

# Laboratory detection and identification of *Aspergillus* species by microscopic observation and culture: the traditional approach

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Molecular and immunologic tests promise better, faster laboratory diagnosis of aspergillosis, but microscopy and culture remain commonly used and essential tools. Procedural changes, as well as adequate training of laboratory professionals, can enhance the value of these traditional tools. Using Blankophor or Calcofluor for microscopic examinations; improving recognition of morphologic characteristics of opportunistic fungi in stained smears of specimens; maximizing the growth rate and production of conidia by *Aspergillus* spp. in culture; and recognizing atypical variants of common aspergilli can improve the laboratory's contribution to rapid diagnosis. Surveys indicate that the number of laboratory professionals is declining as the demand for healthcare is rising. Effective recruitment, retention, and training of personnel must be concurrent with advances in technology.

**Keywords** *Aspergillus*, culture, histopathology, microscopy, training

## Introduction

Traditional methods for diagnosis of aspergillosis and other mycoses are being supplemented by molecular and immunologic approaches. While the advantages of nucleic acid-based tests are obvious, their standardization and clinical utility have not been fully realized [1]. Furthermore, while the galactomannan EIA test for *Aspergillus* antigen is widely available in the US, the standard use of nucleic acid-based tests for identification of clinical isolates appears limited. Reference laboratories offering molecular identification of aspergilli include the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, the Centraalbureau voor Schimmelcultuur (CBS), Utrecht, The Netherlands, and laboratories in the US listed at the online test directory of the Association for Molecular Pathology. Although molecular methods continue to improve and become more readily available, microscopy and culture remain the primary laboratory tools for detecting aspergilli. The 2003 American Society for

Microbiology (ASM) Benchmarking Survey [2] documented that 89% of laboratories performing mycological testing use culture, 16% use serology, and fewer than 5% use molecular tests. Only 3% of reporting laboratories use 'home-brew' molecular testing for microbial pathogens. Given the continued reliance on microscopy and culture, the diagnostic value of these methods must be improved by procedural changes and adequate training of laboratory personnel.

## Microscopy

Microscopic methods, such as wet mounts, Gram stains, and conventional histopathology, provide clues that suggest the presence of *Aspergillus* spp. in tissue. Blankophor or Calcofluor mixed with 10%–20% potassium hydroxide (KOH), stains fungal cell walls and improves detection of fungi. While Calcofluor crystallizes in an alkaline pH, Blankophor does not and it can be stored in a working solution for up to a year [3]. Phenotypic markers detected by histopathologic stains, as well as by Gram stain or wet mounts, provide valuable information for clinically important fungi, especially in the absence of culture (Table 1). However, confirmation of microscopic findings by culture is always desirable and, in most cases involving

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**Table 1** Microscopic markers of selected *Aspergillus* species and other opportunistic fungal pathogens.

Organism (s)	Microscopic markers in slide preparations of specimens prepared by standard procedures*	
	Hyphae	Other features
<i>A. fumigatus</i>	2.5–8 µm wide, septate, hyaline, acute angle branching, tree- or fan-like branching. Stipes may resemble hyphae of zygomycetes	Conidial head uniseriate, columnar, conidia in chains or detached and dispersed. Single or paired conidia may resemble yeast cells
<i>A. niger</i>	See <i>A. fumigatus</i>	Conidial head biseriate, radiate, conidia in chains or detached and dispersed. Single or paired conidia may resemble yeast cells
<i>A. terreus</i>	See <i>A. fumigatus</i>	Small, round, hyaline conidia ('accessory' conidia) attached to the vegetative hyphae
<i>Acremonium</i> , <i>Fusarium</i> , and <i>Paecilomyces</i> spp	See <i>A. fumigatus</i>	Phialides and phialoconidia, specific to the genus, may be found in closed tissue
<i>Scedosporium apiospermum</i>	See <i>A. fumigatus</i>	Typical annelloconidia and annellides may be found in closed tissue
Zygomycetes	10–30 µm wide, aseptate, non-radiating, 90° angle branching. Folds in hyphae may simulate septae	
Dematiaceous moulds	Hyphae with brown pigmentation; walls often not parallel; may appear moniliform (like a 'string of beads')	Brown pigment seen in KOH exam with bright-field illumination; stained with Fontana–Masson and often with Hematoxylin and Eosin

\* Expertise in mould identification is required for accurate evaluation of markers. Results must be confirmed by culture.

opportunistic moulds, essential for definitive identification of the pathogen. Despite the presence of visual clues, identification of aspergilli by microscopy alone may be misleading. Schell [4] reports a case of *Aspergillus niger* sinusitis in which the *A. niger* conidia were confused with the yeast cells of *Candida* spp. and cross sections of the stipes of *A. niger* were confused with the broad hyphae of a zygomycete. Communication between the clinical pathologist and the laboratory mycologist, who routinely identifies filamentous fungi from culture, may improve the diagnostic value of histopathology.

### Recognition of morphologic characteristics

Since aspergilli are ubiquitous in nature, they may commonly contaminate specimens and culture media. Consequently, determining the significance of *Aspergillus* spp. growing in culture is often a challenge when microscopic examination of the specimen is negative. In a survey of *Aspergillus* isolates from liver and kidney transplant recipients, Brown *et al.* [5] found that the presence of more than two colonies in a culture and infection in more than one site predicted significant infection. In granulocytopenic patients with acute leukemia, a single isolation from a lower respiratory specimen must be considered significant [6]. Unambiguous reports of laboratory observations to the physician may reduce the diagnostic dilemma. For

example, the statement, "A total of three colonies of *Aspergillus fumigatus* isolated on two of three plates" provides more information than "Rare *A. fumigatus* isolated".

### Optimizing results of culture

Isolation in culture and phenotypic identification of common clinical isolates of *Aspergillus* spp. is usually quick and easy. However, culture is often described as slow, perhaps creating misconceptions about its value for the detection of aspergilli. *A. fumigatus* is a rapid grower. The typical velutinous, grey-blue-green colonies and uniseriate conidial heads develop within 24–48 h on both fungal media and the sheep blood agar commonly used for bacterial culture. Other aspergilli associated with invasive aspergillosis, specifically, *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus* have growth rates similar to that of *A. fumigatus* when colonies were measured on malt extract agar and Czapek yeast agar after incubation for seven days at both 25°C and 37°C [7]. Because drug resistance of some *Aspergillus* spp. is a threat, full identification, not only of *A. fumigatus*, but also of the less commonly isolated species, is warranted. Laboratory scientists also must recognize atypical isolates of *Aspergillus* spp. Poorly sporulating (white) strains of *A. fumigatus* with decreased susceptibilities to several antifungal drugs were reported recently [8]. These white strains have a genetic sequence different from that of the wild-type,

*A. fumigatus* Fresenius, and failed to develop typical blue-green conidial heads until 10–12 days following incubation. Another challenge is the white mould, *Neosartorya fisheri*, which initially produces sparse, conidial heads resembling those of *A. fumigatus*. However, *N. fisheri* subsequently develops numerous, round, thin-walled cleistothecia, making the differentiation from *A. fumigatus* simple. A dissecting scope is handy for quick location of conidial heads and cleistothecia. Sterile, white, fast-growing or glabrous, mounded, slow-growing isolates of *A. fumigatus* may occur, requiring thermotolerance and exoantigen testing for definitive identification [9]. Khan *et al.* [10] reported an atypical *A. terreus* isolated from lower respiratory specimens of a patient with aspergilloma. Initial colonies were orange and produced a diffusible yellow pigment and small, single cells that were confused with the conidia of *Scedosporium apiospermum*. Precipitating antibodies and typical conidial heads of *A. terreus* produced after 10 weeks of incubation confirmed the identification.

The images and information available in textbooks and on the Internet offer fine educational opportunities for learning to identify *Aspergillus* spp. Hands-on experience, however, remains the most effective teaching tool. The CDC, the National Laboratory Training Network (NLTN), and CBS offer laboratory workshops. When travel off-site is not practical, laboratories are encouraged to use the online tutorial, *Aspergillus* Reference Cultures [11], for in-house training. The reference organisms listed there are available for purchase from major culture collections.

Analysing each step in the culture procedure can lead to improved recovery of aspergilli. Liquefying specimens with Sputolysin or other mucolytic agents has been suggested for recovery of fungi trapped in the mucus of sputum and sinus material recovered from endoscopic surgery [12]. The use of potato dextrose, potato flake, malt extract, inhibitory mould agar, or similar sporulation agars as primary isolation media for *Aspergillus* spp. may speed growth rate and the production of conidia. The addition of antibacterial agents to isolation media helps reduce time to identification by inhibiting bacterial overgrowth and reducing the need for subculture. The initial incubation of fungal media at 35–37°C instead of, or in addition to, 30°C may speed the growth of some aspergilli [13,14]. Similarly, daily inspection of culture media ensures the earliest possible detection. By incubating culture plates in a microaerophilic environment at 35°C, Tarrand [14] found that selected, clinically important *Aspergillus* spp. grew from 12 of 12 broth cultures. Cultures of the same organisms incubated at 25°C

without CO<sub>2</sub> yielded *no* positive results. Further studies would be helpful in clarifying the media and conditions most effective for the recovery and rapid identification of clinically important aspergilli.

With a quick Scotch-tape or tease mount, conidial heads of *Aspergillus* spp. can typically be identified. However, a slide culture may be necessary when sporulation is slow or atypical. The rapid pace of most hospital laboratories dictates the easiest, though not necessarily the most refined, method for performing the slide culture. Riddell's classic slide culture method [15] has been supplemented with other, less labor-intensive techniques [16,17]. A quick method is simply to push an 18 × 18 mm coverslip at a 45 degree angle into a sporulation media, such as potato flake agar. When the mould sporulates, the coverslip is carefully withdrawn from the agar and mounted in a drop of lacto-phenol blue or lacto-fuchsin on a microscope slide. Another drop is placed on top of the small coverslip before completing the assembly with a 22 × 22 mm coverslip.

### Workforce issues

Rapid diagnosis of aspergillosis depends not only on improved methodology but also on an adequate, well-trained workforce. Surveys of mycology practices strongly recommend more training [18–20]. Special emphasis should be placed on accurate identification, direct examination, appropriate use of media, clinical relevance, and cost effectiveness. However, inadequate staffing may compromise both training and the implementation of more clinically relevant procedures. The ASM Benchmarking Survey revealed continuing workforce shortages for some microbiology laboratories in the US. In part, the shortage results from a 53%–56% reduction in CLS training programs over the past 12 years. Thirty-four per cent of the professionals working in microbiology laboratories today are more than 50-years-old. Will younger workers be available to replace them when they retire? While almost 80% of females in the labor force are younger than 30 years, only 10% of female workers in the microbiology laboratory are less than 30 years of age [2]. These data suggest that workforce shortages will continue and possibly exacerbate.

### Conclusion

Improving both traditional and non-traditional diagnostic procedures for mycoses demand concurrent efforts to ensure an adequate workforce and to improve the career mobility, professional

recognition, opportunities for advanced training, compensation and other factors needed to stimulate interest in laboratory science.

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