5 Microbiological Diagnosis of Rhino-Orbito-Cerebral Mucormycosis

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Key Points

- Rhino-orbito-cerebral mucormycosis requires a prompt diagnosis as it is a medical emergency and exhibits high mortality.
- *Mucorales* causing the disease exhibit a wide spectrum of species with the emergence of newer agents.
- Endoscopically obtained tissue samples are preferable.
- Direct microscopy (potassium hydroxide (KOH)/calcofluor white-KOH mount) quickly pinpoints broad aseptate ribbon-like hyphae of *Mucorales*.
- Culture is obtained in only 50% of cases.
- Tissue should be teased instead of grinding as *Mucorales* are very friable.
- Identification of causative agents is vital due to variation in antifungal susceptibility.
- MALDI-TOF and molecular techniques aid in the identification of agents.

5.1 Introduction

Rhino-orbito-cerebral mucormycosis (ROCM), caused by *Mucorales,* is considered a medical emergency due to its ability to cause infarction and necrosis of tissues leading to high mortality. Therefore, early diagnosis is essential for immediate management and maintaining the vitality of the unaffected tissues, thereby improving outcomes. The agents of mucormycosis belong to the phylum *Mucoromycota* subphylum *Mucoromycotina* and order *Mucorales* comprising 261 species and 55 genera (Fig. 5.1) [1]. Of 55 genera, 38 are pathogenic to humans, including commonly encountered *Rhizopus, Lichtheimia* (previously called *Absidia*), *Apophysomyces*, *Mucor*, *Rhizomucor, Saksenaea*, *Cunninghamella, Syncephalastrum, Cokeromyces, Actinomucor* and *Thamnostylum* [1].

5.2 Diagnosis

The diagnosis of ROCM is based on clinical criteria, radiological imaging, microbiological and histopathological examination. The flowchart summarizing the microbiological investigations is given in Fig. 5.2.

The detailed methods are described below.

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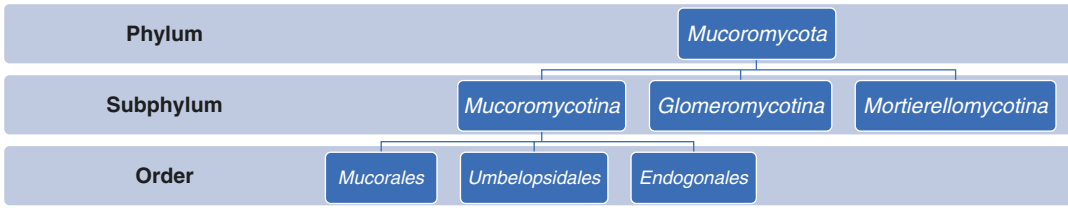

Fig. 5.1 Taxonomical classification of agents causing mucormycosis

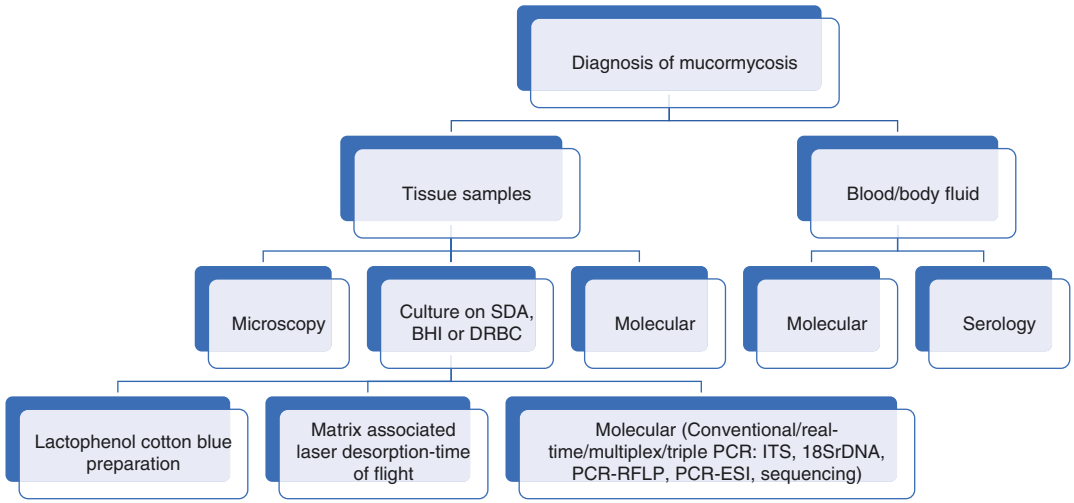

Fig. 5.2 Flowchart showing the various diagnostic modalities for diagnosis of *Mucorales* from clinical samples. *BHI* Brain–heart infusion agar, *DRBC* Dichloran rose Bengal chloramphenicol, *ITS* Internal transcribed spacer, *PCR* Polymerase chain reaction, *PCR-ESI* PCR electrospray-ionization mass spectrometry, *PCR-RFLP* PCR-restriction fragment length polymorphism, *SDA* Sabouraud dextrose agar

5.2.1 Sample Collection and Transport

Type of samples: Endoscopic or Computed Tomography (CT) guided nasal scraping/nasal biopsy, orbital tissue and brain tissue. Swabs are generally not satisfactory since they allow drying of specimens and loss of viability.

Transport: Specimens should be collected aseptically in clean, sterile and properly sealed containers, delivered to the laboratory within 2 h. If processing is to be delayed for more than several hours, it is recommended that specimens be stored under refrigeration at 4 °C.

5.2.2 Sample Processing

The specimens should be handled in a biosafety level-2 (BSL-2) laboratory facility with a Class II biosafety cabinet [2]. The sample should be processed and then inoculated to primary isolation

media within a few hours of collection. Caseous, purulent or bloody areas and necrotic parts of the sample should be selected and included for processing. The processed sample is subjected to microscopy, culture and molecular diagnosis.

5.2.2.1 Microscopy

For a quick presumptive diagnosis of ROCM, direct microscopy is a cheap, rapid and readily available technique. It forms an essential component of national and international experts' recommendations emphasizing septation, angle of branching (45–90°) and hyphal breadth (6–25 μm) [European Confederation of Medical Mycology and Mycoses Study Group Education and Research Consortium (ECMM/MSG ERC)] [3]. However, direct microscopy cannot differentiate amongst different genera or species.

1. *Potassium hydroxide (KOH) mount*: 10–20% KOH wet mount preparation of the specimen is the standard method used in direct microscopy in which characteristic broad, ribbon-like aseptate hyphae of Mucorales are characteristic broad, ribbon-like aseptate hyphae noted under microscope (Fig. 5.3a). KOH, a strong alkali, clears the cell debris and makes fungi clearly appreciable as they are resistant to digestion.
2. *KOH-Calcofluor white (CFW) solution mixture*: CFW stain binds to the cell wall of the fungi (β-1,3 and β-1,4 polysaccharides, specifically cellulose and chitin) and fluoresces bluish-white under a fluorescent microscope, thereby enhancing the visualization of the fungal element in specimens (Fig. 5.3b) [4]. Uvitex 2B and Blankophor are other alternatives. Optimal fluorescence occurs with UV excitation. Hence, the fluorescent microscope needs to have filters of UV range.

5.2.2.2 Culture

The global guidelines by ECMM/MSG ERC strongly recommend culture techniques for identification up to species level and antifungal susceptibility testing [3]. The samples suspected of mucormycosis need to be teased with sterile teasing needles instead of homogenizing due to the highly friable nature of aseptate hyphae. Routinely, the inoculation is done on two tubes of Sabouraud's dextrose agar (SDA) containing antibiotics and one tube of brain heart infusion agar (BHI). One SDA tube and BHI is incubated at 30 °C were another set of SDA at 37 °C. Compared to other moulds, *Mucorales* grow rapidly within 24–48 h. The gross morphology of the colonies classically appears cottony. The incubation at varying temperatures increases

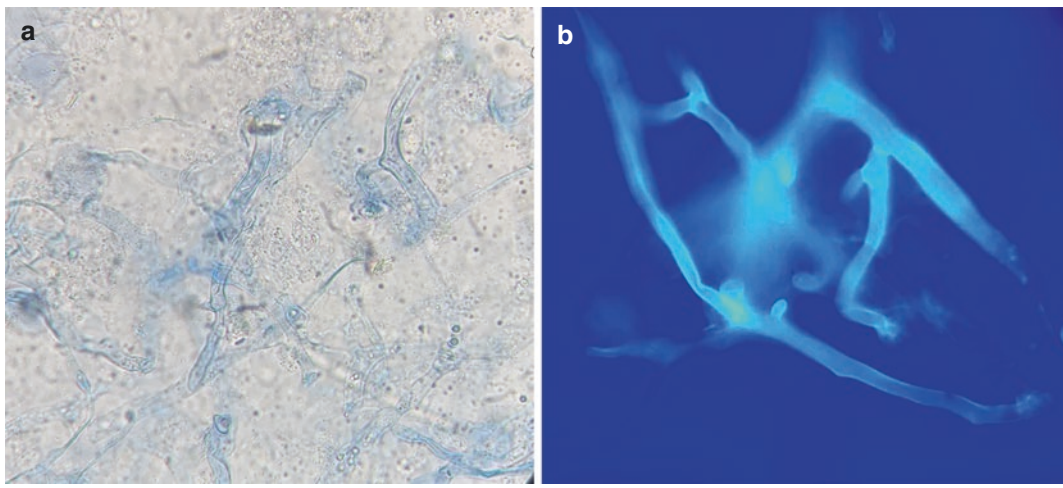

Fig. 5.3 (**a**). KOH mount of nasal scraping showing broad aseptate hyphae of *Mucorales.* (**b**) Calcofluor white (CFW)-KOH mount showing bright bluish-white broad ribbon-like aseptate hyphae of *Mucorales*

the chance of isolation and differentiates between certain members of *Mucorales*. The growth of *Mucorales* from a sterile site is considered confirmed positive, while that from a non-sterile site is judged in combination with clinical and radiological criteria. Despite the ease of sample collection from ROCM cases, culture positivity remains at 50% owing to the frangible aseptate hyphae [5, 6]. Lower culture sensitivity is particularly noted for *R. arrhizus* and *R. homothallicus* indicating their higher vulnerability to damage [7]. Recently, Vaezi et al. demonstrated higher positivity of a microculture assay from blood (28.9% vs 0%) and kidney tissue (98.8% vs 31.1%) of an immunocompetent mouse model of disseminated mucormycosis than conventional methods [8].

The identification of *Mucorales* is based on phenotypic features requiring expertise and genotypic methods. The ECMM-MSG-ERC global guidelines for mucormycosis strongly recommend the identification of *Mucorales* to the species level for epidemiological evaluation [3]. However, identification to the genus level is only marginally supported in deciding the management of patients.

Mucorales are rapidly growing and cottony in appearance, varying from white to grey to blackish colour. *Mucorales* generally produce broad non-septate or sparsely septate hyphae (10–25 μm wide), branching irregularly exhibiting asexual structures like sporangium containing spores and rhizoids apophysis, columellae and sexual structures like zygospores (Fig. 5.3). In the absence of sporulation, especially in *Apophysomyces elegans* and *Saksenaea vasiformis*, slide culture technique in nutrient-deficient media like corn meal agar, potato dextrose agar and water agar with 0.1% of yeast extract enhance the spore formation (Fig. 5.4). The phenotypic characterization is challenging due to overlapping morphological features in different species and many cryptic species [9–13]. The methods used for identification of *Mucorales* listed below include lactophenol cotton blue mount (LCB), matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and PCR sequencing.

Identification of the cultures

(a) *Lactophenol cotton blue mount (LPCB)*: LPCB mount prepared from culture demonstrates microscopic morphology aiding identification of *Mucorales*. Identification features of the commonly associated *Mucorales* are described below (Fig. 5.5) [14–16].

1. *Rhizopus arrhizus*

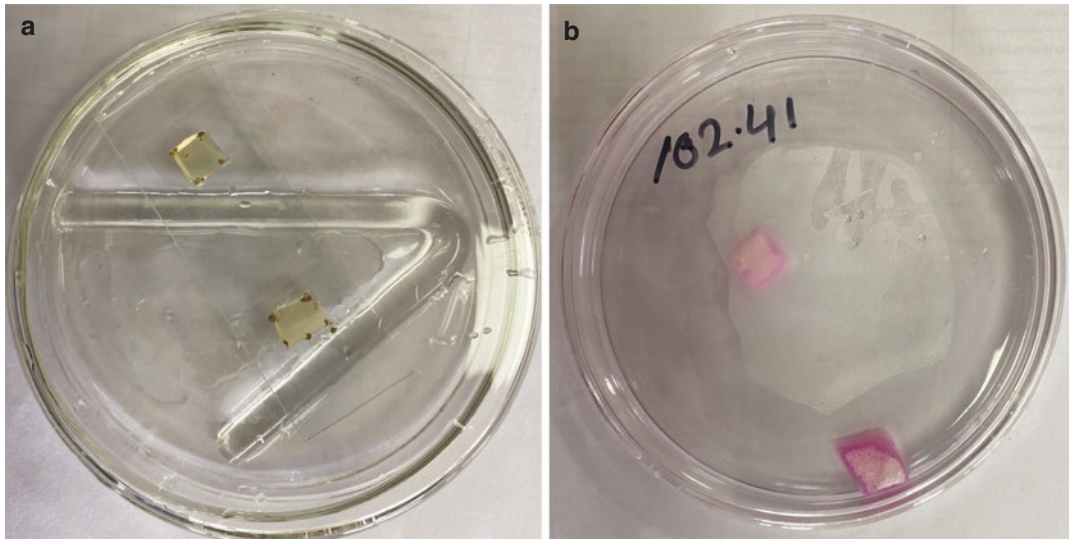

Fig. 5.4 (**a**) Slide culture technique. (**b**) Water agar technique

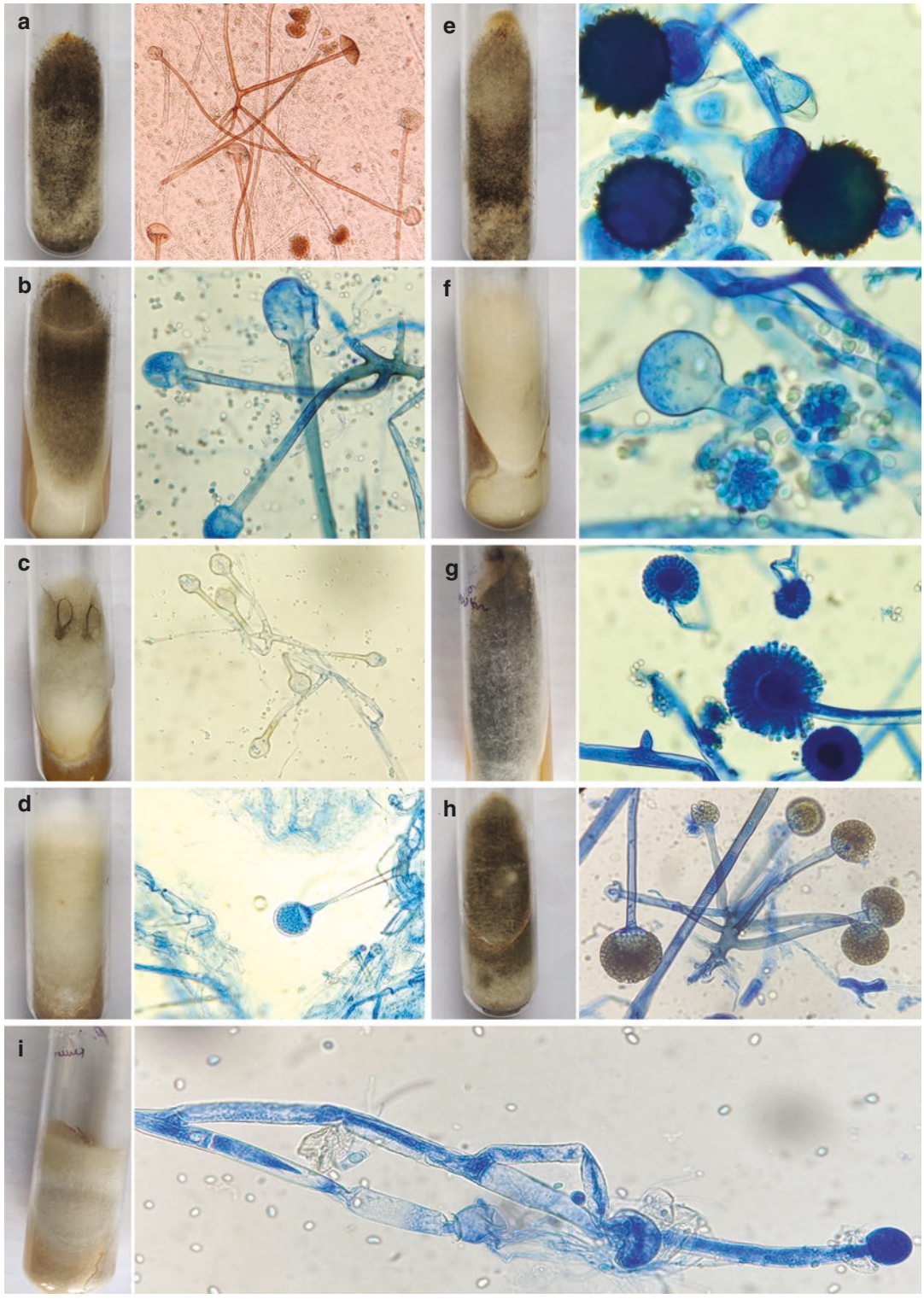

Fig. 5.5 Colony characteristics (left) and microscopic features (right) of (**A**) *R. arrhizus (x100),* (**B**) *R. microspores (x400),* (**C**) *Lichtheimia corymbifera (x100),* (**D**) *Apophysomyces variabilis (x400),* (**E**) *Rhizopus homothallicus (x400),* (**F**) *Cunninghamella bertholletiae (x400),* (**G**) *Syncephalastrum racemosum (x400),* (**H**) *Rhizomucor pusillus (x400)* and (**I**) *Mucor circinelloides (x400)*

Colony Characteristics: (SDA-25–37 °C) Rapidly growing cottony greyish white colonies. *Microscopy*: Sporangiophores are single or in groups, 1–2 mm high (18 μm wide), unbranched; sporangia spherical, brownish grey to blackish; columella covering 50–70% of sporangium; brownish rhizoids; subspherical to rhomboidal brownish sporangiospores (6–8 × 4.5–5 μm) with longitudinal striations.

2. *Rhizopus microsporus*
   Colony Characteristics: (MEA-30 °C) Cottony greyish brown colonies. *Microscopy*: Sporangiophores (8–10 μm wide) 400–500 μm high produced singly or pairs; sporangia spherical, greyish black; columella pyriform covering up to 80% of sporangium; sporangiospores (6 μm) ellipsoidal to spherical (6 and 9 μm in length), with striations.
3. *Lichtheimia corymbifera*
   Colony Characteristics: (MEA, 30 °C) Cottony greyish white rapidly growing colonies. *Microscopy*: Sporangiophores (up to 400 μm) branch repeatedly to form corymbs, sporangia pear-shaped; columella hemispherical or tapering with projections on top and a long conical apophysis; sporangiospores hyaline, smooth-walled, spherical to ellipsoidal.
4. *Apophysomyces variabilis*
   Colony Characteristics (MEA-30 °C): Rapidly growing creamy white cottony colonies turning yellowish over time. *Microscopy*: Sporangiophores (100–400 μm) unbranched, smooth-walled arising singly from a hypha; apex widening to form pyriform apophysis; sporangia pear-shaped; sporangiospores smooth-walled, hyaline to brownish and varying in shapes (cylindrical, oblong, ellipsoidal).
5. *Rhizopus homothallicus*
   Colony Characteristics: (MEA-30 °C) Rapidly growing brownish to greyish cottony colonies. *Microscopy*: Sporangiophores (5–30 μm wide) 2000 μm high; sporangia spherical, greyish black; columella subspherical; sporangiospores ellipsoidal or spherical with striations; homothallic with brownish yellow, spherical, spiny zygospores with unequal suspensors.
6. *Cunninghamella bertholletiae*
   Colony Characteristic: (MEA 37 °C) Rapidly growing greyish white colonies. *Microscopy*: Sporangiophores erect, with a whorl of short lateral branches at the apical region, ending in a swollen vesicle, single-spored sporangiola all over the vesicle attached by denticles, sporangiospores oval to spherical and smooth walled.
7. *Syncephalastrum racemosum*
   Colony Characteristics: (MEA, 25 °C) Greyish rapidly growing cottony colonies. *Microscopy*: Sporangiophores (10–25 μm wide) single or branched, arising from rhizoids, ending round vesicle, covered entirely by merosporangia, greyish cigar-shaped, containing chains of 3–18 spores; merospores smooth-walled, round to oval.
8. *Rhizomucor pusillus*
   Colony Characteristics: (MEA-30 °C) Rapidly growing cottony dark brown colonies. Being thermophilic, it grows up to 54–58 °C. *Microscopy*: Sporangiophores (11–15 μm wide) brownish, sympodially branched; rhizoids short rudimentary; sporangia spherical; columella spherical to pyriform and lacking apophysis; sporangiospores spherical, smooth-walled; homothallic or heterothallic with spherical dark brown spiny zygospores and equal suspensors.
9. *Mucor circinelloides*
   Colony Characteristics: (MEA, 24 °C) Rapidly growing brownish grey, black or yellow colonies. *Microscopy*: Sporangiophores (6 mm high, 17 μm wide) branched, elongated and shorter ones; columellae spherical to ellipsoidal; sporangiospores ellipsoidal and smooth-walled; chlamydospores absent or scanty.

(b) *Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS)*: MALDI-TOF MS with an upgraded database is a remarkably effective technique for identifying *Mucorales* to the tune of 100% at the genus level and 81.1% at the species level [3, 17, 18]. It is a simple, rapid, high-throughput technique for identifying *Mucorales* based on their unique main spectrum profiles (MSPs). However, the database requires continuous upgradation. The ECMM-MSG-ERC global guidelines for mucormycosis moderately support MALDI-TOF use due to its reliability on in-house databases and unavailability at many centres [3].

(c) *Other phenotypic methods*: The success of ID32C (bio Merieux, Marcy l'Étoile, France) and API 50CH (bioMerieux) kits based on carbon assimilation profiles of different *Mucorales* was described by Schwarz et al. [19].

(d) *PCR-sequencing*: The PCR sequencing targeting the internal transcribed spacer (ITS) region is the recommended molecular method for identifying *Mucorales* [3]. It is a cumbersome technique and is mainly available at reference laboratories. The concordance with phenotypic identification is reported to be >90% [20]. Other successful targets used for *Mucorales* identification include 18S rDNA, 28S rDNA, FTR1 and cytochrome b [1, 3].

5.2.2.3 Molecular Diagnosis

The need for molecular technique arises when culture does not yield growth of *Mucorales* (48–68% of direct microscopy positive) or in cases of concurrent infection due to *Aspergillus* and *Mucorales* or when the sparse fragments present in tissue make histopathological differentiation difficult [7, 21–23]. The molecular method is a quicker technique (<48 h) than culture (72–144 h) and histopathological (72–96 h) examination, although direct microscopy is the most rapid (<1 h) [7]. Molecular detection directly from fresh samples and formalin-fixed paraffin-embedded tissues is a promising technique but possesses heterogeneity in target genes (like ITS, 18S rDNA, 28S rDNA, cytochrome B, mitochondrial gene rnl or CotH genes), encompasses different methods with varying sensitivity [PCR and sequencing, semi-nested PCR, RFLP, qPCR and high-resolution melting (HRM) or electrospray ionization mass spectrometry (PCR/ESI-MS)] and lacks validation and standardization (Table 5.1) [6, 8, 24, 25]. Although histopathological examination (HPE) of tissue is an impor-

Table 5.1 Molecular diagnosis of mucormycosis

Sl no	Molecular approach	Target gene/s	Samples	Number of samples	Positivity	Remarks
1	ITS (panfungal) sequencing [38]	ITS	Fresh and FFPE tissues	$N = 8$	8 (100%)	One sample was identified as *R. pusillus* but *Absidia* by culture
2	PCR *Mucorales* specific primer sequencing [39, 40]	18S rDNA of *Mucorales*	Fresh and FFPE tissues	$N = 27$	22, of which one result was discordant with culture at species level	Semi-nested 81% sensitivity
		18S rDNA gene (Muc18S)	Blood and tissue	$N = 12$ (tissue samples) $N = 268$ (serum samples)	91% in paraffin-embedded tissue samples Serum: 100% proven/probable cases, 29% possible cases and 15% in unclassified	Probe-based *Mucorales*-specific real-time qPCR assay

(continued)

Table 5.1 (continued)

Sl no	Molecular approach	Target gene/s	Samples	Number of samples	Positivity	Remarks
3	Real-time PCR (qPCR) followed by high-resolution melt analysis (HRM) [26, 38, 41–43]	ZM1 and ZM3	Fresh and FFPE tissues	*N* = 7	100%	Semi-nested real-time PCR. Melting temperatures: *R. microsporus*, 76.46 °C; *R. oryzae*, 76.59 °C; *M. racemosus*, 76.78 °C; *M. circinelloides*, 76.98 °C; R. *pusillus*, 77.87 °C; *L. corymbifera*, 78.56 °C
		New species-specific real-time PCR assay targeting ITS2 region of ribosomal DNA	BAL	*N* = 99	9/99 (9.09%)	Sensitivity (100%) and specificity (93%); *Rhizopus* spp. (*n* = 6), *R. pusillus* (*n* = 2), and *L. corymbifera* (n = 1); results within 5 h
		Cytochrome *b* gene	Fresh tissue and paraffin-embedded tissue	*N* = 2 (fresh tissue) *N* = 62 (paraffin-embedded tissue)	–	100% sensitivity and specificity for fresh tissue 56% sensitivity and 100% specificity for paraffin-embedded tissue
		rnl gene	Tissue, blood	*N* = 21	15 (71.4% positivity)	LoD: 100 fg mucoralean DNA HRM profile in conidia-spiked blood samples: 10^4 *R. arrhizus*-conidia-spiked blood, equating *R. arrhizus* conidia/PCR reaction
		18S rDNA	Fresh tissue and FFPE	*N* = 6 (fresh tissue) *N* = 1 (FFPE)	100%	Semi nested real-time PCR
4	Multiplex real-time quantitative PCR (qPCR) [44]	Molecular beacon species-specific probes ITS1/ITS2 region with specific probes for *R. oryzae*, *R. microsporus* and *Mucor* spp.	Tissue	*N* = 12	N = 9	Two were negative as the causative agent was not included in the primer set (*R. pusillus* and *C. bertholletiae*)

Table 5.1 (continued)

Sl no	Molecular approach	Target gene/s	Samples	Number of samples	Positivity	Remarks
5	qPCR [45]	28S rDNA	Lung BAL	*N* = 98 *N* = 22	N = 97 N = 22	(99% sensitivity) (100% sensitivity)
6	PCR coupled with electrospray-ionization mass spectrometry (PCR/ESI-MS) [46]	16S-23S rRNA gene (ITS PCR) and 18S PCR	Fresh tissues	*N* = 15	Genus level: 13/15; species level: 12/15	Quantitative real-time PCR and sequencing; results within 6 h; *Cunninghamella* spp. or *Saksenaea vasiformis* misidentified
7	PCR-RFLP [7]	18S ZM1 and ZM3	Tissue	*N* = 50	100%	Identification is possible only up to genus level
8	Triple qPCRs [34, 35, 47, 48]	Acory/Muc1/ RMuc	Blood	*N* = 10	9/10	Limit of detection 3.7 to 15 fg/10 μL; positive up to 68 days before mucormycosis diagnosis; negative result in *Lichtheimia* species
			Blood	*N* = 44	36/44	Retrospective study
			BAL	*N* = 337 suspected patients	15 (5: Proven/ probable mucormycosis, 3: Probable invasive aspergillosis, 6: Invasive fungal disease, 1: No invasive fungal disease)	Sensitivity: 100% Specificity: 97%
			CSF	*N* = 1	1/1 (100%)	Single case
9	PCR-based detection of spore coat protein [27]	*CotH*	Urine	*N* = 4	4/4 (100%)	Sensitivity 90%, specificity 100% for proven mucormycosis; urine samples better than plasma or BAL in mice model
10	Genera-specific qPCR assay targeting *Cunninghamella* [49]	18S rRNA	Serum and BAL	*N* = 1	1/1 (100%)	Single case

Abbreviations: *BAL* Bronchoalveolar fluid, *CSF* Cerebrospinal fluid, *C. bertholletiae*: *Cunninghamella bertholletiae*, *DNA* Deoxyribonucleic acid, *FFPE* Formalin-fixed paraffin-embedded tissue, *ITS* Internal transcribed spacer, *LoD* Limit of detection, *L. corymbifera*: *Lichtheimia corymbifera*, *M. circinelloides*: *Mucor circinelloides*, *M. racemosus*: *Mucor racemosus*, *PCR* Polymerase chain reaction, *qPCR* Real-time PCR, *rDNA* Ribosomal DNA, *rRNA* Ribosomal ribonucleic acid, *R. arrhizus*: *Rhizopus arrhizus*, *R. microsporus*: *Rhizopus microsporus*, *R. oryzae*: *Rhizopus oryzae*, *R. pusillus*: *Rhizomucor pusillus*, *RFLP* Restriction fragment length polymorphism

tant tool for diagnosis of mucormycosis, it cannot identify the genus/species of the etiological agent. Fresh tissues (86–100% sensitivity) are generally preferred samples compared to FFPE tissues (15–90%) [3, 26–28]. Zaman et al. reported a nested PCR technique directly from fresh tissues targeting 18S rDNA with 100% results, whereas ITS sequencing could identify only 54% of the cases [7]. The lower performance of the ITS region in *Mucorales* is probably due to its longer (ITS1 region~300–350 bp) length compared to that in other fungi (~200–250 bp) [29]. Therefore, it is suggested to target the *Mucorales*-specific 18S rDNA region in a semi-nested PCR in tissues positive for aseptate hyphae than the ITS1 region, which performs better in septate fungi and yeasts [30]. The major factor hampering PCR amplification in FFPE tissues is DNA degradation due to histone cross-linking to formalin, which inversely affects the sensitivity over time [31, 32]. Another limitation of the molecular technique from FFPE is the low quality of sequence chromatograms, especially from tissues harbouring colonizing fungi interfering with amplification of target DNA [7, 29]. Jillwin et al. recorded cross-amplification in 14% of nasal/paranasal and cutaneous samples which form the majority of samples in suspected mucormycosis cases [30]. Overall, the analytical sensitivity of molecular methods in fresh tissue samples and FFPE ranges from 97% to 100% and 56% to 80%, respectively [28]. There are no commercially available methods for the same. This remains the focus of the Fungal PCR Initiative Working Group of ISHAM [33]. Apart from the tissue samples, molecular methods have been explored in blood and body fluids (BAL, CSF, urine) for early diagnosis of mucormycosis, especially pulmonary cases where deep tissue sampling is challenging (Table 5.1). Studies have shown detection of *Mucorales* DNA in serum of patients even before 3–68 days of conventional diagnosis [34, 35]. A commercially available, non-FDA-approved kit, MucorGenius (Pathonostics, Maastricht, The Netherlands), is a real-time PCR assay targeting 28S rRNA with a sensitivity of 75% and assay time of 3 h and diagnosing mucormycosis much early than the standard method [3, 24, 36]. It detects *Rhizopus* spp., *Mucor* spp., *Lichtheimia* spp., *Cunninghamella* spp. and *Rhizomucor* spp. The major drawback of these methods is lower sensitivity in patients on antifungal therapy and false-negative results in lower fungal burden [24, 35, 37]. There is still a need to standardize and validate molecular methods from clinical samples. Despite recommendations of screening high-risk patients (e.g. haematological malignancies, burns) for mucormycosis by molecular diagnosis from serum/plasma, its role in the diagnosis of ROCM may be limited.

5.2.2.4 Serological Diagnosis

There is a lack of commercially available antigen biomarkers indicating mucormycosis. The markers like galactomannan are indeed helpful in ruling out the diagnosis of mucormycosis when a high index of fungal infection is clinically suspected [3, 24]. However, one needs to be cautious of the possibility of mixed infections. The ECMM-MSG-ERC global guidelines of mucormycosis do not recommend using (1→3) ß-D-glucan (BDG) to diagnose mucormycosis. Burnham-Marusich et al. developed ELISA [using a panfungal monoclonal antibody (2DA6)] and lateral-flow immunoassay (LFIA), which could detect fucomannan present in the cell wall of numerous fungi, including *Mucorales* [50]. Detection of a serum disaccharide by mass spectrometry (MS) in mucormycosis cases has cross-reaction with other fungal pathogens. Furthermore, *Mucorales*-specific T cells (CD4+CD154+) detected by enzyme-linked immunospot (ELISpot) assay over 24 h seems to be specific for proven mucormycosis cases [51, 52]. Sato et al. identified *Rhizopus*-specific antigen (RSA) by signal sequence trapping and retrovirus-mediated expression (SST-REX) in the mouse model. They evaluated its diagnostic application by developing a monoclonal antibody-based ELISA system which demonstrated higher serum RSA levels in patients with mucormycosis as compared to invasive aspergillosis (15.1 vs 0.53 ng/mL) and negative control (0.49 ng/mL) [50, 53]. Although these tests are still in a nascent stage; their development will be

an asset to non-invasive rapid diagnostics of mucormycosis.

5.2.2.5 Metabolomics-Breath Test

Koshy et al. reported differentiation of infection caused by *R. arrhizus* var. arrhizus, *R. arrhizus* var. delemar and *R. microsporus* and from that of aspergillosis based on breath profile of volatile metabolite, sesquiterpene in mice and human cases tested by gas chromatography/tandem mass spectrometry (GC–MS) [50]. This technique seems easy, non-invasive and can be utilized for screening high-risk patients after complete validation.

5.2.2.6 Antifungal Susceptibility Testing

The ECMM-MSG-ERC global guidelines recommend performing antifungal susceptibility testing using standard methods [broth microdilution by European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the CLSI] for *Mucorales* only in case of non-responders through strong recommendation exists for epidemiological investigation [5, 54, 55]. Commercial methods like E-test are, however, only marginally recommended as their results do not sometimes match with the standard methods. The major hurdle in antifungal susceptibility testing of *Mucorales* is the unavailability of clinical breakpoints, which makes interpretation difficult, although epidemiological cut-off values are available for some species. Though amphotericin B, posaconazole and isavuconazole have good activity against *Mucorales*, few *Cunninghamella* species exhibit higher MICs against amphotericin B, *Rhizopus* species against posaconazole and *Mucor circinelloides* against isavuconazole [5].

5.2.2.7 Environmental Screening

Apart from the clinical samples, *Mucorales* have also been isolated from environmental niches like air and soil. The isolation is pertinent to delineate the spore burden of *Mucorales* in environmental sources. The isolation from the air is preferably performed using DRBC with benomyl medium, which is selective for *Mucorales* [56] (Fig. 5.6). Whole-genome sequencing has also been employed to resolve the dynamics of an outbreak of mucormycosis in a burn unit in France [57].

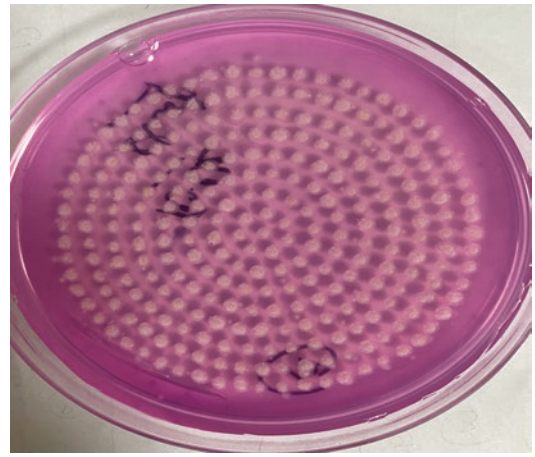

Fig. 5.6 Isolation of *Mucorales* from air on dichloran rose Bengal chloramphenicol (DRBC) with benomyl medium

5.3 Conclusion

The diagnosis of ROCM is considered as most urgent owing to progressive angioinvasion leading to a high fatality rate. High clinical suspicion and microbiological examination of endoscopic tissue biopsies is the prime requirement. Obtaining deep samples might not be possible in patients with neutropenia or thrombocytopenia. The conventional diagnostic techniques like direct microscopy and culture have low sensitivity, though optical brighteners enhance the visual field. The fragile nature of the aseptate hyphae of *Mucorales* affects the yield of culture. There is a complete void of serological markers for *Mucorales* though a negative galactomannan may decrease the likelihood of infection. The molecular techniques are emerging but are available only in reference laboratories and lack standardization.

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