORTC/MSGERC definitions, the sensitivity of PCR was 86.0% (95% confidence interval [CI]: 72.7–93.4%) compared with 63.0% (95% CI: 48.6–75.5%) for serum GM. The PCR sensitivity was not significantly impacted by mold-active prophylaxis confirming recent meta-analyses [4, 5]. Overall, the specificity of PCR was comparable to GM (93.1% vs 93.0%) when testing 130 patients with no evidence of IA and PCR was more useful for both confirming and excluding IA compared with GM. When applying a positivity threshold requiring 2 consecutive PCR-positive results, sensitivity remained excellent, but specificity data were not provided. Given that the specificity for *Aspergillus* PCR utilizing a single positive threshold is already excellent, the requirement for consecutive positive results should enhance this further and supports the current requirement for consecutive blood samples being *Aspergillus* PCR positive before they are considered as an EORTC/MSGERC mycological criterion for IA [1–3].

As the EORTC/MSGERC definitions were developed to standardize the classification of IFD, to allow the enrollment of patients into clinical trials of antifungal therapy and epidemiologic studies as well as the evaluation of diagnostic tests and strategies, confidence in the accuracy of classification is paramount. For instance, misclassification of disease could potentially lead to inaccurate treatment efficacy data or misleading diagnostic performance assessment. In fact, the EORTC/MSGERC definitions are tilted towards greater specificity than sensitivity. Hence, the high specificity reported in the study of Mah et al together with the 95.1% specificity reported by the recently updated Cochrane review of *Aspergillus* PCR testing of blood endorse this approach to diagnosis [1, 5].

Conversely, the accuracy of the classification of IA using *Aspergillus* PCR as the sole mycological criterion in the EORTC/MSGERC definitions when testing BAL fluid has been questioned. In the study of Huygens et al [2], increased mortality was significantly associated with a GM index (GMI) greater than 1.0, whereas mortality associated with PCR positivity was generally lower and not significantly different from patients lacking positive mycological evidence. The authors questioned the utility of PCR testing, stating that it "should be associated with clinical impact and ideally mortality". However, the clinical impact of PCR testing could not be fully assessed as 88% of patient with a positive PCR result had received antifungal therapy so, in effect, there was no comparator arm. Interestingly, 268 patients had chest radiological findings consistent with IA in a high-risk host, satisfying the criteria detailed in the EORTC/MSGERC definitions, of which mycological evidence was found in only 99 (37%) cases being classified as probable IA [2, 3]. Yet, the utility of chest radiology was not queried, despite the high number of possible cases and the fact that radiology underpins the classification of nonproven IA in the EORTC/MSGERC definitions. Undoubtedly, given these numbers, most PCR-positive patients will have

Received 25 June 2023; editorial decision 06 July 2023; accepted 12 July 2023; published online 14 July 2023

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Clinical Infectious Diseases® 2023;77(9):1291–3
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https://doi.org/10.1093/cid/ciad421

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had supporting radiology consistent with IA, thereby increasing its likelihood. Given that 94% of GM-positive patients also received antifungal therapy and mortality was higher, comparable to a positive BAL fluid culture, it could be argued that GM positivity is a later indicator of infection compared with PCR detection, and therefore is associated with a poorer prognosis. However, the use of mortality as a measure of accurate diagnosis may be misleading, particularly when patients have complex underlying conditions such as unremitting leukemia or severe graft-versus-host disease or other life-threatening comorbidities that could drive mortality and, in fact, be confounding factors.

Huygens et al questioned the specificity of *Aspergillus* PCR when testing BAL fluid, which runs counter to the excellent specificity of 90–95% reported in the various meta-analyses of *Aspergillus* PCR testing of BAL fluid [6]. The technical merits of PCR testing over other nonculture tests also needs to be considered. Clearly, the ability to detect genetic markers of azole resistance directly from the specimen is an obvious benefit, overcoming the major limitations of conventional antifungal susceptibility testing such as limited isolate availability and prolonged turnaround time. PCR can also be tailor-made to be pan-fungal, matching, and potentially expanding, on the detection range of culture and will likely be enhanced in the era of metagenomic next-generation sequencing. Nonetheless, the ability of PCR testing to determine the quality of an individual sample through the detection of human housekeeping genes (eg, RNAse P) is often overlooked but helps avoid the reporting of false-negative results. GM testing cannot achieve this, but through the complementary use of PCR, poor-quality samples can be identified and both false-negative PCR and GM results avoided.

We do not necessarily support the notion that the classification of probable IA based on *Aspergillus* PCR positivity in BAL fluid is less reliable than that obtained with any other mycological test result but do accept that the current definition is ambiguous and requires further clarification. For instance, the current classification, “BAL fluid 2 or more duplicate PCR tests positive” could be interpreted in several ways. It could, as described in the study of Huygens et al, be interpreted as the application of 2 different PCR tests on the same DNA extracted from a single BAL fluid sample and, while this is pragmatically efficient, the approach is compromised by technical differences between the PCR tests (eg, PCR efficiency, limit of detection, assay design optimization). Moreover, it does not deal with any contamination that may have compromised that specific DNA eluate. Alternatively, the entire molecular process (DNA extraction and PCR amplification) can be repeated on a different aliquot of the same BAL fluid, and while this introduces a slight delay, it has the potential to minimize the influence of contamination, whether associated with the molecular procedure or airway sampling, and could drive improvements in specificity. Positivity in different BAL fluids will likely increase the likelihood of IA but is not clinically feasible if it requires bronchoscopy being performed at different time points. Testing individual, multiple BAL fluids taken during the same bronchoscopy is an option but demands increased resources and access to all BAL samples. It is preferential to the testing of pooled BAL fluid, where negative BAL fluids potentially dilute positive BAL fluids that were sampled from the focus(es) of infection as identified by chest computed tomography. Indeed, directing PCR testing to BAL fluids associated with the focus of infection may be sufficient to improve confidence in PCR positivity and subsequently the classification of IA when using this mycological criterion.

Alternatively, as discussed by Huygens et al, the application of quantification cycle (Cq) positivity thresholds may be required, the major drawback being that thresholds may vary between PCR assays and centers [5]. However, Huygens et al used one of the most widely used commercial *Aspergillus* PCR assays (Pathonotics *Aspergenius*, Pathonostics, Maastricht, The Netherlands) and reported that the mean Cq values for patients with PCR-positive BAL fluids and additional mycological evidence was lower (33.1 cycles) than that for isolated PCR-positive

Table 1. Potential Amendments to the 2020 EORTC/MSGERC Criteria for *Aspergillus* PCR

Aspergillus PCR
Any one of the following:
Plasma: Two or more consecutive PCR tests positive within 7 d
BAL fluid: Two or more PCR tests positive in separate aliquots of the same BAL fluid[a] Two or more different BAL fluids testing PCR positive[b]
Plasma and BAL fluid: At least 1 PCR test positive in plasma and 1 PCR test positive in BAL fluid within 7 d of each other

The suggestions in this table are intended to help the mycology community arrive at a consensus on using *Aspergillus* PCR for diagnosis.

Abbreviations: BAL, bronchoalveolar lavage; EORTC/MSGERC, European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium; PCR, polymerase chain reaction.

[a]Where separate aliquots of the same BAL fluid are subjected to the full molecular process (DNA extraction and PCR amplification) on different occasions.

[b]Two or more BAL fluids taken during the same bronchoscopy from areas of focal infection as identified by computed tomography of the chest. Two or more BAL fluids taken during different bronchoscopic procedures from an area of focal infection as identified by computed tomography of the chest.

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BAL fluids (36.4 cycles) and a lower threshold was a better predictor of mortality. Interestingly, a similar Cq threshold of 36.8 cycles was deemed optimal when using a well-validated in-house *Aspergillus* PCR to test BAL fluids; and across 2 technical evaluations of *Aspergillus* PCR when testing BAL fluid, the Fungal PCR initiative (FPCRI), a Working Group of the International Society of Human and Animal Mycology, reported Cq thresholds of 36–37 cycles to be required to achieve 100% analytical specificity across 36 datasets [7] (FPCRI; unpublished data). Given that GM positivity in BAL fluid can also be falsely positive, the potential benefit of combining *Aspergillus* PCR and GM positivity should be given serious consideration, where positivity in both samples could improve confidence in the strength of the mycological evidence [8–10]. As shown in Table 1, each definition would benefit from being further qualified, although this would have to be agreed-on through proper consensus.

There has been both positive and negative feedback on the 2020 EORTC/MSGERC definitions covering host factors and radiologic and mycological evidence [11–13]. While increasing the threshold for GM across all sample types to a GMI of 1.0 or greater has been questioned due to its likely negative impact on enrollment into clinical trials, this can be countered by the increased diagnostic rigor associated with the use of elevated positivity thresholds [13]. However, in an era of multiple GM manufacturers with different reaction kinetics and positivity thresholds, stating a threshold that was specific to the Platelia *Aspergillus* sandwich enzyme-linked immunosorbent assay (Bio-Rad, Marnes-La-Coquette, France) needs to be re-considered to permit classification when GM positivity has been derived through an alternative source. Given their consensus design, differing of opinions will undoubtedly arise about the EORTC/MSGERC definitions, so it is essential that we continue to review them in a timely manner and update them as and when required to ensure they reflect, and remain relevant to, current clinical practice.

Note

Potential conflicts of interest. P. L. W. performed diagnostic evaluations and received meeting sponsorship from Bruker, Dynamiker, and Launch Diagnostics; speaker's fees, expert advice fees, and meeting sponsorship from Gilead; speaker's and expert advice fees from F2G and speaker's fees from MSD and Pfizer: speaker's fees and performed diagnostic evaluations for Associates of Cape Cod and IMMY; speaker's fees from Qiagen; expert advice fees from Mundipharma; is a founding member of the Fungal PCR Initiative and involved with developing the *Aspergillus* PCR criterion in the 2020 EORTC/MSGERC definitions. J. P. D. is a consultant for F2G, Gilead Sciences, and Shionogi Europe BV and is a founding member of the Fungal PCR Initiative. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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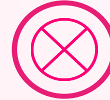
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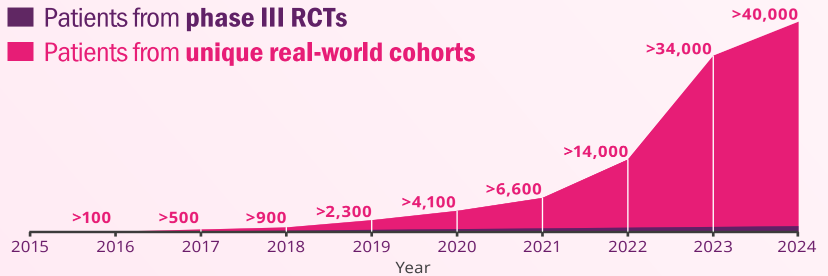

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ABBREVIATIONS

3TC, lamivudine; **CD4,** cluster of differentiation 4; **DTG,** dolutegravir; **FDA,** United States Food and Drug Administration; **FTC,** emtricitabine; **HIV,** human immunodeficiency virus; **ITT-E,** intention-to-treat exposed; **NRTI,** nucleoside/nucleotide reverse transcriptase inhibitor; **RCT,** randomised controlled trial; **RNA,** ribonucleic acid; **TAF,** tenofovir alafenamide fumarate; **TDF,** tenofovir disoproxil fumarate; **XTC,** emtricitabine.

FOOTNOTES

*Data extracted from a systematic literature review of DTG+3TC real-world evidence. Overlap between cohorts cannot be fully excluded.

**The reported rate reflects the sum-total of resistance cases calculated from GEMINI I and II (n=1/716, through 144 weeks), STAT (n=0/131, through 52 weeks), and D2ARLING (n=0/106, through 24 weeks).[5-7]

†GEMINI I and II are two identical 148-week, phase III, randomised, double-blind, multicentre, parallel-group, non-inferiority, controlled clinical trials testing the efficacy of DTG/3TC in treatment-naïve patients. Participants with screening HIV-1 RNA ≤500,000 copies/mL were randomised 1:1 to once-daily DTG/3TC (n=716, pooled) or DTG + TDF/FTC (n=717, pooled). The primary endpoint of each GEMINI study was the proportion of participants with plasma HIV-1 RNA <50 copies/mL at Week 48 (ITT-E population, snapshot algorithm).[13]

‡STAT is a phase IIIb, open-label, 48-week, single-arm pilot study evaluating the feasibility, efficacy, and safety of DTG/3TC in 131 newly diagnosed HIV-1 infected adults as a first line regimen. The primary endpoint was the proportion of participants with plasma HIV-1 RNA <50 copies/mL at Week 24.[6]

§D2ARLING is a randomised, open-label, phase IV study designed to assess the efficacy and safety of DTG/3TC in treatment-naïve people with HIV with no available baseline HIV-1 resistance testing. Participants were randomised in a 1:1 ratio to receive DTG/3TC (n=106) or DTG + TDF/XTC (n=108). The primary endpoint was the proportion of participants with plasma HIV-1 RNA <50 copies/mL at Week 48.[7] Results at week 24 of the study.

||The reported rate reflects the sum-total of resistance cases calculated from TANGO (n=0/369, through 196 weeks) and SALSA (n=0/246, through 48 weeks).[8,9]

¶TANGO is a randomised, open-label, trial testing the efficacy of DOVATO in virologically suppressed patients. Participants were randomised in a 1:1 ratio to receive DOVATO (n=369) or continue with TAF-containing regimens (n=372) for up to 200 weeks. At Week 148, 298 of those on TAF-based regimens switched to DOVATO. The primary efficacy endpoint was the proportion of subjects with plasma HIV-1 RNA ≥50 copies/mL (virologic non-response) as per the FDA Snapshot category at Week 48 (adjusted for randomisation stratification factor).[8,13]

#SALSA is a phase III, randomised, open-label, non-inferiority clinical trial evaluating the efficacy and safety of switching to DTG/3TC compared with continuing current antiretroviral regimens in virologically suppressed adults with HIV. Eligible participants were randomised 1:1 to switch to once-daily DTG/3TC (n=246) or continue current antiretroviral regimens (n=247). The primary endpoint was the proportion of subjects with plasma HIV-1 RNA ≥50 copies/mL at Week 48 (ITT-E population, snapshot algorithm).[9]

PM-GBL-DLL-BNNR-240004 May 2024