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ADVANCES IN DIAGNOSIS OF INVASIVE FUNGAL INFECTIONS (O MORRISSEY, SECTION EDITOR)

Histopathology in the Diagnosis of Invasive Fungal Diseases

Henrik Elvang Jensen [1]

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Abstract

Purpose of Review The classical diagnostic principles for applying histopathology for the diagnosis of invasive fungal diseases are reviewed. Although several new molecular based techniques have recently been developed, the histopathological identification of fungal elements together with a typical tissue reaction remains the golden standard for stating a diagnosis of invasive mycosis. Therefore, and due to the risk of false negative and false positive results obtained from cultivation as well as the non-culture based diagnostic test for invasive fungal infections, an examination should always complement histopathology in the diagnosis of invasive fungal diseases.

Recent Findings The application of molecular in situ identification techniques, i.e., immunohistochemistry and in situ hybridization, for morphologically observed fungal elements in tissue sections, has indeed improved the diagnostic accuracy of histopathology for the diagnosis of invasive fungal diseases.

Summary Because the specific molecular techniques applied in the histopathological diagnosis of invasive mycoses are directed toward specific targets, the panel of specific immunoglobulins/probes to be used on tissue sections should be directed from the histomorphology of the fungal elements as detected by conventional histopathological methods.

Keywords Histopathology · Immunohistochemistry · In situ hybridization · Tissue · Mycoses · Fungi

Introduction

Stating a diagnosis of a fungal invasive disease is ideally based on specific clinical symptoms, demonstrating typical fungal elements within lesions together with a matching host response, and by subsequent demonstration of the invasive fungal agent in culture. However, this ideal situation is only rarely achieved when dealing with the huge variety of invasive fungal diseases present [1•] Therefore, obtaining a correct diagnosis often depends on the application of a number of laboratory techniques apart from cultivation and carrying out a histopathological examination. Depending on the suspicion of an infecting agent, different approaches may be relevant, e.g., detection of (1) specific fungal antigens in different fluids like urine, bronchoalveolar lavage specimens, serum, and spinal fluid as in cases of specific *Histoplasma* and *Cryptococcus* antigen and galactomannan in aspergillosis; (2) antibodies toward specific fungal antigens can be used in cases suspected for, e.g., histoplasmosis; (3) metabolites from fungi like D-arabinitol in *Candida* cases are also useful; and (4) markers as (1→3)-β-d-glucan for fungal cell walls, which is present within the wall of almost all fungi, with the exception of Mucorales, may be useful for initial screening when an invasive mycosis is suspected [2]. During the last two decades, polymerase chain reaction (PCR)-based techniques applied on blood and other clinical specimens including fresh and formalin-fixed tissues have become more and more reliable for the diagnosis of a number of mycotic infections [3, 4]. Although, series of new diagnostic tests have been and are being developed for stating the diagnosis of invasive mycoses, an examination should always complement histopathology in order to avoid false negative and false positive results from cultivation as well as the diagnostic tests based on non-culture techniques.

Due to the high specificity of the non-culture founded techniques, they are generally not available for a number of the

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✉ Henrik Elvang Jensen
elvang@sund.ku.dk

[1] Section of Pathology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

more rare and new pathogenic fungi causing invasive lesions. Moreover, it is well-known that isolation of fungi from solid organs may be quite challenging, and apart from being a slow process, it quite often results in a sterile outcome. Moreover, at gross inspection mycoses are often not suspected and are more likely presumed to be neoplasms or an inflammation of uncertain etiology. Furthermore, the whole tissue specimen is often fixed for histopathological examination, whereby being unsuitable for cultivation [5]. In such cases, the application of histopathology together with different in situ techniques and PCR-based techniques are mandatory in order to obtain an etiological diagnosis if not repeating biopsies are to be sampled, if possible at all [1•]. However, it should be kept in mind that the molecular-based techniques only will detect those targets for which they are designed, sufficient material is present, and no inhibition occurs [6]. Even though the characteristics of fungal elements with respect to morphology (for yeast like cells, e.g., the size, shape, the way of budding, and thickness of cell wall, and for hyaline hypae forming fungi the contours of the walls, the pattern, and orientation of branching together with the frequency of septation) may support a tentative diagnosis, it is often not possible, however, to state a specific and reliable diagnosis founded on details observed histologically due to similarities of morphology between the tissue forms of more fungal genera, observation of atypical and bizarre fungal forms [7]. Moreover, the use of antimycotic agents may often also alter the morphology of invasive fungal elements [8–10].

Application of histopathology is essential for several reasons in order to state a diagnosis of invasive mycoses. Within 24 hours or less, a specific diagnosis may be obtained for some fungi by the in situ demonstration of genus and/or species specific morphological patterns of fungal elements [11–13, 14••]. Especially, in cases of the opportunistic pathogenic fungi, it is essential for upholding the significance of an isolate to demonstrate its coherent morphology by histopathological demonstration of specific characteristics [14••]. Moreover, the histopathological observation of tissue invasion together with an intravital reaction from the host clearly documents the significance of an isolate, i.e., pathogenic, or its presence is due to colonization as a part of the normal mycobiota (e.g., *Candida albicans*) or a contaminant from the environment (e.g., *Aspergillus* spp.) [15]. The nature of the inflammatory reaction and the exact localization of fungal elements within the different tissues of an organ is also helpful in order to rule out if a mycotic disease is due to a state of hypersensitivity or represents a true invasive mycosis (e.g., allergic bronchopulmonary aspergillosis contra invasive pulmonary aspergillosis) [16]. Finally, careful histopathological examinations may indicate and sometimes actually reveal the presence of infections by more infectious agents (e.g., different fungal agents and/or fungi together with bacteria) simultaneously or state the presence of a non-fungal related lesion/disease, which may show similar clinical symptoms as different kinds of invasive mycoses. In several situations, obtaining a specific diagnosis is crucial not only for an optimal therapy, but also for the study of, e.g., pathological and epidemiological aspects of specific invasive fungal infections [5].

Histological Demonstration of Invasive Fungi

In order to detect fungi in tissue sections, several histochemical stains are available [17, 18]. In most patho-diagnostic laboratories, the primary stain used initially on all tissue sections is hematoxylin and eosin (H&E) as it in a perfect way demonstrates the inflammatory response by the host, reveals the Splendore–Hoeppli (asteroid bodies) reaction, and demonstrates if fungal elements are colorless (hyaline) or phaeoid (pigmented–dematiaceous) [19]. H&E is the stain of choice for the demonstration of the hematoxylinophilic or amphophilic nuclei of yeast-form cells that are multinucleated, especially those of *Blastomyces dermatitidis*, *Lacazia loboi*, and *Paracoccidioides brasiliensis*. Some fungi, e.g., the *Aspergillus* spp. and Mucorales, are hematoxylinophilic and usually well demonstrated within tissue sections stained with H&E, but several fungi are only poorly or not at all stained, and this accounts for, e.g., *Candida* spp. However, lesions containing fungal elements, which are not or only poorly stained, will often be disclosed as an invasive mycosis by careful examination due to the outlines of the elements. Moreover, the presence of a necrotizing, granulomatous, or pyogranulomatous inflammation should always alert the pathologist to think of an invasive fungal infection [5]. As the H&E staining has limitations, more special stains reacting with most fungal genera should be applied for an optimal demonstration of the fungal morphology in tissue sections [1•].

For the demonstration of by far the most fungal elements in tissue sections, stains like Gomori's methenamine silver (GMS), the periodic acid–Schiff reaction (PAS) and Gridley's fungus (GF) procedures are useful. By the three stains, the reactivity of hydroxyl groups within the cell walls is responsible for the coloration of the fungal elements. By the GMS procedure, fungal cell walls stain brownish–black, whereas by PAS and the GF procedures, fungi are colored magenta. By the special stains, also normal elements within tissues will be stained, e.g., basement membranes, as normal tissue elements also contain hydroxyl groups. The best contrast is obtained by the GMS stain, which also will, although to a different level, stain non-viable and old fragments that are not as well demonstrated by the PAS and GF procedures. A drawback of using the special stains, especially the GMS stain, is the masking of the innate color of fungal elements, i.e., it cannot be determined whether it is a hyaline or phaeoid fungus. This is crucial to determinate the diagnosis of a mycosis caused by pigmented fungi, e.g., phaeohyphomycosis,

chromoblastomycosis, and black-grain eumycotic mycetomas [16]. When using the special stains, it is also difficult to evaluate the inflammatory response developing in connection with the infection. Stains for mucin, e.g., Alcian blue and Mayer's mucicarmine stain, are essential stains for the demonstration of the capsule of mucopolysaccharide surrounding cryptococci. However, by these stains the capsule-free cryptococci will not be stained. Mucin stains also stain the walls of *B. dermatitidis* (Fig. 1), *P. brasiliensis* (Fig. 2), and *Rhinosporidium seeberi* (Mesomycetozoa) (Fig. 3) to a variable extend [1•]. However, these microorganisms are non-encapsulated and morphologically very different from *C. neoformans* and should therefore not be mixed up with each other.

The identification of capsule-free cryptococci (i.e., "dry" variants) can be identified by the presence of melanin-like substances derived from dihydroxyphenylalanine within the cell wall [20–23]. If agents of phaeohyphomycosis are non-pigmented or only lightly pigmented, stains for melanin can be used to confirm the presence of melanin in their cell walls [16, 24]. For screening of scrapings, swabs, body fluids, paraffin-embedded or fresh-frozen tissue sections optical brightening histochemistry is often used. For this purpose, different whitening agents such as Calcofluor White M2R, Blankophor, and Uvitex 2B, which will fluoresce under ultraviolet light, are typically applied [5, 25–28].

Histological Identification of Invasive Fungi

Due to the diversity in morphology, the contents of hydroxyl groups in the walls and their size, most standard histological stains will disclose the presence of fungal elements within tissue sections and to different levels deduct their identification by the initial screenings of slides by pathologists [1•, 13, 14••, 29]. From the appearance of fungi in tissue sections, it is practical, apart from being either hyaline or phaeoid, to group them within one of the following non-taxanomic groups in order to differentiate between the histomorphological diagnosis of invasive fungal diseases: (1) yeast cells, (2) hyphae forming, (3) spherules with endosporulation, and (4) mycetomas (granules) (Tables 1, 2, 3, and 4). When the fungal elements have been recognized to be within one of the groups, the correct identification to genus and sometimes also species level can be considered.

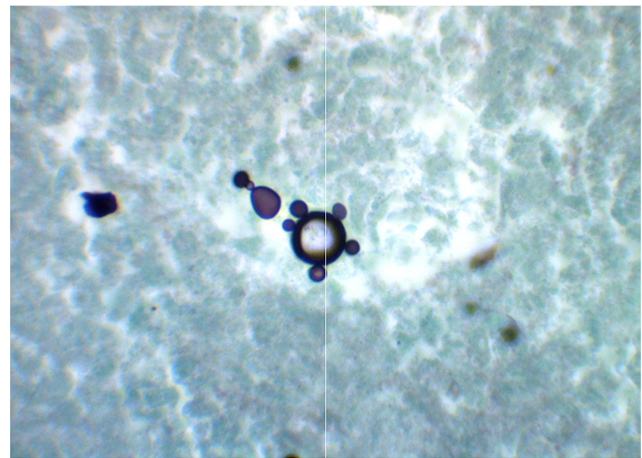

Fig. 2 The formation of "steering wheel" budding is characteristic for cells of *Paracoccidioides brasiliensis*. (GMS stained)

With reference to Table 1, a yeast cell having a thick wall, showing a single broad-based bud and containing more nuclei, can only be *B. dermatitidis* (Fig. 1). Concerning fungi forming hyphae, it is more difficult to categorize the fungi in a reliable way because several of these are similar in tissue sections (Fig. 4; Table 2). Furthermore, compared with yeast cells, hyphae forming fungi in sections are more prone to alterations due to the specific tissue being infected, the age of the hyphae, and the inflammatory response by the host [1•, 10, 14••].

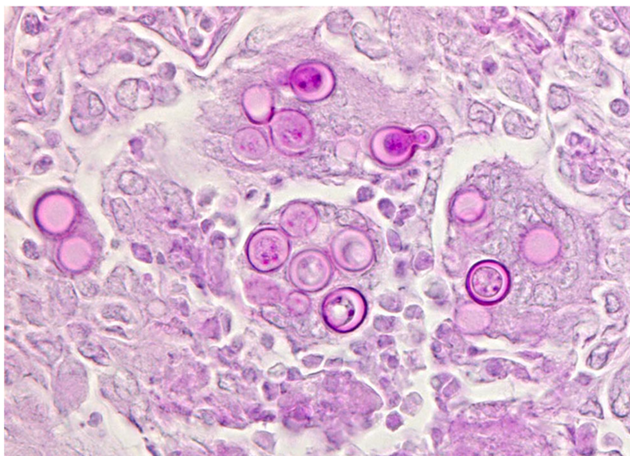

Fig. 1 *Blastomyces dermatitidis* organisms are characterized by thick cell walls, formation of a single broad-based bud, and multiple nuclei. (PAS stained)

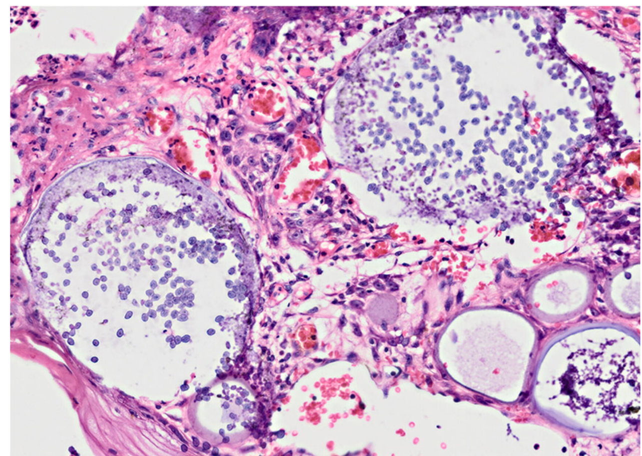

Fig. 3 The formation of large, mature spherules with endospores are characteristic for *Rhinosporidium seeberi* (Mesomycetozoa). (H&E stained)

Table 1 Morphology of fungi that occur as yeast-like cells in tissue

Feature	*Histoplasma capsulatum* var. *capsulatum*	*Histoplasma capsulatum* var. *duboisii*	*Blastomyces dermatitidis*	*Cryptococcus neoformans*	*Talaromyces (Penicillium) marneffei*	*Paracoccidioides brasiliensis*	*Lacazia (Loboa) loboi*	*Sporothrix-schenckii* var. *schenckii*	*Candida* spp.	*Candida glabrata*	*Pneumocystis jirovecci*
Size (μm)	2–4	6–12	7–15 Microforms, 2–4 μm in diameter, also may occur in tissue	2–20	2.5–5	5–60	5–12	2–10	3–6	2–5	2-10
Shape	Spherical or oval	Oval	Spherical	Pleomorphic	Spherical, oval or elongated	Spherical	Spherical	Pleomorphic, cigar-shaped cells are characteristic	Spherical or oval	Spherical or oval	Spherical, oval or crecentic
Number of buds	Single	Single	Single	Single and rarely multiple	None	Multiple; “steering wheel” forms	Multiple; chains	Single and rarely multiple	Single; chains	Single	None
Attachment of buds	Narrow	Narrow	Very broad	Narrow	Not applicable	Narrow	Narrow; tubular	Narrow	Narrow	Narrow	Not applicable
Schizogony	–	–	–	–	+	–	–	–	–	–	+
Thickness of cell wall	Thin	Thick	Thick	Thin	Thin	Variable	Thick	Thin	Thin	Thin	Thick in “cysts”; thin in “trophozoites”
Pseudohyphae and/or hyphae	Rare	Absent	Rare	Rare	Absent	Rare	Absent	Rare	Characteristic	Absent	Absent
Number of nuclei	Single	Single	Multiple	Single	Single	Multiple	Single	Single	Single	Single	Multiple in “cysts”; single in “trophozoites”
Mucicarmine reaction	–	–	±	+; some strains may be capsule deficient and non-carminophilic	–	±	–	–	–	–	–

Table 2 Morphology of fungi that occur as hyaline hyphae in tissue

Feature	*Aspergillus* spp.	*Fusarium* spp.	*Scedosporium* spp.; *Pseudallescheria boydii*	Mucorales (*Mucor*, *Rhizopus*, *Lichtheimia*, *Rhizomucor*, *Gunninghamella*)
Width (μm)	3–6	3–8	2–5	6–25
Contours	Parallel	Parallel	Parallel	Irregular
Pattern of branching	Dichotomous	Dichotomous or right angle	Dichotomous and/or haphazard	Haphazard
Orientation of branches	Parallel or radial	Random and parallel	Random and parallel	Random
Frequency of septation	Frequent	Frequent	Frequent	Absent or infrequent

Especially fungi and Mesomycetozoa species, which produce spherules with endosporulation, usually can be specifically identified in tissue sections (Table 3; Fig. 3). Apart from these and when several and typical elements are present, a number of invasive mycoses can be identified based on histomorphological criteria: blastomycosis, coccidioidomycosis, cryptococcosis, histoplasmosis capsulati, histoplasmosis duboisii, lacaziosis (lobomycosis), paracoccidioidomycosis, talaromycosis (penicilliosis) marneffei, sporotrichosis schenckii, sporotrichosis luriei, and pneumocystosis. In still other invasive fungal diseases, more species of a genus may be responsible for the infection, and in these situations, identification to the level of genus is possible: candidosis and trichosporonosis. From Table 2, it is apparent that a clear-cut diagnosis of aspergillosis, fusariosis, and scedosporiosis can in far most cases not be based on the morphology of hyphae in tissue sections (Fig. 4) [10]. Furthermore, in several cases even mucormycosis and candidosis have been mixed up with aspergillosis, fusariosis, and scedosporiosis and vice versa [7, 14••]. In cases where typical fungal elements are not present or they cannot be generically identified, the invasive mycoses can be diagnosed to a certain level: chromoblastomycosis, hyalohyphomycosis, phaeohyphomycosis, and mucormycosis [1•].

Formation of conidial heads in tissue sections may help the identification and sometimes even to the level of species [29]. In addition, the presence of calcium oxalate crystals along the formation of hyphae or in the form of generalized oxalosis can help in the diagnosis of invasive aspergillosis due to *Aspergillus niger* [29].

In mycetomas, which are caused by an actinomycete (branched, filamentous bacterium) or fungi (eumycete), the pathologist should be able to classify it as hyaline (white grained) or phaeoid (black grained) (Table 4). When only fragments and/or bizarre elements are present in tissue sections, typically due to hypoxia, necrosis, or antifungal therapy, the pathologist can only conclude that an invasive mycoses is present, and in order to obtain a more specific diagnosis, other in situ identification techniques [14••] or PCR techniques must be applied [30, 31].

In Situ Identification of Invasive Fungi by Immunohistochemical and Hybridization Techniques

Immunohistochemistry

Essential to the use of immunohistochemistry is to get access to primary reagents characterized with respect to specificity [5]. Both direct and indirect protocols are used for obtaining immunohistochemical diagnoses of mycoses, and for visualization of specific reactions, different techniques have been used, i.e., fluorochromes, gold-silver complexes, and enzymes

Table 3 Morphology of fungi and mesomycetozoa that occur as large spherules in tissue

	Coccidioides immitis and *posadasii*	*Rhinosporidium seeberi* (Mesomycetozoa)	*Chrysosporium parvum* var. *crescens*
External diameter of spherule (μm)	20–200	100–350	200–400
Thickness of spherule wall (μm)	1–2	3–5	20–70
Diameter of endospores	2–5	6–10	None
Hyphae or arthroconidia	Rare	None	None
Special stain reactions			
GMS	+	+	+
Mucicarmine	–	+	–

Table 4 Morphology of fungal and non-fungal mycetomas

Mycetomatous pathogens		
Acremonium spp., *Aspergillus nidulans*, *Curvularia geniculata*, *Exophiala jeanselmei*, *Leptosphaeria senegalensis*, *Madurella grisea*, *M. mycetomatis*, *Neotestudina rosatii*, *Pseudallescheria boydii*, *Pyrenochaeta romeroi*, and others	Eumycotic	Granules, 0.2 mm to several millimetres in diameter, composed of broad (2–6 μm), hyaline, or phaeoid, septate hyphae that often branch, and form chlamydoconidia
Actinomadura madurae, *A. pelletieri*, *Nocardia* spp., *Nocardiopsis dassonvillei*, *Streptomyces somaliensis*, and others	Actinomycotic	Granules, 0.1 to several mm diameter, composed of delicate gram-positive filaments, 1 μm wide, which are often branched, and beaded

[5, 32, 33]. In addition, avidin-biotin enzyme complex (ABC) methods are used [10]. Using enzyme-based techniques are preferred compared with fluorochrome techniques as the host reaction toward the fungi can be evaluated at the same time as the immunoreactivity [1•, 5, 10]. Furthermore, enzyme-based techniques provide permanent slides, and access to fluorescence microscopes is not needed.

Application of immunohistochemistry together with in situ hybridization techniques is in cases, where no typical or characteristic morphological hall markers are present, the only means of establishing an accurate in situ etiological diagnosis in formalin-fixed tissue sections [10]. When fungal forms are found to point into different directions with respect to morphology not only within the same lesion but also in different organs, it should obviously determine whether the fungi belong to a single or more taxa. In these situations, the use of dual/multiple immunostaining and in situ hybridization techniques are useful for obtaining a trustable diagnosis [1•, 10]. Moreover, in tissues, reactivity based on different specific primary reagents is used for the in situ identification of the different *Cryptococcus neoformans* serotypes [34].

Unfortunately, many of the specific reagents for immunohistochemistry are not commercially available because most of them are based on polyclonal reagents, where heterologous absorption with cross-reacting antigens is mandatory in order to render them specific [10]. However, for the most important causes of invasive mycoses, monoclonal antibodies are available [1•, 7].

Specific identification of the most important fungi causing invasive infection has been established immunohistochemically for *Candida* spp. [7, 35–38], *Aspergillus* spp. [7, 39], Mucorles [7], *Fusarium* spp. (Fig. 5) [7, 39, 40], *Blastomyces dermatitidis* [41], *Coccidioides immitis* [42], *Cryptococcus neoformans* var. *neoformans* and *C. neoformans* var. *gatti* [34], *Paracoccidioides brasiliensis* [43], *Histoplasma capsulatum* (tissue forms of *capsulatum* and *duboisii* varieties) [44], Talaromyces (*Penicillium) marneffei* [45], *Pneumocystis jirovecci* [46], *Pseudallescheria boydii* [47], *Scedosporium* spp. [7], *Sporothrix schenckii* var. *schenckii* [48], and *Trichosporon* [49].

In Situ Hybridization

Fluorescence in situ hybridization (FISH) is effective for specific identification of several species of fungi causing invasive

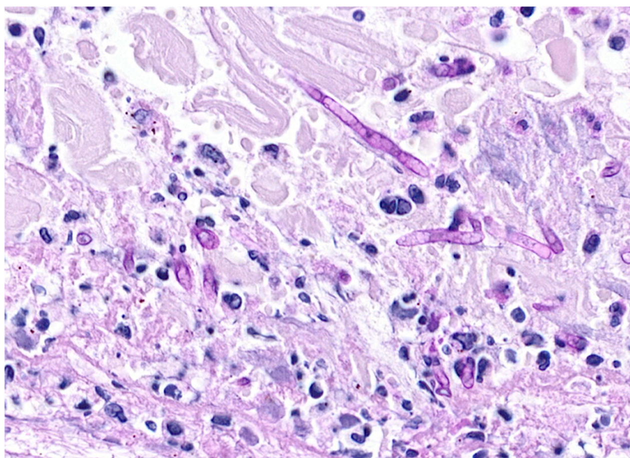

Fig. 4 Mycotic panophthalmitis. From the morphology of fungal elements, the etiology cannot be determined. It might be aspergillosis, fusariosis or scedosporidiosis. (H&E stained)

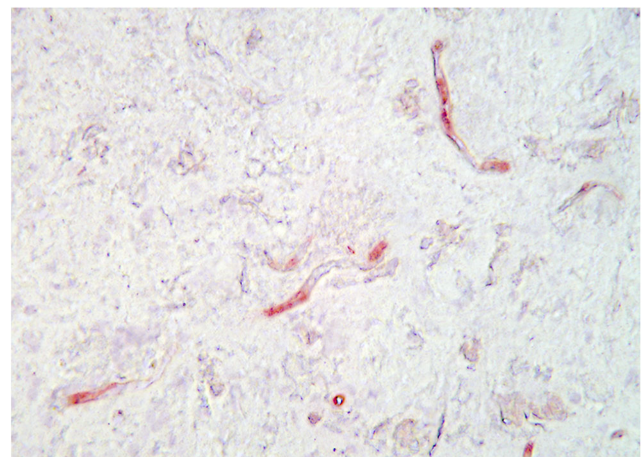

Fig. 5 Mycotic panophthalmitis (same case as in Fig. 4). Immunohistochemical staining of fungal elements with a specific monoclonal antibody raised towards *Fusarium solani*. (immunohistochemistry)

mycoses. By the method, fluorescently labeled oligonucleotide probes are targeting complementary specific sequences of nucleic acids within the fungi [14••]. By FISH, both DNA and RNA sequences can be detected [50]. Due to the natural amplification of rRNA molecules FISH techniques are usually based on ribosomal RNA (rRNA) targeting probes [30, 31]. Similar to other microorganisms, the rRNA of fungi is to a high degree conserved between species; however, it contains enough variable sequences allowing an identification at the level of species. The FISH technique can be completed in a few hours at a low cost when specific probes are available [51, 52]. Because rRNA is fragile and rapidly disintegrates when irreversible damage has occurred, especially viable cells are identified. As fungi identified by FISH depend on specifically designed probe sequences, only the targeting organisms will be detected, i.e., unknown species will be negative [6]. Therefore, universal (pan-fungal) probes with advantage should be applied initially for screening of sections for fungal elements [30, 53•].

The classical DNA probes consist of fluorescently labeled oligonucleotide probes or polynucleotides, targeting a specific sequence of fungal rRNA. Peptide nucleic acid (PNA) probes are also used for in situ identification of fungi [54]. Compared with the classical probes, the benefits of using the PNA probes, which are oligomers of single bases linked by a peptide backbone, are due to their higher affinity to hybridize to complementary sequences and that they do not degrade [54, 55]. The reaction with probes labeled directly can be evaluated in the microscope just after hybridization. When probes indirectly labeled are applied, an enzyme or a reporter molecule is bound to the probe and a brighter signal often is obtained. Within tissues, different fungal species can be identified by labeling each type of probe with different fluorophores.

Several types of probes have been successfully used for the detection of fungi: oligonucleotide DNA, PNA (peptide nucleic acids; DNA mimics with a peptide backbone) [54], and LNA (a mix of DNA and locked nucleic acid (LNA)-modified nucleotides in which the 2′ oxygen and the 4′ carbon are linked through a methylene unit) [56, 57•]. Especially, the PNA and LNA nucleotides are preferred as they strongly hybridize to their complementary RNA and DNA nucleotides and are establishing temperature stable hybrids. During recent years, a number of probes targeting the most important causes of invasive mycoses have been evaluated, and the sequences, published: *Candida* spp. [30, 31, 58], Mucorales [30, 53•], *Aspergillus* spp. (Fig. 6) [59], *Fusarium* spp. [56, 59, 60], *Blastomyces dermatitidis* [58], *Coccidioides immitis* [58], *Cryptococcus neoformans* var. *neoformans* and *C. neoformans* var. *gatti* [58], *Paracoccidioides brasiliensis* [61], *Histoplasma capsulatum* (tissue forms of *capsulatum* and duboisii varieties) [58], *Talaromyces* (*Penicillium*) *marneffei* [62], *Pneumocystis jirovecci* [63], *Pseudallescheria boydii* [59], *Scedosporium* spp. [64], *Sporothrix schenckii* var. *schenckii* [58], and *Trichosporon* spp. [65•].

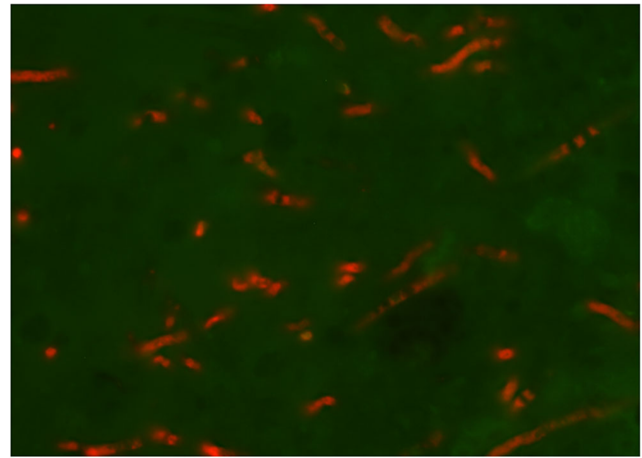

Fig. 6 Pulmonary aspergillosis due to *Aspergillus fumigatus* stained by in situ hybridization with a specific probe targeting the 28S rRNA region. (FISH in situ hybridization)

Apart from tissue sections, both immunohistochemical and in situ hybridization methods can be used to identify fungi in other specimens, e.g., smears of exudates, bronchial washings, bronchoalveolar lavage fluid, blood, bone marrow, cerebrospinal fluid, and in sputum.

Conclusions

Although several non-cultural based molecular techniques have been developed in recent years, the application of histopathology remains a golden standard for stating a diagnosis of invasive fungal diseases. The use of histopathology is essential, as it discriminates between false positive and false negative diagnoses obtained by all other techniques including cultivation. Therefore, the etiological significance of a culture isolate and results from non-cultural molecular techniques should always be determined by a careful histopathological evaluation. For the diagnosis of some invasive mycoses, where the etiology cannot be cultured (e.g., lacaziosis), the identification of typical fungal elements is essential. Certain fungal diseases are caused by agents that can be specifically identified in tissue sections due to their distinctive morphology. Although a tentative diagnosis can be provided from the morphology of fungal elements within tissues, a clear-cut diagnosis cannot always be established based on morphological details due to similarities of morphology among tissue forms of several fungal genera and the presence of sparse or atypical fungal elements. Moreover, antimycotic agents often also will alter the morphology of invasive fungi. Although a specific disease name cannot be appointed in such instances, the

pathologist can nevertheless often conclude that an invasive fungal infection is present and then apply a panel of immunohistochemical in situ hybridization techniques or PCR techniques on the tissue specimens in order to obtain a specific diagnosis.

Declarations

Conflict of Interest The author declares no conflict of interest relevant to this publication.

Human and Animal Rights and Informed Consent This article does not contain any studies with human and animal subjects performed by the author.

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•• Of major importance

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