Diagnostic Modalities for Pulmonary Aspergillosis: A Systematic Literature Review of KOH Microscopy, Culture, Galactomannan Assay, and PCR-Based Molecular Techniques

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Abstract:
Background:
Pulmonary aspergillosis is a group of disorders that include allergic reactions as well as a severe infection of the lungs (invasive pulmonary aspergillosis or IPA), which is life-threatening and mainly occurs in people with weakened immune systems. Diagnosis is still not easy and may be missed because of the non-specific clinical symptoms and radiological changes. These methods are still widely used in practice, but have drawbacks in terms of turnaround time and sensitivity, and are based on conventional microscopy using potassium hydroxide (KOH) and fungal culture. In the last few years, several new technologies have been developed which have potential for helping with early detection, including galactomannan assays and using polymerase chain reaction (PCR) based molecular techniques.
Objective:
This is a systematic literature review, including the performance of diagnosis and the advantages and disadvantages of the existing practical approach. The advantages and disadvantages of KOH microscopy, fungal culture and galactomannan assays are discussed. molecular diagnosis of pulmonary aspergillosis.
Methods:
The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) were used. Relevant studies were searched via electronic databases from January 2004 to June 2026. All studies that compare KOH microscopy, fungal cultures, galactomannan assays or PCR were included. The data extracted and synthesized consisted of data on study characteristics, diagnostic performance, and methodological quality.
Results:
These studies showed that the diagnostic utility of the traditional, serological and molecular methods used to diagnose pulmonary aspergillosis varied. The sensitivity of conventional techniques like KOH microscopy and fungal culture was low, but they are helpful for preliminary screening, species identification and antifungal susceptibility testing. In all studies, the performance of serum galactomannan was poorer than that of bronchoalveolar lavage (BAL) galactomannan, with sensitivity of 63–78%, and specificity of 85–93%, versus 87–92%, and 89–98%, respectively. The molecular assays produced excellent diagnostic accuracy, with in individual studies, sensitivity up to 90.2% and specificity up to 96.4% for PCR-based assays of BAL, which allowed for earlier detection of Aspergillus infection. Overall, the evidence is supportive of an integrated approach to using microbiological, serological and molecular techniques for an increased diagnostic yield.
Conclusion:
Galactomannan assays and molecular methods are valuable for early detection and diagnostic accuracy and can be used together with the traditional methods used for routine diagnosis in the laboratory. For individuals with clinical suspicion of pulmonary aspergillosis, a multimodal approach to diagnosis—comprising microbiology, serology and molecular testing—may be best for achieving clinical results.
Keywords:
Pulmonary aspergillosis, Invasive pulmonary aspergillosis, Aspergillus, Galactomannan, PCR, Molecular diagnostics, Fungal culture, KOH microscopy, Systematic literature review, Diagnostic accuracy.
How to cite this article: Singh S, Sujatha R, Afaq N, Singh RP. Diagnostic Modalities for Pulmonary Aspergillosis: A Systematic Literature Review of KOH Microscopy, Culture, Galactomannan Assay, and PCR-

Based Molecular Techniques. Int J Drug Deliv Technol. 2026;16(70s): 1682-1697. DOI: 10.25258/ijddt.16.70s.183

1. Introduction:

1.1 Burden of Pulmonary Aspergillosis:

Aspergillus species are an important group of fungi, the pulmonary form of aspergillosis being the most common, and other species are now beginning to be recognized as important human pathogens namely A. flavus, A. terreus and A. niger.. Allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis (CPA) and invasive pulmonary aspergillosis (IPA) are varieties of the disease spectrum, of which the latter is the most severe type of opportunistic fungal infection in immunocompromised patients (Patterson et al., 2016). Aspergillus plays an important role in fungal diseases worldwide and in the cost of healthcare. There are estimated to be 100000 or hundreds of thousands of people in the world who suffer from CPA, including those with other underlying conditions such as tuberculosis, chronic obstructive pulmonary disease (COPD), sarcoidosis and others. Despite the use of supportive care and antifungal therapy, invasive pulmonary aspergillosis continues to be a disease with high morbidity and mortality (Patterson et al., 2016). There are some groups of patients where pulmonary aspergillosis is more common. People who have had haematological malignancies, people who have undergone haematological stem cell transplantation (HSCT), solid organ transplantation, long-term corticosteroid treatment or critically ill intensive care unit (ICU) patients (Patterson et al., 2016; Matteo et al., 2021). The COVID-19-associated pulmonary aspergillosis (CAPA) syndrome is another recent example of the increasing impact of Aspergillus infections in the critically ill patient with viral pneumonia (Koehler et al., 2021).

1.2 Challenges in Diagnosis:

Aspergillus is a difficult diagnosis to make as the clinical signs are non-specific and there is considerable overlap with many other forms of pneumonia and inflammatory lung diseases. Laboratory signs and symptoms such as fever, cough, dyspnea, chest pain and hemoptysis are insufficient by themselves to make a definite diagnosis and must be correlated with radiological and laboratory findings (Patterson et al., 2016). Nodules, cavitary lesions, halo signs and air-crescent signs may be one of the signs of invasive aspergillosis, but they are not specific. There are other pulmonary diseases that may cause some of these signs (Patterson et al., 2016). Therefore, multiple biological, serological, molecular and radiological parameters can be necessary for diagnosis. Another significant clinical problem is delayed diagnosis, as early antifungal therapy has been shown to have a strong association with good outcome. Early diagnosis of invasive disease can stop further invasive disease spread, early dissemination and prolonged hospitalisation and death (Patterson et al., 2016; Koehler et al., 2021).

1.3 Conventional Diagnostic Methods:

Traditional diagnostic assays have been the basis for the diagnosis of pulmonary aspergillosis for many years and remain a vital part of standard clinical practice. The diagnosis and identification of Aspergillus species from respiratory specimens is mainly based on direct microscopic examination and fungal culture. Traditional methods are widely used and fairly inexpensive, but lack high diagnostic value, are time consuming, and require specimen quality and/or laboratory expertise.

1.3.1 KOH Microscopy:

One of the oldest and most commonly used tests used for initial detection of fungal elements in respiratory specimens is potassium hydroxide (KOH) microscopy. The method enables the immediate identification of septate hyphae in clinical specimens, and is particularly useful in areas with limited resources. The advantages of KOH microscopy are: It is easy to do, inexpensive and it requires a short turnaround time.

However, the KOH microscopy technique is not particularly sensitive, and depends on specimen quality and the experience of the examiner. Further, the microscopic characteristics are unreliable in distinguishing the Aspergillus species from other moulds of similar microscopic characteristics (Patterson et al., 2016).

1.3.2 Fungal Culture:

Cultural growth of fungi is the essential part of the diagnosis of aspergillosis in which fungal species can be identified and antifungal susceptibility testing can be done. Selective fungal media are routinely used to culture respiratory specimens, including sputum, bronchoalveolar lavage (BAL) fluid and tissue biopsies.

However, the use of culture for diagnosis is limited by relatively low sensitivity and long incubation times. Viable organisms may not grow in the laboratory or may exist as a small population in a negative culture and infection cannot be excluded. Further, it is important to interpret a positive culture as the Aspergillus species can colonise the respiratory tract without being a cause of invasive disease (Patterson et al., 2016).

1.3.3 Strengths and Limitations:
Traditional culture-based diagnostic tools are still important tools for routine fungal diagnosis, but these are not sufficiently sensitive and do not provide a timely diagnosis, which has led to the development of non-culture-based diagnostic tools that are able to detect fungal diseases earlier.

1.4 Emerging Diagnostic Approaches:
The diagnosis of fungal infections is improving and new diagnostic strategies have been developed to enhance early, accurate diagnosis of pulmonary aspergillosis. These newer techniques are based on the detection of fungal antigens and nucleic acids, rather than microscopic identification and fungal growth, as is required by traditional techniques. The introduction of galactomannan assays and molecular techniques, including PCR, has proved to be more sensitive and quicker to diagnose, especially in high-risk patients.

1.4.1 Galactomannan Assay:
Galactomannan is a polysaccharide component of their cell wall, which is secreted in the active growth phase of Aspergillus species. Galactomannan antigen detection in serum and BAL has become an important diagnostic technique for the diagnosis of invasive aspergillosis, and is part of the current definitions of invasive fungal disease (IFD) (Mercier et al., 2021).
The published literature demonstrates that BAL gn, although not invariably, more often has a better diagnostic performance than serum gn, particularly when the patient is not neutropenic. This is a more sensitive assay than culture-based assays and has been incorporated as an added useful complementary biomarker in high-risk patient population (Mercier et al., 2021).

1.4.2 PCR-Based Diagnostics:
The direct detection of Aspergillus by PCR is possible by examining clinical samples. The sensitivity, along with the specificity of PCR assays, has been greatly improved by molecular technology and has proved to be a valuable complement to conventional microbiological techniques.
Real-time PCR platforms have rapid turnaround and can be used to quantify fungal burden. Furthermore, molecular assays may be useful to identify mutations associated with azole resistance, which may aid in therapeutic decisions (Patterson et al., 2016).

1.4.3 Molecular Characterisation:
The molecular characterisation of Aspergillus spp. Based on gene targets like 18S rRNA gene, 28S rRNA gene, ITS regions, , β-tubulin gene and calmodulin gene has helped to enhance the identification of the species at the level of species and epidemiological investigations. These molecular tools can be used to help explain the diversity of Aspergillus, its transmission and resistance to antifungals (Patterson et al., 2016).

1.5 The need for a Comparative Systematic Literature Review:
Many studies have evaluated the diagnostic value of KOH microscopy, fungal culture, galactomannan assays, and PCR techniques, but considerable differences have been reported in the reported sensitivity and specificities of these tests. The data published in the literature are conflicting because of the differences in the study population, the type of specimen, the diagnostic criteria and the laboratory procedures (Mercier et al., 2021).
Furthermore, there is no agreement for an algorithm of diagnosis for all patient groups in regard to pulmonary aspergillosis. Thus, a comprehensive evaluation of all the conventional, serological and molecular diagnostic methods is required to aid in clinical decision making on the basis of evidence and to decide on the optimal diagnostic approach.

1.6 Review Objective:
This review aims to assess the diagnostic performance, merits, drawbacks and clinical utility of the use of KOH microscopy, fungal culture, galactomannan assays and PCR-based techniques for the diagnosis of pulmonary aspergillosis. In addition, this review also covers recent developments in the molecular characterisation and future directions of enhancing Aspergillus diagnostics.

2. Methodology:
2.1 Study Design:
The study was performed as SLR with a particular protocol and the diagnostic value of conventional and molecular diagnostic techniques in the diagnosis of PA was reviewed and compared. The review focussed on the use of potassium hydroxide (KOH) microscopy, culture of fungus, galactomannan assays and molecular techniques (PCR). This review method was developed to be transparent, repeatable and to find all relevant evidence.

2.2 PRISMA Guidelines:
The Systematic Review Reporting Items for Meta-Analyses (PRISMA) 2020 statement was followed for the review protocol and the reporting of systematic reviews (Page et al., 2021). Process of study selection and eligibility assessment, data extraction and reporting methodology were designed to be the same as PRISMA guidelines. In addition, methodologic problems unique to

diagnostic test accuracy reviews were incorporated as appropriate (McInnes et al., 2018).

2.3 Research Question:

The purpose of this review was to provide an answer to the following research question:

- What is the diagnostic efficacy of KOH microscopy, fungal culture, galactomannan assays and molecular techniques in diagnosing pulmonary aspergillosis?

It is developed according to the PICOS framework:
It is formulated by using the PICOS framework:

Population (P): Patients who have been suspected of having pulmonary aspergillosis CPA or IPA.

Intervention/Index Test (I): KOH microscopy, fungal culture, galactomannan assays, conventional PCR and real-time PCR.

Comparator (C): Based on diagnostic criteria (e.g. EORTC/MSGERC, histopathology confirmation, composite clinical diagnosis or comparison with other diagnostic modalities).

Outcomes (O): Diagnostic sensitivity, specificity, PPV, NPV, diagnostic accuracy, AUC.

Study Design (S): Diagnostic accuracy studies and cohort studies, cross-sectional and multicenter validation studies.

2.4 Literature Search Strategy:

A thorough literature review was conducted in several electronic databases such as PubMed/MEDLINE, Scopus, Web of Science and Embase. The databases selected for this work were selected to cover the following areas of pulmonary aspergillosis: biomedical research, clinical studies, and diagnosis. Literature published between January 2004 and June 2026 was included in the search and all relevant literature that compared all the conventional diagnostic techniques (e.g., KOH microscopy and fungal culture) and the non-culture-based techniques (e.g., galactomannan assays and PCR-based molecular techniques) were sought.

The following keywords were used in the search strategy: MeSH and free text terms related to pulmonary aspergillosis and diagnostic tests. The following terms were used as search terms: “pulmonary aspergillosis,” “invasive pulmonary aspergillosis,” “chronic pulmonary aspergillosis,” “Aspergillus,” “KOH microscopy,” “fungal culture,” “galactomannan,” “polymerase chain reaction,” “PCR,” “molecular diagnosis,” “diagnostic accuracy,” “sensitivity,” and “specificity.” For each database, the search strategy was modified, depending on the indexing system and search capabilities of the database.

Boolean operators (AND, OR) were used to appropriately combine the search terms. The articles and review papers relevant to the investigation were also hand-searched from reference lists of included articles to search for further articles.

Here's an example of a PubMed search strategy:
("pulmonary aspergillosis" OR "invasive pulmonary aspergillosis" OR "chronic pulmonary aspergillosis")
AND
Culture ORgalactomannan,OR PCR OR "polymerase chain reaction")
AND
(diagnosis AND OR diagnostic OR sensitivity OR specificity).

2.5 Eligibility Criteria:

To ensure that the studies had relevance, eligibility criteria were devised to the review objective and that sufficient data were presented in the studies to evaluate the diagnostic usefulness of the conventional and molecular diagnostic tests for pulmonary aspergillosis. Studies were reviewed using a priori screening of inclusion/exclusion criteria consistent with study design, population characteristics, diagnostic modalities evaluated and outcome measures reported.

- **Inclusion Criteria:**

Studies in peer-reviewed journal articles that were published between January 2004 and June 2026. Those involving human participants at risk for PA were included. Eligible studies evaluated any of the following: KOH microscopy, fungal culture, galactomannan assays, conventional PCR assays or real-time PCR assays. Studies were only included if the diagnostic performance measure reported at least one diagnostic performance outcome (sensitivity, specificity, PPV or NPV, diagnostic accuracy or AUC) that was relevant to the review objective.

- **Exclusion Criteria:**

Case reports, case series, narrative reviews, editorials, letters to the editor, conference abstracts and expert opinion articles were not included in the study. Studies did not include those that relied on animal models or in vitro. In addition, articles which did not provide adequate diagnostic performance data, addressed only non-pulmonary Aspergillus infections, or were published in a language other than English were excluded from the review.

2.6 Study Selection Process::

All records in the databases were imported into the reference management software and
duplicate records were removed. Titles and abstracts were independently screened and
evaluated for eligibility according to pre-defined eligibility criteria. All the potentially eligible

studies were given a full-text review. Studies were included based on agreement by reviewers; differences were resolved by discussion. A PRISMA flow diagram was used to document the process of study selection to show the number of records identified, screened, excluded and included in the final review.

2.7 Extraction of Data:

A standardised data collection form was used for data extraction that was specially designed for this review. All the studies included the name of the author(s), year of publication, country of study, study design, patient population, sample size, diagnostic modality evaluated, specimen type and the reference standard used. In addition, different diagnostic performance measures, including sensitivity, specificity,PPV, NPV, and other outcome measures, were recorded. Key findings of each of the studies were also developed for the comparison of diagnostic modalities. All the data extracted were checked crosswise against each other and independently to minimise the error in the data extraction process and to ensure the accuracy of the data.

2.8 Quality Assessment:

Risk of bias and applicability concerns of included studies were assessed using Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) as a validated tool recommended for the risk of bias and applicability assessment in diagnostic accuracy studies (Whiting et al., 2011). The QUADAS-2 consists of four domains; patient selection, index test, reference standard and flow and timing. All domains were assessed for potential risk of bias and the domains patient selection, index test, and reference test were also assessed for concerns about applicability to review questions. The researchers were categorized as having low, high or unclear risk of bias based on pre-established criteria. Results of quality assessment were then taken into account in the interpretation and synthesis of the evidence.

2.9 Data Synthesis:

The outcome was then qualitatively synthesized to compare the diagnostic efficacy of KOH microscopy, fungal culture, galactomannan and molecular testing including PCR.

The diagnostic results, such as sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy, were presented overall in the different studies. Special consideration was given to the differences between the serum and the bronchoalveolar lavage (BAL) galactomannan assays as well as the differences between the conventional and real-time PCR methodologies.

The pros and cons of each modality, as well as the features of diagnostic performance, were summarized in comparative tables. If there were several studies with comparable data, these were compared where possible to determine trends and sources of variation.

3. Results:

3.1 Study Selection:

A systematic study selection process was carried out in line with the PRISMA 2020 guidelines. The total number of records identified by database searching was estimated to be a total of 3,624: PubMed (n = 1,876); Embase (n = 1,396); the Cochrane Library (n = 352), and other registers and sources (n = 64). 2,560 unique records were screened after the 1,128 records were duplicated.

After screening for title and abstract, 2,212 records were excluded and 348 full-text articles were then screened for eligibility based on the predetermined criteria. Of these, 134 articles were excluded after full-text review because they were not directly related to Aspergillus diagnostics, 76 articles because of inappropriate population or clinical setting, 52 articles because of inappropriate study design, 38 articles because of insufficient diagnostic outcome data, 17 articles were review/editorial/letters and 10 articles were duplicate/overlapping data set.

Lastly, 21 studies were selected and then the information was considered appropriate for qualitative synthesis, as shown in the PRISMA flow **Figure 1.**

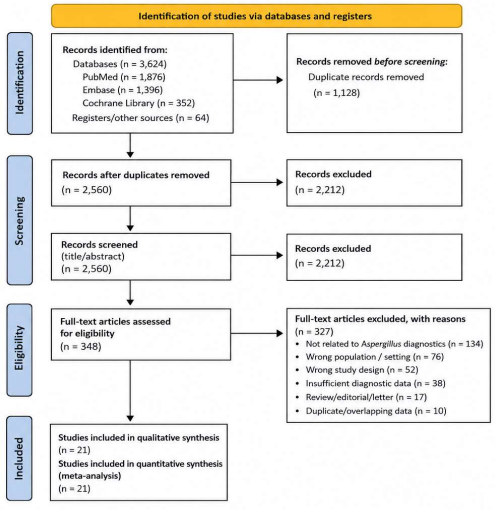

Figure 1. Prisma flow diagram

3.2 The characteristics of the included studies are summarised in this section:

The studies included in this review assessed conventional or non-culture methods of diagnosis. The conventional methods used were mostly KOH

microscopy and fungal culture, with non-culture-based methods such as serum galactomannan (GM), bronchoalveolar lavage (BAL) galactomannan, conventional PCR and real-time PCR as summarised in **Table 1.**

Table 1: Characteristics of Diagnostic Modalities

Method	Advantages	Limitations	Diagnostic Performance
KOH Microscopy	Rapid, inexpensive, widely available	Operator dependent, poor sensitivity	Low–moderate
Fungal Culture	Species identification and susceptibility testing	Slow growth, low sensitivity	Moderate
Serum Galactomannan	Non-invasive, enables early diagnosis	False positives and lower sensitivity in some populations	Good
BAL Galactomannan	High diagnostic accuracy	Requires bronchoscopy	Excellent
Conventional PCR	Rapid Aspergillus DNA detection	Methodological variability	Good–excellent
Real-Time PCR	High sensitivity, quantitative detection	Cost and technical expertise	Excellent

The 21 studies reviewed in this systematic review evaluated various diagnostics for PA from 2004 to 2024. Evidence included in the package was systematic reviews, meta-analyses, prospective (and multicenter) diagnostic accuracy studies, assay validation studies and international guideline or consensus documents. The research took place in different geographical settings in Europe, Asia, Australia and other overseas countries and the results are applicable to a broad range of settings. Diagnostic techniques consisted of conventional microbiological methods (KOH microscopy and culture), antigen detection assays (mostly galactomannan assays on serum and on bronchoalveolar lavage (BAL) samples) and molecular testing (blood, BAL and respiratory specimens were analysed with conventional and real-time PCR for Aspergillus spp.). The sample size of the studies varied greatly, from small prospective cohort studies to large multicentre and pooled meta-analytic studies. In general, the studies included herein and in **Table 2**, covered the changing diagnostic picture of pulmonary aspergillosis in detail, thus allowing for comparison of the traditional, antigen-based, and molecular diagnostic approaches.

Table 2: Characteristics of Studies Included in the Systematic Review of Diagnostic Methods for Pulmonary Aspergillosis

Study	Year	Country/Region	Study Design	Sample Size	Diagnostic Modality	Specimen Type
Leeflang et al.	2015	Multiple countries	Cochrane systematic review	5,660	Serum galactomannan	Serum
Zou et al.	2012	Multiple countries	Systematic review & meta-analysis	2,268	BAL galactomannan	BAL
Mengoli et al.	2009	Multiple countries	Systematic review & meta-analysis	1,618	PCR	Blood/serum/plasma
Avni et al.	2012	Multiple countries	Systematic review & meta-analysis	1,585	BAL-PCR vs BAL galactomannan	BAL
Sun et al.	2011	Multiple countries	Bivariate meta-analysis	~1,200+	PCR	BAL

Heng et al.	2015	Multiple countries	Systematic review & meta-analysis	783	BAL galactomannan ± PCR	BAL
Xu et al.	2025	China	Comparative diagnostic accuracy study	589	GM-EIA vs GM-LFA vs Aspergillus PCR vs mNGS	Blood + BAL
D'Haese et al.	2012	Belgium	Diagnostic validation study	150	BAL galactomannan	BAL
Yang et al.	2024	China	Retrospective diagnostic study	237	BAL galactomannan + mNGS comparative evaluation	BAL
Cuenca-Estrella et al.	2009	Spain	Prospective diagnostic study	119	Real-time PCR	Whole blood
Krishna et al.	2024	India	Prospective clinicomycological study	82	KOH microscopy + culture	Respiratory specimens
Urabe et al.	2017	Japan	Prospective diagnostic study	53	PCR + galactomannan + β-D-glucan	BAL
Eigl et al.	2017	Austria	Prospective diagnostic study	Not reported	BAL + blood galactomannan + PCR	BAL + blood
Oliveira et al.	2023	Portugal	Prospective molecular diagnostic study	Not reported	Aspergillus PCR detection	Respiratory samples
van Grootveld et al.	2021	Netherlands	Prospective ICU study	Not reported	PCR + culture	Tracheal aspirate
Xess et al.	2004	India	Observational prevalence study	154 clinical isolates	Culture-based Aspergillus detection	Clinical samples
Shrimali et al.	2013	India	Cross-sectional sputum isolation study	50	Culture-based Aspergillus isolation	Sputum
Mortensen et al.	2011	Denmark	Prospective laboratory-based study	Not reported	Aspergillus culture + azole resistance analysis	Respiratory samples
Johansen et al.	2011	Denmark	Prospective respiratory study	Not reported	Culture + clinical impact assessment	Respiratory tract samples

Halliday et al.	2005	Australia	Diagnostic assay validation	Not reported	Nested real-time PCR	Clinical specimens
Trovato et al.	2025	Italy	Retrospective molecular diagnostic study	Not reported	Quantitative real-time PCR (qPCR)	Respiratory specimens

3.3 Evaluation of the diagnostic value of the KOH microscopy:

KOH microscopy continued to be one of the most readily available diagnostic tests, especially in resource-limited areas. By using this method the fungal hyphae could be seen quickly, and antigen-based and molecular methods were more sensitive. A few studies showed that microscopy was not very useful for distinguishing species of Aspergillus from other filamentous fungi and was not effective in detecting infection in the case of low fungal burden. Thus, KOH microscopy was not a definitive diagnostic test, but rather was used as a screening test.

3.4. The ability of culture-based methods in diagnosis:

The importance of fungal culture to the identification of species and assessment of the sensitivity of fungi to antifungal agents remained important. Culture was not very sensitive and incubation periods were long. There were often negative culture results in clinically confirmed invasive pulmonary aspergillosis (IPA). Culture was a helpful test in confirming the diagnosis, but was not very sensitive for early diagnosis and thus often used in conjunction with the detection of antigen or molecular techniques.

3.5. The diagnostic performance of the galactomannan assays were assessed as follows:

One of the most popular non-culture-based diagnostic tests for pulmonary aspergillosis is galactomannan (GM) assays. The assay measures the presence of the galactomannan polysaccharide constituents of the fungal cell wall which are released during fungal growth allowing an earlier diagnosis than conventional microbiological methods. Galactomannan testing can be performed on various types of specimens, such as serum, bronchoalveolar lavage fluid (BAL) and other specimens, and the diagnostic performance can vary based on specimen type, patient population, and positivity cut-off. The studies in the included literature consistently showed that galactomannan assays offer greater sensitivity and aid in the timely clinical decision making**.**

3.5.1 Serum Galactomannan:

A high level of serum galactomannan was superior to conventional microscopy and culture. Leeflang et al. (2015) did a Cochrane systematic review of 54 studies and 5,660 patients and found pooled sensitivities of 78%, 71% and 63% at optical density index (ODI) threshold values of 0.5, 1.0, and 1.5, respectively; and specificities of 85% to 93% depending on the threshold.

Further studies have confirmed the value of serum galactomannan as an early marker of IPA, especially in neutropenic patients and haematological malignancies. Pooled sensitivity and specificity estimates for proven or probable IPA were reported to be ~76% and ~92%, respectively, by Mercier et al. (2023).

3.5.2 Bronchoalveolar Lavage Galactomannan:

However, BAL galactomannan was always superior to serum testing. Heng et al. (2015) examined 16 studies containing 783 haematology patients, and found that the overall sensitivity was 92% and overall specificity was 98% with an ODI of 1.5.

Similarly, Zou et al (2012) analyzed 30 studies and reported the pooled sensitivity and specificity of 0.87 and 0.89, respectively, for an ODI level of 0.5.

In a clinical study of non-neutropenic subjects, found anAUC of 0.961 for BAL galactomannan, with a sensitivity 91.7%, and specificity of 92.5%, which were significantly superior to serum galactomannan.

3.6 Diagnostic Performance of PCR Based Molecular Techniques:

In addition to the current use of conventional culture-based diagnosis, polymerase chain reaction (PCR)–based molecular diagnostics have also developed as potentially valuable tools for early diagnosis of pulmonary aspergillosis, allowing direct identification of the Aspergillus DNA from clinical specimens prior to the appearance of conventional culture results. The sensitivity and turnaround time of PCR is better than traditional methods, especially in patients with low levels of fungus or those already on antifungal treatment. The studies reviewed in this paper assessed both conventional and real-time PCR techniques in various specimen types such as blood, serum, bronchoalveolar lavage (BAL), sputum, and respiratory samples. Overall, molecular diagnostics had good diagnostic performance and were additional to galactomannan assays to increase diagnostic confidence.

3.6.1 Conventional PCR:

The advantage of PCR-based diagnostics was that they were much more sensitive than the traditional culture techniques. The molecular detection of Aspergillus was helpful in demonstrating the presence of Aspergillus directly in respiratory samples and blood work, leading to timely diagnosis.

Avni et al. (2012) conducted a systematic review and meta-analysis of 19 studies that showed a pooled sensitivity and specificity of BAL-PCR of 90.2% and 96.4%, respectively, for the diagnosis of proven or probable IPA. Further studies showed that the use of PCR in addition to galactomannan testing led to a marked improvement in the diagnostic yield, especially within high-risk haematological groups (White et al., 2010).

3.6.2 Real-Time PCR:

The real-time PCR also had other benefits, in that it could detect fungal DNA quickly and in quantity. Based on several of the studies, it was more reproducible and more accurate in diagnosis than the conventional PCR.

Blood-based PCR showed sensitivities from 57% to 84% and specificities from 58% to 95%, while the Fungal PCR Initiative (FPCRI)/ISHAM Working Group carried out an extensive umbrella review of 107 primary studies, which had sensitivities of 57% to 91% and specificities of 92% to 97% (Cruciani et al., 2023).

Another key aspect of the review was the standardization of PCR protocols, which greatly improved the accuracy of diagnosis and allowed for the incorporation of PCR into current diagnostic strategies for invasive aspergillosis (Cruciani et al., 2023).

3.6.3 Gene Targets Used for Detection:

Commonly tested molecular targets for the detection and characterization of Aspergillus species were the 18S ribosomal RNA gene, 28S gene, ITS regions and calmodulin gene. These genetic markers have been extensively used for the detection, species identification, phylogenetic analysis and differentiation of closely related Aspergillus species. These are the targets which are used mainly in ITS regions, while β-tubulin and calmodulin regions show better discriminatory power to identify fungal species at the right level.

These targets provided information to determine and identify Aspergillus isolates. ITS regions and the β-tubulin genes were particularly useful for the identification of closely related species of Aspergillus, while the ribosomal RNA targets proved to be of some assistance for general detection.

3.7 Comparative Analysis of Diagnostic Modalities:

The performance characteristics of the evaluated diagnostic methods (KOH microscopy, fungal culture, galactomannan assays and molecular diagnostic methods (PCR) were assembled and summarized for direct comparison. The comparison highlights the different sensitivities and specificities, as well as the diagnostic significance and application of the modalities. Overall, though, conventional methods were not very sensitive but were still useful for microbiological confirmation, whereas the galactomannan and PCR-based assays were highly sensitive and had high yield in early diagnosis of pulmonary aspergillosis. The comparative results are given in **Table 3.**

Table 3: Comparative Diagnostic Performance of Diagnostic Modalities for Pulmonary Aspergillosis

Diagnostic Method	Sensitivity (%)	Specificity (%)	Advantages	Limitations
KOH Microscopy	Variable; generally low	Moderate	Rapid, inexpensive	Low sensitivity, observer dependent
Fungal Culture	Variable; moderate	High when positive	Species identification, susceptibility testing	Long turnaround time
Serum Galactomannan	63–78	85–93	Non-invasive, early diagnosis	False positives possible
BAL Galactomannan	87–92	89–98	Excellent diagnostic performance	Requires bronchoscopy
Conventional PCR	77–90	93–96	Rapid DNA detection	Lack of methodological standardization
Real-Time PCR	57–91	92–97	Highly sensitive and quantitative	Cost and technical expertise

In general, the evidence showed that PCR and molecular diagnostics and BAL galactomannan had the highest diagnostic accuracy for pulmonary

aspergillosis. Although conventional microscopy and culture were important due to their accessibility and microbiological confirmation, they were less effective due to low sensitivity and were not effective as a single diagnostic test. Overall, the evidence favours the implementation of a combined diagnostic algorithm using microbiological, serological, and molecular testing to maximize diagnostic yield and help early therapeutic interventions.

3.8. Risk of Bias Assessment:

Risk of bias assessment was used to assess methodological quality and included studies varied in methodological quality. Studies investigating the diagnostic and validation studies tended to score lower on the items of risk of bias, particularly on the use of pre-defined diagnostic procedures, the use of pre-defined laboratory procedures, and the clarity of the specimen collection used. In several studies, galactomannan and PCR-based assays were evaluated, and in most of these, the applicability of the index test was not a concern, although some of these studies did not include information about how patients were selected or about blinding of outcome assessment. However, some studies showed an inconclusive risk in the reference standard domain due to diagnostic definition heterogeneity and differences in the diagnostic criteria for PA.

Overall, the methodological quality of the systematic reviews and meta-analyses included in this review was high, but there were some potential areas of concern regarding the populations included in the studies, the sample types collected (serum, BAL, blood, and respiratory samples), and the diagnostic thresholds across studies. Consensus guidelines and review articles were used to aid in the interpretation of the diagnostic approaches included, but were not used to perform quantitative synthesis. Overall, the data was found to be of moderate methodological quality and sufficiently applicable to the evaluation of the diagnosis of pulmonary aspergillosis. The risk of bias assessment **(Table 4) presented the overall methodological quality of the** included studies, which was found to be of moderate quality.

Table 4. Risk of Bias Assessment of Included Studies

Study	Study Type	Risk of Bias	Main Concerns
Leeflang et al., 2015	Cochrane systematic review	Low	Comprehensive methodology; minor heterogeneity in included populations
Zou et al., 2012	Systematic review & meta-analysis	Low	Diagnostic threshold variability across included studies
Mengoli et al., 2009	Systematic review & meta-analysis	Low	Variation in PCR methods across studies
Avni et al., 2012	Systematic review & meta-analysis	Low	Moderate heterogeneity in BAL diagnostic thresholds
Sun et al., 2011	Bivariate meta-analysis	Low	Variation in PCR assay standardization
Heng et al., 2015	Systematic review & meta-analysis	Low	Population heterogeneity and combined testing approaches
Xu et al., 2025	Comparative diagnostic study	Low–Moderate	Retrospective design and possible selection bias
D'Haese et al., 2012	Diagnostic validation study	Low	Minor concern regarding BAL positivity threshold
Yang et al., 2024	Retrospective diagnostic study	Moderate	Retrospective recruitment and single-center design
Cuenca-Estrella et al., 2009	Prospective diagnostic study	Low–Moderate	Limited external generalizability
Krishna et al., 2024	Prospective clinicomycological study	Moderate	Small sample size and single-center recruitment
Urabe et al., 2017	Prospective diagnostic study	Moderate	Small cohort and disease-specific population

Eigl et al., 2017	Prospective diagnostic study	Low–Moderate	Limited sample reporting and institutional setting
Oliveira et al., 2023	Prospective molecular study	Low–Moderate	Restricted to high-risk CPA population
van Grootveld et al., 2021	Prospective ICU study	Moderate	ICU-specific population limits applicability
Xess et al., 2004	Observational prevalence study	Moderate	Non-comparative design and prevalence-focused outcomes
Shrimali et al., 2013	Cross-sectional study	Moderate	Small sample and absence of molecular confirmation
Mortensen et al., 2011	Laboratory-based prospective study	Moderate	Specialized population and laboratory focus
Johansen et al., 2011	Prospective respiratory study	Moderate	Observational design and limited external validity
Hallidaay et al., 2005	Diagnostic assay validation	Moderate	Early assay development with limited clinical validation
Trovato et al., 2025	Retrospective molecular diagnostic study	Moderate	Retrospective design and incomplete sample characterization

4. Discussion:

4.1 Summary of key findings:

The aim of this SLR was to assess the accuracy of the conventional microscopy, fungal Diagnosis of PA in the laboratory using culture, galactomannan assays and molecular methods.The results show that there are significant differences in the diagnostic performance of the examined modalities. Conventional methods like KOH microscopy and fungal culture are also helpful as they are easy to use and inexpensive, and provide a microbiological confirmation, however they have insufficient sensitivity and they are not helpful in situations where contamination has occurred after antifungal therapy or in patients with low fungal burden (Patterson et al., 2016).

Non-culture techniques like bronchoalveolar lavage (BAL), galactomannan and molecular assays (PCR) have been found to be more sensitive and specific in some studies than the other methods. They can be used to help identify infection earlier and could potentially have a significant impact on clinical outcomes by early antifungal treatment (Mercier et al., 2021; Avni et al., 2012).

These data support the prevailing point of view that any one method should never be employed in all clinical situations. However, integrated diagnostic tools (as a combination of microbiological, serological and molecular methods) appear to be the most practical tool to achieve maximum diagnostic success.

4.2 Clinical Utility of KOH Microscopy and Culture:

Despite all this technology, KOH microscopy continues to be a valuable, quick and inexpensive screening tool, particularly in under-resourced areas where other diagnostic technology may not be available. A direct microscopic technique that will give information about the presence of fungal hyphae within minutes and can be used as a means to aid preliminary clinical decisions. The lack of distinction of Aspergillus species from other filamentous fungi in microscopy, however, significantly restricts its diagnosis specificity (Patterson et al., 2016).

Likewise, fungal culture is still crucial for the identification of species and susceptibility testing to antifungal agents. With the emergence of increasing azole resistance of Aspergillus spp., culture-based diagnosis is of particular importance. It is not very sensitive, however, and limited incubation periods restrict its use as a stand-alone diagnostic test (Denning et al., 2015).

Therefore, while KOH microscopy and culture are included in the routine in the lab, they may be considered more as an additional rather than a replacement to the newer methods.

4.3 Diagnostic Value of Galactomannan Assays:

The galactomannan assay is the most commonly validated non-culture test for invasive pulmonary aspergillosis. Galactomannan antigen is produced during the active growth of the fungus, which can be detected before the fungus turns positive on

culture, thus providing earlier diagnosis (Mercier et al., 2021).

We demonstrated in the current review that BAL galactomannan had a higher success rate compared to serum galactomannan in several studies. With the use of meta-analysis, sensitivities above 90% and specificities around 98% are reported for appropriate cut-offs (Heng et al., 2015). In the present study, BAL Galactomannan has been shown to be useful as a front line diagnostic test in bronchoscopic patients.

While galactomannan testing has advantages, there are some disadvantages. A few β-lactam antibiotics, diet ingredients and other fungal infections have been reported to cause false-positive results. On the other hand, there is a possibility of false negatives in patients who are taking antifungal medications before sampling (Mercier et al., 2021). Therefore, any results obtained should be looked at in the context of the entire clinical picture.

4.4 Role of PCR in Early and Accurate Diagnosis:

Pulmonary aspergillosis is an entity which is growing in significance and is now being diagnosed by emerging very useful molecular diagnostics, which are PCR based. Diagnosis by PCR directly from respiratory specimens or blood can be made before results of conventional culture are known (Avni et al., 2012).

From the results of this review, it could be concluded that PCR assays are very efficient as a diagnostic tool as they are very sensitive and very specific especially real-time PCR. Besides pathogen detection, PCR-based techniques can measure fungal burden and detect mutations that are linked to resistance to antifungals, which can aid in personalised treatment approaches (Patterson et al., 2016).

As the use of PCR became more widespread in international diagnostic guidelines, its clinical use became more and more confident. There are, however, still inter-laboratory variations, which were caused by differences in DNA extraction procedure, target genes and amplification systems, and positivity cut-off (White et al., 2010).

4.5 Molecular Characterisation of Aspergillus Species:

The molecular characterisation of Aspergillus species has greatly improved the identification and classification of these species. Traditional morphological approach has not been able to distinguish between closely related species and the molecular approach can provide greater resolution and reproducibility.

There are several genetic targets which have been used primarily for detection and characterisation of Aspergillus:

- **18S rRNA Gene**: This gene is highly conserved and is generally used in detecting large groups of fungi. It is conserved and can be amplified with sensitivity in a number of Aspergillus species, but may be difficult to discriminate at the species level.
- **28S rRNA Gene:** The 28S rRNA gene yields better phylogenetic resolution and has been widely used in fungal taxonomy and in molecular diagnostics. All clinically relevant Aspergillus species can be differentiated by sequencing of the 28S regions.
- **Internal Transcribed Spacer (ITS) Regions:** The ITS Regions are the primary fungal DNA barcode used and are extensively used for fungal species identification. They are highly variable among different species and can be used to distinguish closely related isolates of Aspergillus (Schoch et al., 2012).
- **β-tubulin**:ITS sequencing can be insufficiently discriminatory and β-Tubulin sequencing has become an important supplementary marker to help identify Aspergillus. This marker has been very useful to distinguish the various cryptic species of the genus Aspergillus.
- **Calmodulin Gene:** Good species level resolution; a marker that is becoming more widely recognized as a good taxonomical marker for Aspergillus. Its-based methods have been found to be less discriminatory than several studies.

Together, these molecular targets have opened up new possibilities for epidemiological studies, outbreak surveillance and antifungal resistance surveillance.

4.6 Challenges and existing limitations in diagnostics:

While significant advances in the diagnosis of fungal diseases have been achieved, there are still some difficulties to the diagnosis of pulmonary aspergillosis. There are no clinical symptoms which are specific but are common to bacterial pneumonia, tuberculosis and other respiratory infections. In addition, the radiographic findings are also suggestive, but not definitive (Patterson et al., 2016).

Other limitations are due to sampling methodology, laboratory methodology, lack of universal PCR

standardization and differences in diagnostic cut-off value for galactomannan assays. In addition, many diagnostic research studies have been performed on high-risk populations, making it difficult to extrapolate to larger populations of patients.

4.7 Future directions in Aspergillus Diagnostics:
Further studies should focus on increasing the diagnostic accuracy, and reduce the turnaround time as well as the laboratory variation.

- **Multiplex PCR:** Potentially multiplex PCR platforms will result in the simultaneous detection of multiple fungal pathogens, which will help enhance the cost-effectivity and efficient diagnosis. These systems are particularly important in patients with severe lung infections and are complex.
- **Molecular epidemiology:**Integration of molecular epidemiological approaches can contribute to an understanding of transmission patterns, environmental reservoirs and regional antifungal resistance patterns.
- **Gene sequencing**:Next-generation sequencing (NGS) and whole genome sequencing (WGS) are exciting technologies capable of revolutionising the diagnostics of Aspergillus by identifying, profiling and characterising the pathogen and its resistance, directly from clinical specimens.
- **Combined (galactomannan, PCR, culture and radiological) diagnostic:** These algorithms are more effective than single tests, and there is growing evidence in support of these algorithms. A patient group specific approach would be the focus of future diagnostic algorithms.
- **Standardisation of Molecular Methods:** There is a need for international efforts in PCR protocol, target, and reporting criteria to improve the reproducibility of molecular tests, and facilitate wider clinical use (White et al., 2010).
- Afaq et al.in 2024 conducted a microbiological study evaluating fungal infections using conventional direct microscopy and culture-based identification methods. The investigators emphasized the diagnostic importance of appropriate clinical specimen processing, direct microscopic examination, fungal culture, and morphological identification of fungal isolates. Their findings demonstrated that conventional mycological methods remain useful for the initial detection and identification of fungal pathogens, including Aspergillus species; however, the diagnostic yield may be limited by low fungal burden and culture negativity. The study highlighted the importance of correlating direct microscopy with fungal culture and clinical findings for accurate diagnosis. These observations support the present systematic review, which demonstrates that conventional microscopy and culture remain essential baseline diagnostic tools but should preferably be complemented by antigen-based and molecular techniques to improve the early and accurate diagnosis of pulmonary aspergillosis.

4.8 Strengths and limitations:
One of the most significant merits of this review is that they have evaluated the conventional diagnosis as well as the molecular diagnosis of pulmonary aspergillosis. The review provides a general overview of the existing diagnostic approach and applies the following methods: microscopy, culture, antigen detection and molecular methods.
A few caveats, however, should be noted. There was significant variation among the studies included in the review in terms of the patients studied, the type of specimens examined, the diagnostic criteria used, and the methods of assay. The diagnostic performance reported here might be influenced by the cutoff values for galactomannan and the design of the PCR. In addition, differences in the quality of the studies, such as publication bias, could not be excluded.

5. Conclusion:
Pulmonary aspergillosis is an important cause of morbidity and mortality, especially in immunocompromised patients and in the critically ill. Early and correct diagnosis is important for prompt antifungal treatment, which can have superior clinical outcomes.
From then on, there is still a broad place for the traditional methods of diagnosis (KOH microscopy, fungal culture) in the clinical routine because of their accessibility and capacity to microbiological diagnosis. However, they tend to be not very sensitive, so that they are not used as single diagnostic tests.
Galactomannan assay, especially BAL galactomannan, greatly enhances the early detection and shows excellent diagnostic utility in various patient population groups. The other advantages of molecular methods for the detection of pathogens are their rapid and precise diagnosis, the capability to distinguish the species and, potentially, the detection of antifungal resistance markers. The molecular characterisation of Aspergillus, using the 18S rRNA, 28S rRNA, ITS

regions, β-tubulin and calmodulin genes, has greatly enhanced the identification of Aspergillus and epidemiological studies. Emerging technologies such as next-generation sequencing and multiplex PCR and integrated diagnostic algorithms are expected to further improve the accuracy of diagnosis.

In conclusion, the available evidence shows that a combination of traditional microbiologic testing, antigen detection testing and molecular testing is warranted. In the management of patients with suspected pulmonary aspergillosis, a combination of approaches is likely to be most diagnostic and may result in better clinical outcomes.

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