The diagnosis of mucormycosis by PCR in patients at risk: a systematic review and meta-analysis

Lottie Brown,[a,b] Lena Tschiderer,[c] Alexandre Alanio,[d,e] Rosemary A. Barnes,[f,x] Sharon C-A Chen,[g] Massimo Cogliati,[h] Mario Cruciani,[i] J. Peter Donnelly,[j] Ferry Hagen,[k,l,m] Catriona Halliday,[n] Lena Klingspor,[o] Katrien Lagrou,[p,q] Willem Melchers,[r] Laurence Millon,[s,t] Florent Morio,[u] Elena Salvador,[v] Giacomo Stroffolini,[v] Markus Ruhnke,[w] Stephanie Toepfer,[x] Karin van Dijk,[y] Andrew M. Borman,[z,aa] María José Buitrago,[ab] Rebecca Gorton,[ac] Jürgen Löffler,[ad] Riina Rautemaa-Richardson,[ae,af] Boualem Sendid,[ag] Peter Willeit,[c,ah,ai] P. Lewis White,[aj] and Michaela Lackner[x,*]

[a]St George's Hospital, St George's NHS Foundation Trust, London, UK
[b]Institute of Infection and Immunity, City St George's University of London, London, UK
[c]Institute of Clinical Epidemiology, Public Health, Health Economics, Medical Statistics and Informatics, Medical University of Innsbruck, Innsbruck, Austria
[d]Institut Pasteur, Université Paris Cité, National Reference Center for Invasive Mycoses and Antifungals, Translational Mycology Research Group, Mycology Department, Paris, F-75015, France
[e]Laboratoire de Parasitologie-mycologie, AP-HP, Hôpital Saint-Louis, Paris, F-75010, France
[f]School of Medicine, Cardiff University, Cardiff, UK
[g]Centre for Infectious Diseases and Microbiology Laboratory Services, Institute of Clincial Pathology and Medical Research, New South Wales Pathology, Westmead Hospital and The University of Sydney, Sydney, Australia
[h]Medical Mycology Laboratory, Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milano, Italy
[i]Fungal PCR Initiative, A Working Group of the International Society of Human and Animal Mycology, Verona, Italy
[j]EAPCRI Foundation, De Hoefkamp 1096, Nijmegen, 6545MD, the Netherlands
[k]Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands
[l]Institute for Biodiversity and Ecosystems Dynamics, University of Amsterdam, Amsterdam, the Netherlands
[m]Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, the Netherlands
[n]Clinical Mycology Reference Laboratory, Centre for Infectious Diseases and Microbiology Laboratory Services, Institute for Clinical Pathology and Medical Research – New South Wales Health Pathology, Westmead Hospital, Westmead, NSW, Australia
[o]Division of Clinical Microbiology, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden
[p]Department of Microbiology, Immunology and Transplantation, Laboratory of Clinical Microbiology, KU Leuven, Leuven, Belgium
[q]Department of Laboratory Medicine, National Reference Center for Mycosis, University Hospitals Leuven, Leuven, Belgium
[r]Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, the Netherlands
[s]Chrono-environnement UMR6249, CNRS, Franche-Comté University, Besançon, F-25000, France
[t]Parasitology-Mycology Department, Besançon University Hospital, Besançon, France
[u]CHU de Nantes, Cibles et Médicaments des Infections et de l'Immunité, Nantes Université, IICiMed, UR1155, Nantes, 44000, France
[v]Department of Infectious-Tropical Diseases and Microbiology, IRCCS Sacro Cuore Don Calabria Hospital, Via Don A. Sempreboni, 5, Verona, 37024, Italy
[w]Helios Klinikum Aue, Klinik fürHämatologie/Onkologie & Palliativmedizin, Aue, Germany
[x]Institute for Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria
[y]Department of Medical Microbiology and Infection Prevention, Amsterdam University Medical Center, Location AMC, Amsterdam, the Netherlands
[z]UKHSA Mycology Reference Laboratory, Southmead Hospital, Bristol, UK
[aa]MRC Centre for Medical Mycology, University of Exeter, UK
[ab]Mycology Reference Laboratory, National Centre for Microbiology and CIBERINFEC, ISCIII -CIBER de Enfermedades Infecciosas, Instituto de Salud Carlos III, Madrid, Spain
[ac]Department of Infection Sciences, Health Services Laboratories, London, UK
[ad]Medizinische Klinik II, Labor WÜ4i, Universitätsklinikum Würzburg, Germany
[ae]Mycology Reference Centre Manchester and Department of Infectious Diseases, Manchester University NHS Foundation Trust, Wythenshawe Hospital, Manchester, UK

*Corresponding author. Institute for Hygiene and Medical Microbiology, Medical University of Innsbruck, Schöpfstrasse 41, Innsbruck, 6020, Austria.
E-mail addresses: Michaela.Lackner@i.med.ac.at (M. Lackner), lottiebrown1995@gmail.com (L. Brown), lena.tschiderer@i-med.ac.at (L. Tschiderer), alexandre.alanio@pasteur.fr (A. Alanio), barnesra@cf.ac.uk (R.A. Barnes), Sharon.Chen@health.nsw.gov.au (S.C.-A. Chen), massimo.cogliati@unimi.it (M. Cogliati), crucianimario@virgiliio.it (M. Cruciani), p.donnelly@usa.net (J.P. Donnelly), f.hagen@westerdijkinstitute.nl (F. Hagen), Catriona.Halliday@health.nsw.gov.au (C. Halliday), Lena.Klingspor@ki.se (L. Klingspor), katrien.lagrou@uzleuven.be (K. Lagrou), Willem.Melchers@radboudumc.nl (W. Melchers), lmillon@chu-besancon.fr (L. Millon), florent.morio@chu-nantes.fr (F. Morio), elena.salvador@sacrocuore.it (E. Salvador), giacomo.stroffolini@gmail.com (G. Stroffolini), Markus.Ruhnke@helios-gesundheit.de (M. Ruhnke), Stephanie.toepfer@i-med.ac.at (S. Toepfer), k.vandijk1@amsterdamumc.nl (K. van Dijk), Andy.Borman@nbt.nhs.uk (A.M. Borman), Buitrago@isciii.es (M.J. Buitrago), Rebecca.Gorton@hslpathology.com (R. Gorton), loeffler_j@ukw.de (J. Löffler), riina.richardson@manchester.ac.uk (R. Rautemaa-Richardson), boualem.sendid@univ-lille.fr (B. Sendid), peter.willeit@i-med.ac.at (P. Willeit), Lewis.White@wales.nhs.uk (P.L. White).

[af]Division of Evolution, Infection and Genomics, Faculty of Biology, Medicine and Health, University of Manchester, UK
[ag]INSERM U1285, CNRS UMR 8576, Université de Lille, CHU Lille, Institut de Microbiologie, Service de Parasitologie-Mycologie, Lille, F-59000, France
[ah]Ignaz Semmelweis Institute, Interuniversity Institute for Infection Research, Vienna, Austria
[ai]Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK
[aj]Public Health Wales Mycology Reference Laboratory and Cardiff University Centre for Trials Research, UHW, Cardiff, UK

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Summary

Background This systematic review and meta-analysis aimed to examine the performance of polymerase chain reaction (PCR) assays for diagnosing mucormycosis.

Methods A standardised search was conducted from conception to December 3rd 2024 using PubMed, Embase, Global Health, and Cochrane library. Original studies that used PCR-based methods on any human specimen to diagnose mucormycosis were analysed for eligibility. Using a bivariate meta-analysis, the diagnostic performance of PCR was examined against the European Organisation for Research and Treatment of Cancer–Mycoses Study Group Education and Research Consortium 2020 (EORTC-MSGERC) definitions of proven and probable invasive mould disease, which was modified to include all patients at risk of mucormycosis. The study protocol was registered on the PROSPERO database (CRD42023478667).

Findings Of 4855 articles, a total of 30 met inclusion criteria, including 5920 PCR reactions on 5147 non-duplicate specimens from 819 cases of proven/probable mucormycosis and 4266 patients who did not meet the EORTC-MSGERC 2020 criteria. According to specimen type, sensitivity of PCR varied ($p < 0.001$) whereas specificity was similar ($p = 0.662$). Bronchoalveolar lavage fluid offered the highest sensitivity of 97.5% (95% CI 83.7–99.7%), specificity of 95.8% (95% CI 89.6–98.4%), positive likelihood ratio (LR+) of 23.5, and negative likelihood ratio (LR–) of 0.03. Tissue provided sensitivity of 86.4% (95% CI 78.9–91.5%), specificity of 90.6% (95% CI 78.1–96.3%), LR+ of 9.2, and LR– of 0.15. Blood provided reduced sensitivity of 81.6% (95% CI 70.1–89.4%), specificity of 95.5% (95% CI 87.4–98.5%), DOR of 95, LR+ of 18.3, and LR– of 0.19. Formalin-fixed paraffin-embedded specimens yielded the lowest sensitivity of 73.0% (95% CI 61.0–82.3%), highest specificity of 96.4% (CI 95% 87.5–99.0%), LR+ of 20.2, and LR– of 0.28. The covariates best explaining heterogeneity of the overall analysis were specimen type, study design (cohort *versus* case-control) and disease prevalence while patient population (COVID-19 *versus* other) and PCR (conventional *versus* quantitative) had less impact on heterogeneity.

Interpretation This meta-analysis confirms the high performance of PCR for diagnosing mucormycosis and supports the instatement of PCR detection of free-DNA in blood, BALF and tissue into future updated definitions and diagnostic guidelines for mucormycosis.

Funding None.

Keywords: Mucorales; Mucormycosis; PCR; Fungal; Diagnosis

Introduction

Mucormycosis is a severe and often fatal angio-invasive fungal disease caused by a group of ubiquitous, environmental moulds from the order Mucorales. Clinical manifestations of mucormycosis are diverse including cutaneous, rhino-orbital, cerebral, pulmonary, gastrointestinal, and disseminated disease. Susceptible populations include those with uncontrolled diabetes mellitus and compromised immunity of any form, including haematological malignancy, solid organ transplant, and the use of immunomodulating therapy.[1] The incidence of mucormycosis has risen in recent years, partly driven by the COVID-19 pandemic which led to a major outbreak of rhino-orbital-cerebral mucormycosis in India, primarily among patients with poorly controlled diabetes mellitus or receiving systemic corticosteroids.[2] Cutaneous mucormycosis may occur in healthy individuals following trauma or burns injuries, and outbreaks following natural disasters are recognised.[3,4] The overall mortality rate of mucormycosis remains very high (46% in a recent meta-analysis), particularly in those with cerebral involvement or disseminated disease.[1] The prognosis can be improved with early diagnosis and treatment.[5]

For decades, the diagnosis of mucormycosis has relied on histopathology, microscopy, and culture of

Research in context

Evidence before this study

Conventional diagnostic tests for mucormycosis including direct examination, culture, and histopathology are insensitive with cultures missing around 50% of cases and requiring up to 3–7 days to grow. Fungal biomarkers like serum galactomannan and β-D-glucan (BDG) are of no use for the detection of Mucorales species and while in development, currently no Mucorales-specific antigen test is available. In recent years, significant advances have been made in PCR-based diagnosis of mucormycosis. In case-control and cohort studies, pan-Mucorales qPCR assays, targeting the conserved rDNA (mainly 18S, 28S rDNA, and internal transcribed spacer (ITS) regions), have demonstrated good sensitivity for detecting Mucorales in fresh tissue samples and bronchoalveolar lavage fluid (BALF). However, these samples are often not feasible in critically unwell patients (e.g., haematological patients with coagulopathy) or may be delayed due to the need for specialist expertise or equipment. Detection of circulating Mucorales by PCR on blood specimens (including serum, plasma, and whole blood) has demonstrated potential as an alternative to BALF or tissue biopsies for the early diagnosis of mucormycosis, significantly reducing the time to diagnosis when compared with conventional methods. Though specificity of PCR on blood specimens is very high, sensitivity appears to be lower than for BALF/tissue specimens. A standardised search of PubMed, Embase, Global Health, and Cochrane library from conception to December 3rd 2024 found no systematic reviews or meta-analyses published previously on the performance of PCR for the diagnosis of mucormycosis and identified a data gap, which has undermined our understanding of this test that is critical to overcoming the current diagnostic limitations of mucormycosis.

Added value of this study

This rigorous and comprehensive meta-analysis presents comparative diagnostic performance of conventional and quantitative PCR (qPCR) of various specimen types and in patient populations against EORTC–MSGERC 2020 definitions of proven and probable invasive mould disease, as applied to mucormycosis. In a bivariate meta-analysis of 30 articles, overall sensitivity, specificity, and DOR were high. Sensitivity of PCR varied significantly according to specimen type, with BALF providing highest sensitivity, followed by tissue, blood, and FFPE specimens. While specificity was similar across all specimen types, it was likely compromised by the sub-optimal sensitivity of the reference test. There was no significant difference in performance of qPCR and conventional PCR in our analysis, though data for conventional assays were limited. Where available, qPCR is recommended over conventional assays due to the key technical advantages of rapid identification to genus/species level, quantification of fungal burden and reduced opportunity for contamination. Across the cohort studies, there was a very broad range of disease prevalence due to varying pre-test probabilities of disease, including very high prevalence in studies of rhino-orbital-cerebral mucormycosis (≥50%) associated with the COVID-19 pandemic.

Implications of all the available evidence

The findings of our systematic review and meta-analysis have implications on diagnostic guidelines for mucormycosis. Across all specimen types, PCR positivity is indicative of mucormycosis, but requires clinical interpretation, dependent on clinical and radiological evidence, and the strength of qPCR signal (indicative of fungal load), particularly in specimens where there is a risk of mucosal surface contamination (e.g., tissue from the nasal cavity or sinuses, respiratory tract specimens). PCR on BALF provides high sensitivity and specificity and is our recommended test for suspected pulmonary mucormycosis. PCR on blood and BALF is recommended for testing of high-risk patients (i.e., burns, haematological malignancy, traumatic injury with environmental contamination, outbreaks associated with COVID-19 and influenza), due to high negative predictive value (NPV) which can exclude infection when there is low pre-test probability. Where tissue biopsy or BALF are not feasible, blood PCR provides a non-invasive alternative with adequate diagnostic performance. Where possible, PCR on fresh tissue is recommended over FFPE specimens due to higher sensitivity. PCR negativity on FFPE specimens cannot exclude mucormycosis. However, the role of PCR on FFPE specimens is primarily to provide an identification on tissue where fungal elements have been seen but culture is negative, for which high specificity is key. qPCR is recommended over conventional PCR due to the technical benefits of quantification of burden and reduced risk for carryover contamination. PCR should not replace conventional methods which remain crucial for formal identification, antifungal susceptibility testing, and typing for delineation of outbreaks.

tissue biopsy and respiratory samples. Histopathology can identify invasive disease and distinguish between Mucorales and other invasive moulds like *Aspergillus*, but cannot differentiate between different Mucorales species and diagnostic accuracy is heavily dependent on specimen quality and operator skill.[6] Culture allows genus and species identification, but is positive in only 50% of cases and can require an average of 3–7 days for the mould to grow.[5] Tests to detect serum galactomannan and β-D-glucan (BDG) are of no use for the detection of Mucorales and, while in development, currently no Mucorales-specific antigen test is available.[7]

In recent years, significant advances have been made in PCR-based diagnosis of mucormycosis. Alongside

Aspergillus and pan-fungal PCR, Mucorales PCR on bronchoalveolar lavage fluid (BALF) or tissue formed part of the 2018 European QUALity (EQUAL) Score for diagnosis of invasive fungal diseases (IFD).[8] However, In the updated 2020 European Organisation for Research and Treatment of Cancer—Mycoses Study Group Education and Research Consortium (EORTC-MSGERC) consensus definitions for IFD, classical histopathology, and cultures remain the main, recommended methods for the diagnosis of mucormycosis and other rare moulds.[9] A combined approach of PCR and sequencing is yet only recommended for microscopy-positive tissue biopsies. The most recent global guideline (2019) from the European Confederation of Medical Mycology (ECMM) in cooperation with the Mycoses Study Group Education & Research Consortium (MSGERC) also makes a "moderate" recommendation for the use of molecular methods for the identification of the causative agent in biopsy specimens.[10]

Pan-Mucorales qPCR assays, targeting the conserved rDNA locus (mainly 18S, 28S rDNA, and ITS regions), have demonstrated good sensitivity for detecting Mucorales in fresh tissue samples and BALF.[11] When these invasive samples are not feasible, detection of circulating Mucorales by PCR on peripheral blood specimens has demonstrated potential as a non-invasive tool for the early diagnosis of mucormycosis.[12] Though the specificity of blood PCR is very high, sensitivity appears to be lower than for other specimen types.[13] The aim of this study was to undertake a systematic review and meta-analysis of the available literature to examine the comparative diagnostic performance of PCR on different specimens in patients with proven/probable mucormycosis, with a view to informing clinicians on the best strategy for the diagnosis of mucormycosis and accurate interpretation of PCR results.

Methods

This systematic review and meta-analysis were conducted by the Fungal PCR Initiative (FPCRI), a working group of the International Society for Human and Animal Mycology (ISHAM).

Search strategy and selection criteria

A standardised search was conducted from January 1st, 1946 to December 3rd, 2024 using PubMed, Embase, Global Health, and Cochrane Library (search terms in Supplementary Figure S1). No language restrictions were applied to the search and non-English language studies were translated. Additional relevant articles from the reference sections were reviewed and study authors were contacted for missing data.

Original studies that used PCR-based methods on any human specimen were analysed for eligibility. Studies were eligible if: (1) PCR test results were compared with the reference diagnosis made using standard laboratory methods in line with EORTC/MSGERC 2020 definition of proven or probable invasive mould disease, as applied to mucormycosis, including all those with known host factors for mucormycosis (e.g., diabetes mellitus, burns, traumatic injury) which are not stated in EORTC/MSGERC 2020 definitions; (2) results of the index test were reported as false positive, true-positive, false-negative, and true-negative, or this data could be derived from the study if not specifically stated; and (3) evaluation of the test(s) was performed in cohort studies of clinically relevant patient populations, defined as individuals at high risk of mucormycosis, typically where there is clinical suspicion of the disease, and case-control studies, which evaluated specimens from a group of patients known to have mucormycosis, and from a separate group of subjects without evidence of disease. Both prospective and retrospective studies were included. Studies evaluating pan-fungal PCR and sequencing were excluded.

Duplicated studies identified from different databases were removed before screening. Pairs of authors independently screened articles for eligibility and selected articles for full-text review, with disagreements resolved by a third author new to the specific article and blinded to the previous reviews. Abstract screening was facilitated using the web-based software Rayyan.[14] Pairs of authors independently extracted data from the studies included and assessed risk of bias by use of the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) tool, with any disagreements resolved by third author.[15,16] Our study adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis for Diagnostic Test Accuracy (PRISMA-DTA) guideline.[17] The PROSPERO protocol is available online (https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42023478667).

Statistical analysis

Using the GRADE approach, certainty of evidence was rated as high, moderate, low, or very low based on four domains namely, risk of bias, indirectness, inconsistency, and imprecision.[18] The QUADAS-2 tool was used to assess risk of bias and indirectness. To evaluate inconsistency, pre-specified analyses of covariates were conducted to investigate potential sources of heterogeneity and downgraded when the inconsistency in the accuracy estimates could not be explained. For imprecision, 95% confidence intervals (CI) of sensitivity and specificity estimates were assessed and downgraded where heterogeneity of the results could not be explained. Publication bias was not formally evaluated because there is no appropriate test with adequate statistical power to reliably assess this in the context of diagnostic test accuracy systematic reviews.[19] A leave-one-out meta-analysis approach was performed to

understand the impact of each individual study on the overall pooled effect estimate.

For included studies, either individual data or summary estimates of sample size, number of true positive, true negative, false positive, and false negative results of PCR in each study were extracted. The sensitivity, specificity, diagnostic odds ratio (DOR, a measure of overall diagnostic performance), and positive and negative likelihood ratio (LR+, LR–) of PCR tests and the 95% CI were calculated, as were the positive predictive values (PPV) and negative predictive values (NPV). The main statistical approach consisted of a bivariate meta-analysis of sensitivity and specificity using a multilevel mixed-effects logistic regression approach for binomial responses with an unstructured covariance matrix. Analyses were performed with Stata version 15.1 (Stata version 15.1; StataCorp LLC, Lakeway Drive, Texas: USA; https://www.stata.com/) using the commands 'meqrlogit' (Multilevel mixed-effects logistic regression [QR decomposition]) and 'metadta'.[20] Meta-regression was undertaken to investigate differences across the subgroups. Co-variates were pre-specified and included specimen type (tissue, blood, FFPE specimens, BALF or mixed, where a study pooled multiple specimen types), PCR type (qPCR, conventional), and study type (case-control, cohort). Additional co-variates of prevalence of proven/probable mucormycosis according to reference test (prevalence <5%, 5–<20%, 20–<50%, and ≥50%) and patient cohort (COVID-19 only *versus* other) were specified post-hoc. Corresponding covariates for sensitivity and specificity were added to the model assuming equal variances for the random effects of the logit sensitivities and the logit specificities. P-values were obtained from likelihood ratio tests and were considered statistically significant if <0.05. Heterogeneity was evaluated by visual inspection of forest plots of sensitivity and specificity of PCR by co-variates.

Ethics

This meta-analysis used publicly available, anonymised data and did not require ethical approval.

Role of the funding source

There was no funding source.

Results

Of the 4855 unique references identified, 133 potentially relevant articles were selected for full-text review. After full-text review, 103 studies were excluded for various reasons (Fig. 1, flow chart) and 30 studies published between 2007 and 2024 met the inclusion criteria for the systematic review and meta-analysis (Supplementary Figure S2).[21–46]

A total of 5920 PCR reactions using 5147 non-duplicate specimens from 819 cases of EORTC/MSGERC 2020 proven/probable mucormycosis and 4266 cases that did not meet the criteria for proven/probable mucormycosis were included. Studies were from 11 different countries, mostly cohort (n = 24, 80.0%) and single centre in design (n = 24, 80.0%). The reference standard was proven mucormycosis in 13 studies (43.3%), where all cases were confirmed by culture, and proven/probable mucormycosis in 17 studies (56.7%), where the diagnosis was confirmed by microscopy or culture. The reported prevalence of mucormycosis in the cohort studies ranged from 0.4% to 68.4%. Study characteristics are summarised in Supplementary Table S1.

Haematological malignancy was the most common underlying disease (n = 871, 17.1%), followed by COVID-19 (n = 613, 12.1%), diabetes mellitus (n = 529, 10.4%), organ transplantation (n = 249, 4.9%), and solid malignancy (n = 198, 3.9%). Underlying disease was not specified for over half of the included patients (n = 2905, 57.1%). Specimens tested by PCR were BALF (n = 1997, 38.8%), blood (n = 1307, 25.4%), tissue (n = 712, 13.8%), FFPE specimens (n = 512, 9.9%), or mixed (n = 619, 12.0%). In studies of "mixed" specimens (n = 8), insufficient data were provided to perform analysis based on specimen type. There were variations in the PCR formats used: qPCR was used in 21 studies (n = 5004, 84.5%) and conventional PCR was used in 9 studies (n = 916, 15.4%). Details of the PCR assays used are summarised in Supplementary Table S2.

A total of thirty-four 2 × 2 tables reporting true-positive, false-positive, false-negative, and true-negative cases were obtained from the 30 articles. QUADAS-2 assessment demonstrated that most included studies were of overall good or acceptable quality. Several studies were unclear in their reporting of patient selection and conduct of the index and reference test (i.e., use of blinding). All seven case-control studies were classified as "high risk of selection bias" regarding patient selection. Concerns regarding applicability were seldom found (Supplementary Figures S2 and S3). The certainty of the evidence varied from low in case-control studies to moderate in cohort studies, moderate for both qPCR and conventional PCR, moderate in studies evaluating BALF and blood, and low in studies evaluating tissue and FFPE specimens, for which there were comparatively fewer data (Supplementary Table S3).

Sensitivity and specificity of PCR according to specimen type, PCR assay, and study design (Table 1). Overall pooled sensitivity of PCR was 90.3% (95% CI 84.5–94.1%) and specificity was 95.0% (95% CI 91.9–96.9%). According to specimen type, sensitivity of PCR varied significantly (p < 0.001), whereas specificity was similar (p = 0.662). BALF offered the highest sensitivity of 97.5% (95% CI 83.7–99.7%), specificity of 95.8% (95% CI 89.6–98.4%), diagnostic odds ratio (DOR) of 915, LR+ of 23.5, and LR– of 0.03. Tissue provided a sensitivity of 86.4% (95% CI 78.9–91.5%), specificity of 90.6% (95% CI 78.1–96.3%), DOR of 70,

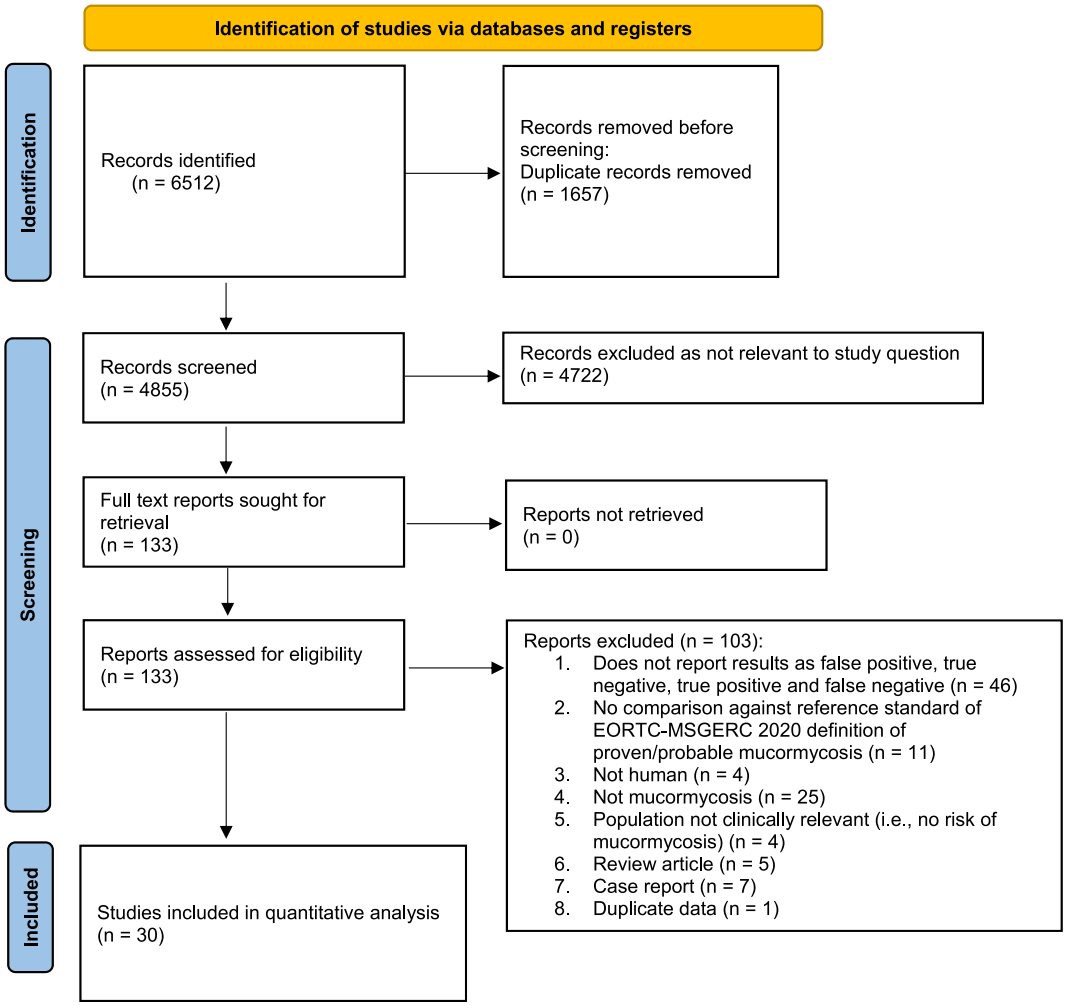

***Fig. 1:* Study selection**. Abbreviations: EORTC, European Organisation for Research and Treatment of Cancer; FN, false negative; FP, false positive; PCR, polymerase chain reaction; TN, true negative; TP, true positive.

LR+ of 9.2, and LR– of 0.15. Blood provided reduced sensitivity of 81.6% (95% CI 70.1–89.4%), specificity of 95.5% (95% CI 87.4–98.5%), DOR of 95, LR+ of 18.3, and LR– of 0.19. A subgroup analysis of plasma and serum was performed but showed no significant difference in sensitivity or specificity (p = 0.208 and 0.607, respectively, Supplementary Figure S5). FFPE specimens yielded the lowest sensitivity of 73.0% (95% CI 61.0–82.3%), highest specificity of 96.4% (CI 95% 87.5–99.0%), DOR of 72, LR+ of 20.2, and LR– of 0.28. Compared to tissue, the sensitivity of FFPE specimens was lower (p = 0.034) but differences in specificity did not reach statistical significance (p = 0.232). There were no significant differences in sensitivity and specificity of qPCR and conventional PCR (p = 0.957, 0.374, respectively), with qPCR providing sensitivity of 90.4% (95% CI 83.2–94.7%) and specificity of 95.6% (95% CI 92.4–97.6%), compared to conventional PCR with sensitivity of 90.2% (95% CI 79.3–95.7%) and specificity of 93.0% (95% CI 83.8–97.1%).

Regarding study design, reported sensitivity of PCR was higher in cohort studies (93.0%, 95% CI 88.1–96.0%) compared to case-control studies (78.8%, 95% CI 64.1–88.5%, p = 0.008), whereas specificity was not significantly different (94.1%, 95% CI 90.1–96.6% for cohort studies compared to 97.5%, 95% CI 92.1–99.2% for controls, p = 0.17). In a post-hoc analysis, sensitivity was significantly different according to disease prevalence (p = 0.020), whereas specificity did not change (0.426). The point estimate of specificity was lower for COVID-19 populations (85.5%, 95% CI 67.2–94.5%) compared to populations with other conditions (94.7%, 95% CI 91.4–96.8%), though this did not reach statistical significance (p = 0.069). Sensitivity was not significantly different according to population (p = 0.689).

	No. of assays	No. of positive/ negative specimens	Sensitivity (95% CI)	Specificity (95% CI)	DOR (95% CI)	LR+ (95% CI)	LR− (95% CI)
Specimen type							
Mixed	8	345/527	0.977 (0.944–0.990)	0.961 (0.882–0.988)	1021 (256–4064)	24.9 (8.0–7.7)	0.02 (0.01–0.06)
Tissue	8	328/504	0.864 (0.789–0.915)	0.906 (0.781–0.963)	61 (22–168)	9.2 (3.8–22.1)	0.15 (0.10–0.23)
Blood (overall)	6	118/1189	0.816 (0.701–0.894)	0.955 (0.874–0.985)	95 (29–308)	18.3 (6.4–52.6)	0.19 (0.11–0.32)
Serum	3	65/919	0.8753 (0.749–0.943)	0.949 (0.671–0.994)	130 (8.5–1984)	17.1 (2.0–145.8)	0.13 (0.06–0.30)
Plasma	3	53/270	0.742 (0.563–0.865)	0.978 (0.766–0.998)	126 (5.6–2834)	33.3 (2.3–473.1)	0.26 (0.14–0.50)
FFPE specimens	5	238/355	0.730 (0.610–0.823)	0.964 (0.875–0.990)	72 (19–272)	20.2 (5.7–71.8)	0.28 (0.19–0.41)
BALF	7	37/2279	0.975 (0.837–0.997)	0.958 (0.896–0.984)	915 (100–8394)	23.5 (9.1–60.5)	0.03 (0.00–0.19)
PCR							
qPCR	24	700/4184	0.904 (0.832–0.947)	0.956 (0.924–0.976)	207 (91–472)	20.8 (11.8–36.6)	0.10 (0.06–0.18)
Conventional	10	366/670	0.902 (0.793–0.957)	0.930 (0.838–0.971)	121 (37–400)	12.8 (5.4–30.5)	0.11 (0.05–0.23)
Study design							
Case-control study	9	283/518	0.788 (0.641–0.885)	0.975 (0.921–0.992)	143 (36–574)	31.3 (9.6–101.6)	0.22 (0.12–0.39)
Cohort study	25	783/4336	0.930 (0.881–0.960)	0.941 (0.901–0.966)	213 (95–477)	15.8 (9.3–27.1)	0.07 (0.04–0.13)
Prevalence							
≥50%	9	590/448	0.935 (0.862–0.971)	0.934 (0.830–0.976)	203 (65–634)	14.14 (5.3–37.5)	0.07 (0.03–0.15)
>20–50%	9	309/547	0.788 (0.647–0.883)	0.919 (0.821–0.966)	42 (17–107)	9.74 (4.4–21.6)	0.23 (0.14–0.39)
5–20%	6	102/640	0.919 (0.788–0.972)	0.973 (0.917–0.992)	413 (102–1673)	34.3 (11.0–106.9)	0.08 (0.03–0.23)
<5%	10	65/3219	0.963 (0.866–0.990)	0.959 (0.912–0.981)	601 (139–2605)	23.5 (10.9–50.8)	0.04 (0.01–0.15)
Population[a]							
Other	26	625/2790	0.918 (0.852–0.956)	0.947 (0.914–0.968)	199 (88–451)	17.3 (10.6–28.2)	0.09 (0.05–0.16)
COVID-19	5	291/327	0.896 (0.737–0.964)	0.855 (0.672–0.945)	51 (12–224)	6.2 (2.5–15.3)	0.12 (0.04–0.33)

Pooled diagnostic performance with reference to EORTC/MSGERC definitions of proven/probable mucormycosis. Abbreviations: 95% CI, 95% Confidence Interval; BAL, broncho-alveolar lavage fluid; CI, confidence interval; DOR, diagnostic odds ratio; FFPE, formalin-fixed paraffin-embedded; LR+, positive likelihood ratio; LR−, negative likelihood ratio; No., number; PCR, polymerase chain reaction; qPCR, quantitative PCR. [a]Three studies were excluded from this analysis because they included a mixed population and did not provide sufficient data to evaluate performance based on subgroups of patients with COVID-19.

Table 1: **Pooled sensitivity, specificity, and diagnostic odds ratio of polymerase chain reaction (PCR) test according to specimen type, PCR, study design, prevalence and population.**

PPVs and NPVs were also calculated to understand how diagnostic accuracy may change according to disease prevalence (Fig. 2). Forest plots according to specimen type showed moderate variability of effect sizes and related CIs, with sensitivity being more heterogeneous than specificity (Fig. 3). An overview of all subgroup analyses performed is summarised in Fig. 4. The covariates best explaining the heterogeneity of the overall analysis were the type of specimens evaluated, study design, and prevalence, while patient population (COVID-19 *versus* other) and PCR type had less impact on sensitivity and specificity (Supplementary Figures S5–S9). In our leave-one-out sensitivity analysis, there was minimal fluctuation in the effect estimates when each study was excluded (Supplementary Table S4).

Discussion

This meta-analysis presents a rigorous and comprehensive analysis of the comparative diagnostic performance of both conventional and qPCR on four different specimen types from 819 patients with proven/probable mucormycosis according to EORTC/MSGERC 2020 definitions and 4266 patients who did not meet these criteria. In our analysis, COVID-19 was reported as the second most common underlying disease, after haematological malignancy and surpassing diabetes mellitus and organ transplantation, although the presence of classical risk factors within the COVID-19 cohort was not specified within the papers. This highlights the changing epidemiology of mucormycosis and the expanding population of susceptible patients since the COVID-19 pandemic. Across the cohort studies, there was a very broad range of disease prevalence due to varying pre-test probabilities of disease, with some studies consisting entirely of patients with clinical and radiological evidence highly indicative of mucormycosis and other studies screening high-risk individuals with no specific clinical features (Supplementary Table S1). There was a very high prevalence of rhino-orbital-cerebral mucormycosis in some studies which were performed during the COVID-19 pandemic.

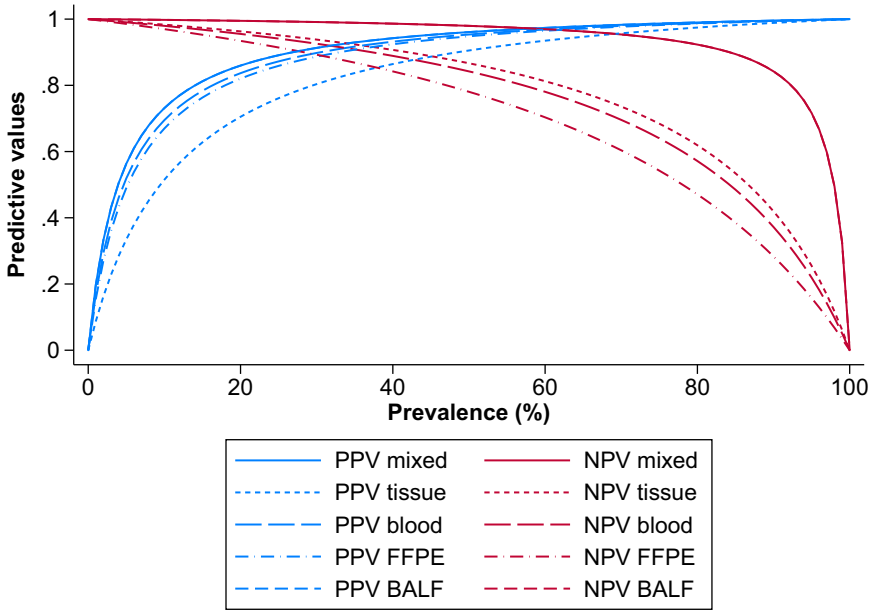

Fig. 2: **Positive predictive values (PPVs) and negative predictive values (NPVs) presented as probability curves for quantitative polymerase chain reaction on each specimen type**. Probability curves based on the predictions of the bivariate meta-analysis model. Positive and negative predictive values of Mucorales PCR are highly dependent on pre-test probability as determined by the patient's risk factors, clinical and radiological features. Understanding the impact of the pre-test probability on the interpretation of any test, including PCR, is key. Abbreviations: BALF, bronchoalveolar lavage fluid; FFPE, formalin-fixed paraffin-embedded; positive predictive values, PPV; negative predictive values, NPV.

The site of infection determines the appropriate choice of specimen for the PCR test. Of all specimen types, most data were available for PCR analysis of BALF, which provides the highest sensitivity and high specificity and is our recommended test for the diagnosis of pulmonary mucormycosis. In the presence of consistent clinical signs, the LR+ and LR− for BALF are sufficient to both include and exclude pulmonary mucormycosis. For other types of mucormycosis, tissue biopsy at the site of infection should be obtained wherever possible. PCR on tissue provides high sensitivity although specificity is reduced, possibly due to the detection of surface contamination of the mucosal membranes in the upper respiratory tract when testing nasal cavity and sinus tissue.[21,24,36,47] The point estimate of specificity was reduced in COVID-19 populations, which largely presented with rhino-orbital-cerebral mucormycosis, though statistical significance was not reached ($p = 0.069$) and data were limited and related to an outbreak setting. Corroboration of PCR positivity with histological or microscopic evidence of fungal elements within tissue is strongly recommended. Tissue homogenisation, the presence of PCR inhibitors in nasal secretions, and the use of different sections of biopsy for histopathology, culture, and molecular testing may also affect performance. Where available, the use of fresh tissue for PCR is recommended rather than FFPE tissue.[48] The specificity of FFPE and tissue specimens were similar but the sensitivity of FFPE was compromised, likely due to DNA degradation by formalin treatment and leakage of cellular contents from the fragile, pauci-septate hyphae during preparation/sectioning.[49] Mucorales-specific mitochondrial DNA appears to be more resistant to DNA degradation by formalin and warrant further investigation.[50] The use of thicker FFPE tissue specimens (50 μm) has been shown to improve sensitivity.[31]

Although sampling closer to the site of fungal growth offers higher sensitivity, tissue sampling is not always feasible in critically ill patients (e.g., haematology patients with thrombocytopaenia) and bronchoscopy may be delayed due patient's condition or the need for specialist equipment and expertise. PCR on blood demonstrated good sensitivity and excellent specificity, while providing a non-invasive, low-risk, and less technically demanding approach to sampling and is our recommended test for screening high-risk patients.[33] In our analysis, PCR on blood was used to diagnose a wide range of mucormycosis presentations, including pulmonary, rhino-orbital-cerebral, and disseminated infection, but it was not possible to evaluate diagnostic performance for individual presentations due to a lack of stratified data presented in the studies. As blood-based PCR utilises a readily available specimen type, it is suitable for screening high-risk individuals (e.g., critical burns, haematological malignancy), to enable

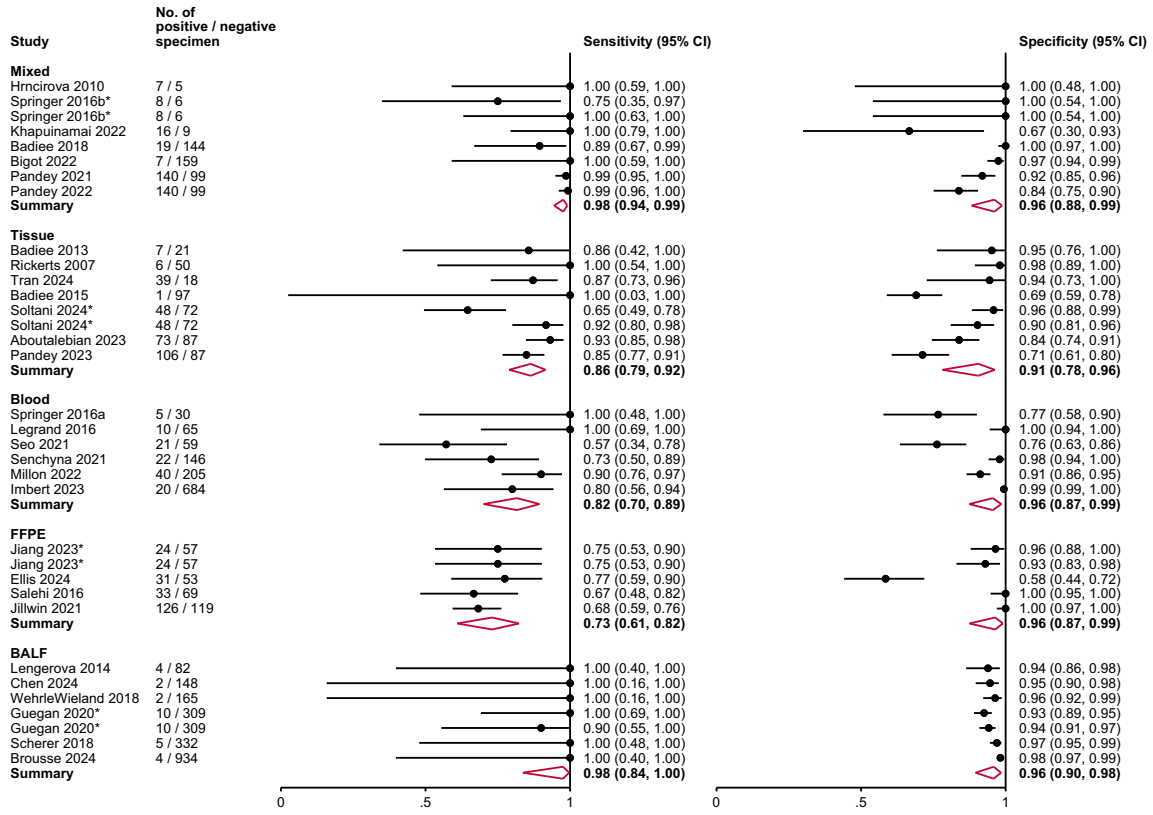

Fig. 3: **Forest plots for sensitivity and specificity of polymerase chain reaction by specimen type**. Abbreviations: BALF, bronchoalveolar lavage fluid; CI, confidence interval; FFPE, formalin-fixed paraffin-embedded; No., number. *Studies Springer 2016b and Jiang 2023 reported findings of two different assays.

early recognition of mucormycosis and monitoring of treatment response.[5,12,33,35,51] For these uses, quantification of the fungal load is key to distinguishing between contamination/colonisation and genuine infection and monitoring the burden of circulating Mucorales DNA as a marker for treatment response.[13]

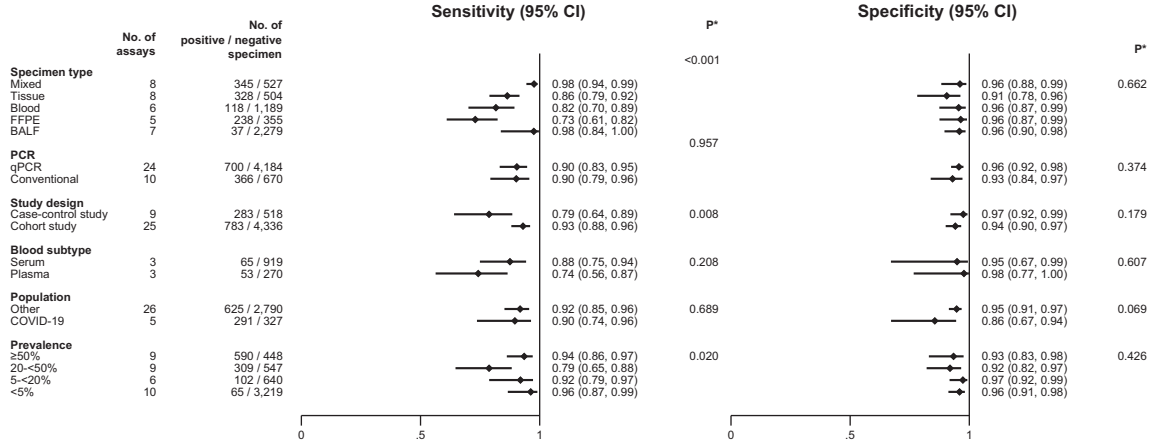

Fig. 4: **Forest plots for pooled sensitivity and specificity of polymerase chain reaction according to specimen type, PCR type, study design and prevalence**. Abbreviations: BALF, bronchoalveolar lavage fluid; CI, confidence interval; FFPE, formalin-fixed paraffin-embedded; No., number; PCR, polymerase chain reaction; qPCR, quantitative PCR. *P-values are from Likelihood-ratio test. Three studies were excluded from this analysis because they included a mixed population.

Our analysis yielded no statistically significant differences in sensitivity or specificity when comparing qPCR with conventional PCR. Only ten studies evaluated conventional PCR assays, which limited the statistical power to investigate differences according to quantitative or conventional PCR. Where available, qPCR should be used in place of conventional assays, due to the key technical benefits of quantification of fungal burden and reduced contamination risk. Currently, there are only a few commercially available assays for the detection of Mucorales in clinical samples. Most studies in our analysis utilised various DNA extraction kits and methods as well as in-house PCR assays, with variations in the gene target, PCR chemistry, -platforms, -volume, and -protocols, which will affect the concentration of fungal DNA and diagnostic yield. For blood PCR, sensitivity was significantly reduced in one study which utilised only 200 µL of plasma.[43] Previous studies have demonstrated improved sensitivity of PCR by collection of large volume serum/plasma samples (≥1 mL) and combining the cell pellet and supernatant for BALF.[12,41,42,52] As PCR is increasingly used as a diagnostic tool for mucormycosis, further work is needed to develop international standards for Mucorales PCR.

Regarding study design, the point estimate of specificity for case-controls was higher than cohort studies, possibly due to recall bias associated with the case-control design, though data was limited to 9 studies, and statistical significance was not reached ($p = 0.179$). The pooled sensitivity of case-control studies was reduced compared to cohort studies, but this appeared to be driven by specimen type, with five of the nine case-controls examining PCR on FFPE specimens. Similarly, sensitivity varied significantly according to disease prevalence, but this was also likely driven by specimen type: four of the nine studies reporting a prevalence of between 20 and 50% examined FFPE specimens.

These data do not consider the burden of fungal disease as determined by an individual positive qPCR test, where higher burdens are generally associated with an increased likelihood of disease compared to colonisation or contamination. Analysis of PPVs/NPVs indicates that PCR on blood or BALF can be used as a screening test in low-prevalence populations, where negativity can confidently exclude mucormycosis (Fig. 2). PCR positivity will likely require clinical interpretation, partially dependent on the pre-test probability of disease (based on clinical and radiological evidence), the strength of the PCR signal (indicative of fungal burden), and the specimen type. However, this multicomponent approach is commonplace in medical mycology, where perfect diagnostic tests are rare and combining multiple laboratory diagnostic tests with clinical evidence typical of disease is common practice. The development of antigen tests to complement molecular methods for the diagnosis of mucormycosis are needed. Mixed infections, particularly with *Aspergillus*, are common (reported in up to a third of patients) and therefore Mucorales PCR should be performed in combination with PCR and antigen tests for other relevant pathogens.[53]

This review has several limitations. Firstly, fewer data were available for FFPE and tissue specimens which may have limited the power to assess their diagnostic performance and necessitated the pooling of different types of tissue (nasal cavity/sinuses *versus* deep sites) and is a possible source of heterogeneity. Secondly, the sensitivity of microscopy and culture as the reference test is suboptimal which may have led to misclassification of cases and generally compromised specificity of PCR. Many of the studies reported false-positive Mucorales PCR from patients where there was a strong clinical suspicion of mucormycosis and positive response to treatment, but negative microscopy.[26,27,35,43] The accuracy of microscopy and histology is highly dependent on the expertise and experience of the operator. The heterogeneity of sample collection techniques within specimen types such as sample volume and quality, and operator skill when performing bronchoscopy or tissue biopsy could not be considered in the analyses due to lack of information. Technical parameters associated with optimal analytical performance have recently been identified.[52] Our analysis identified significant heterogeneity in the methodology of DNA extraction and PCR which will have likely impacted diagnostic yield. A small proportion of specimens included in our analysis were not unique: four studies evaluated multiple assays using the same specimens and two studies evaluated multiple specimens from the same patients. To provide a complete overview of the literature, these specimens were included in our analysis though they are not independent. Finally, reporting was variable across studies. Underlying disease was not specified in over half of cases. It was not possible to perform a stratified analysis according to underlying disease (except for COVID-19), patient demographics or medication use as the studies seldom presented sufficient data for comparative analysis according to these factors. Reporting of patient selection and conduct of the index test was unclear and is a possible source of bias in our analysis. The very high prevalence of disease in some cohort studies and artificially high prevalence in case-controls may be a source of selection bias. There is a clear need for further studies evaluating the diagnostic accuracy of standardised PCR methods against the reference standard, with comparative performance according to underlying disease, specimen type, and timing of treatment initiation.

Nevertheless, we can conclude that the overall diagnostic performance of Mucorales PCR is high. Our findings provide a framework for clinicians to interpret Mucorales PCR on different specimens and PCR techniques and support the instatement of PCR detection of

free-DNA in blood, BALF, and tissue into future updated definitions of mucormycosis and diagnostic guidelines.[9,54]

Contributors

Study conception and design: M. L., F. H., L. B., R. A. B., J. P. D., P. L. W., J. L., and M. Cr. Data curation and formal analysis: All authors. Methodology: L. B., L. T., P. W., M. L., F. H., R. A. B, J. P. D., P. L. W., J. L., and M. Cr. Project administration: L. B., M. L., and F. H. Supervision: M. L., F. H., R. A. B, J. P. D., P. L. W., J. L., P. W., and M. Cr. Visualization: L. B., M. Cr., and L. T. Writing–original draft: L. B., and L. T. Writing–review & editing: All authors. L. B., L. T., and M. Cr. verified the underlying data.

Data sharing statement

Data in this systematic review with meta-analysis were extracted from published and preprint studies available on the internet. Request for the meta-analysis data can be made by contacting the corresponding author.

Declaration of interests

S. C.-A. A. has received united educational grants from F2G Ltd and travel grants from Asia Fungal Working Group. K. v D has received honoraria from Pfizer and participated on advisory boards for Pfizer and Gilead. A. A. has received honoraria from Gilead. R. R-R. has received honoraria from Pfizer, Mundipharma/NAPP, Scynexis, and Gilead. K. L. has consulted for Mundipharma, received grants from TECOmedical, received honoraria from Gilead, Pfizer, Mundipharma, and FUJIFILM Wako chemicals Europe GmbH, received travel support from Gilead, Pfizer, and AstraZeneca, and participated on advisory boards for Pfizer. P. L. W. has performed diagnostic evaluations and received meeting sponsorship from Associates of Cape Cod, Bruker, Dynamiker, and Launch Diagnostics; speaker fees, expert advice fees, and meeting sponsorship from Gilead; and speaker and expert advice fees from F2G. J. P. D. has received personal fees from F2G, Gilead Sciences, and Pfizer. J. L. leads the European Aspergillus PCR Initiative (EAPCRI). P. W. has received consultancy fees from Novartis Pharmaceuticals. M.L. has received research grants from Gilead and Pfizer. All other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.eclinm.2025.103115.

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