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Review Article

Review Article

Pathogenicity patterns of mucormycosis: epidemiology, interaction with immune cells and virulence factors

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Abstract

Fungi of the basal lineage order Mucorales are able to cause infections in animals and humans. Mucormycosis is a well-known, life-threatening disease especially in patients with a compromised immune system. The rate of mortality and morbidity caused by mucormycosis has increased rapidly during the last decades, especially in developing countries. The systematic, phylogenetic, and epidemiological distributions of mucoralean fungi are addressed in relation to infection in immunocompromised patients. The review highlights the current achievements in (i) diagnostics and management of mucormycosis, (ii) the study of the interaction of Mucorales with cells of the innate immune system, (iii) the assessment of the virulence of Mucorales in vertebrate and invertebrate infection models, and (iv) the determination of virulence factors that are key players in the infection process, for example, high-affinity iron permease (FTR1), spore coat protein (CotH), alkaline *Rhizopus* protease enzyme (ARP), ADP-ribosylation factor (ARF), dihydrolipoyl dehydrogenase, calcineurin (CaN), serine and aspartate proteases (SAPs). The present mini-review attempts to increase the awareness of these difficult-to-manage fungal infections and to encourage research in the detection of ligands and receptors as potential diagnostic parameters and drug targets.

Key words: Zygomycosis, Etiology, Phagocytes, Phylogeny, Systematics and Taxonomy.

Introduction

From a systematic point of view, the Mucorales represent the most prominent order amongst the zygosporic fungi, formerly summarized in the class Zygomycetes.[1] The name Zygomycetes is derived from the Greek word "zygos" for balance. In both ancient and modern Greek, a "*zygos*" is a tool that balances two different elements, a yoke, which refers to the yoke-shaped suspensors resulting in a zygospore during fusion of two gametangia (gametangiogamy) in sexual conjugations. The sexual interaction involves various ways of communication at both, morphogenetic and chemical level. Sexual spores (zygotes which mature to zygospores; Fig. 1A) are formed upon a cooperative synthesis of the chemotactic, sexual pheromone trisporic acid followed by hyphal conjugation between two compatible mating partners (+ and -).[2] Most of the species are heterothallic, few species are self-fertile (homothallic).[3–5] Zygospores rarely disperse and sporulate in the environment and thus do not play any role in pathogenesis. Most Zygomycetes reproduce clonally (asexually) via the formation of nonmotile (aplanosporic) mitospores (3–11 μm in diameter). which are soil-, air-, feed- and food-borne and produced in multi- or few spored sporocarps (sporangia and sporangiola/merosporangia, respectively Fig. 1B–D).[6] The Zygomycetes was first described as "Phylum des Zygomycètes" (phylum Zygomycota) by Whittaker.[7]

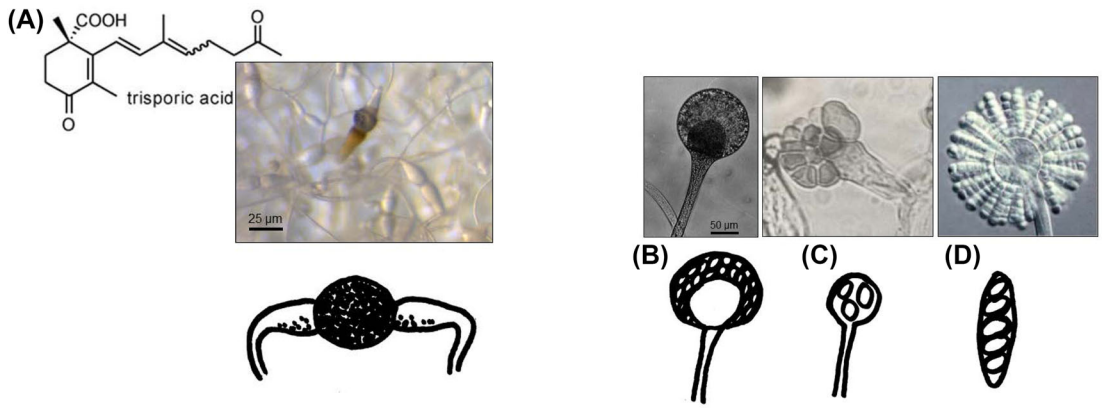

Figure 1. Major morphological structures of the zygomycetes: A: Zygospore and the sexual pheromone trisporic acid. B: Multi-spored sporangium (most abundant form). C: Few-spored sporangiolum (small sporangium; *Cokeromyces*, *Cunninghamella*, *Mycotypha*). D: Merosporangium (cylindrical, uniserate, few-spored sporangium, *Syncephalastrum*). This Figure is reproduced in color in the online version of *Medical Mycology*.

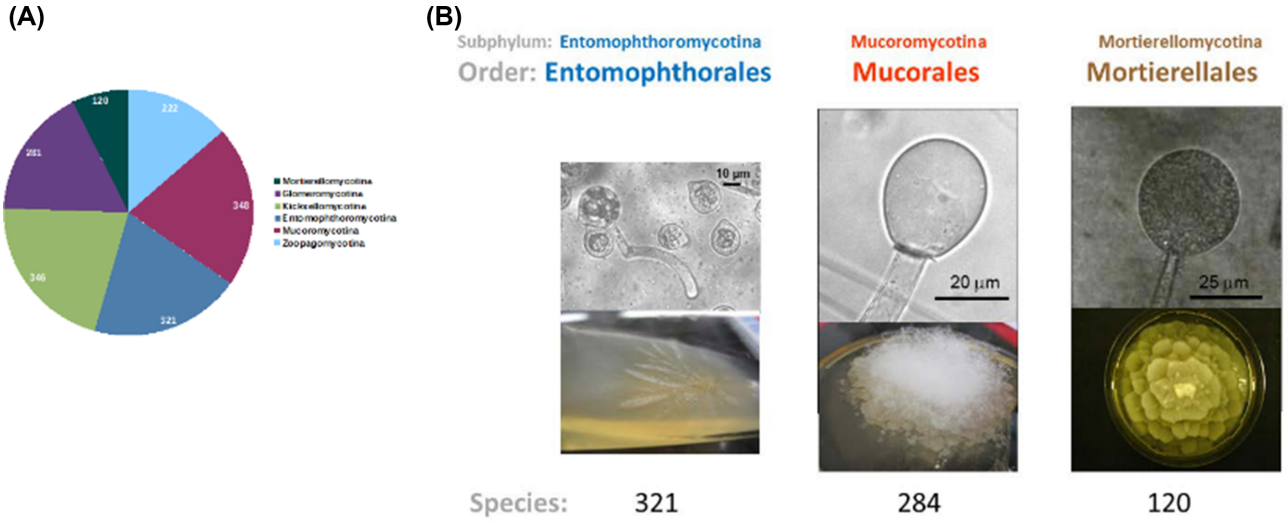

Figure 2. A: Species distribution among the subphyla of the zygomycetes ("Zygomycota," Speciesfungorum at www.indexfungorum.org as of 27 Sept 2018). B: Systematics and morphology of three clinically relevant orders of the zygomycetes Entomophthorales: *Conidiobolus coronatus*, Mucorales: *Mucor mucedo*, Mortierellales: *Mortierella parvispora*. The numbers below the photographs indicate species counts (Speciesfungorum at www.indexfungorum.org as of 27 Sept 2018). This Figure is reproduced in color in the online version of *Medical Mycology*.

They represent the first terrestrial fungi appearing on Earth.[8] The use of genes has largely accelerated the research on fungal phylogenetics influencing and revolutionizing the systematics of the fungi. Multi-gene phylogenies provided evidence for the paraphyletic relationships among the most basal lineages of terrestrial fungi,[9–11] leading to the disintegration of the Zygomycota into five subphyla: *Entomophthoromycotina*, *Kickxellomycotina*, *Mucoromycotina Zoopagomycotina*,[12] and *Mortierellomycotina*[13] (Fig. 2A). Due to attempts to improve the phylogenetic backbone resolution by the application of genome-wide phylogenomic approaches,[14–16] the subphyla were raised to phylum level.[17] Among the zygomycetes, three orders appear to be predominant and were reported to be connected with zygomycotic infections in humans and animals, the Entomophthorales, the Mucorales, the Mortierellales[18] (Fig. 2B). The Entomophthorales and the Mucorales are known to play an important role in the development of zygomycoses in humans, which are termed entomophthoromycosis and mucormycosis, respectively.[8] While the entomophthoromycosis are slowly progressing and locally confined in immunocompetent patients, the mucoralean fungi can rapidly invade and disseminate in deep tissues especially in immunocompromised patients.[19] Two species count for typical causative agents of entomophthoromycosis: *Conidiobolus coronatus* and *Basidiobolus ranarum*[20]; 25 species of the genera *Actinomucor*, *Apophysomyces*, *Cokeromyces*, *Cunninghamella*, *Lichtheimia*, *Mucor*, *Mycotypha*, *Rhizomucor*, *Rhizopus*, *Saksenaea*, and *Syncephalastrum*[18] have been reported to cause infections in human and represent seven out of 14 family branches as displayed in the phylogenetic tree of the order Mucorales (Fig. 3). With exception of the sporangiola-forming genera

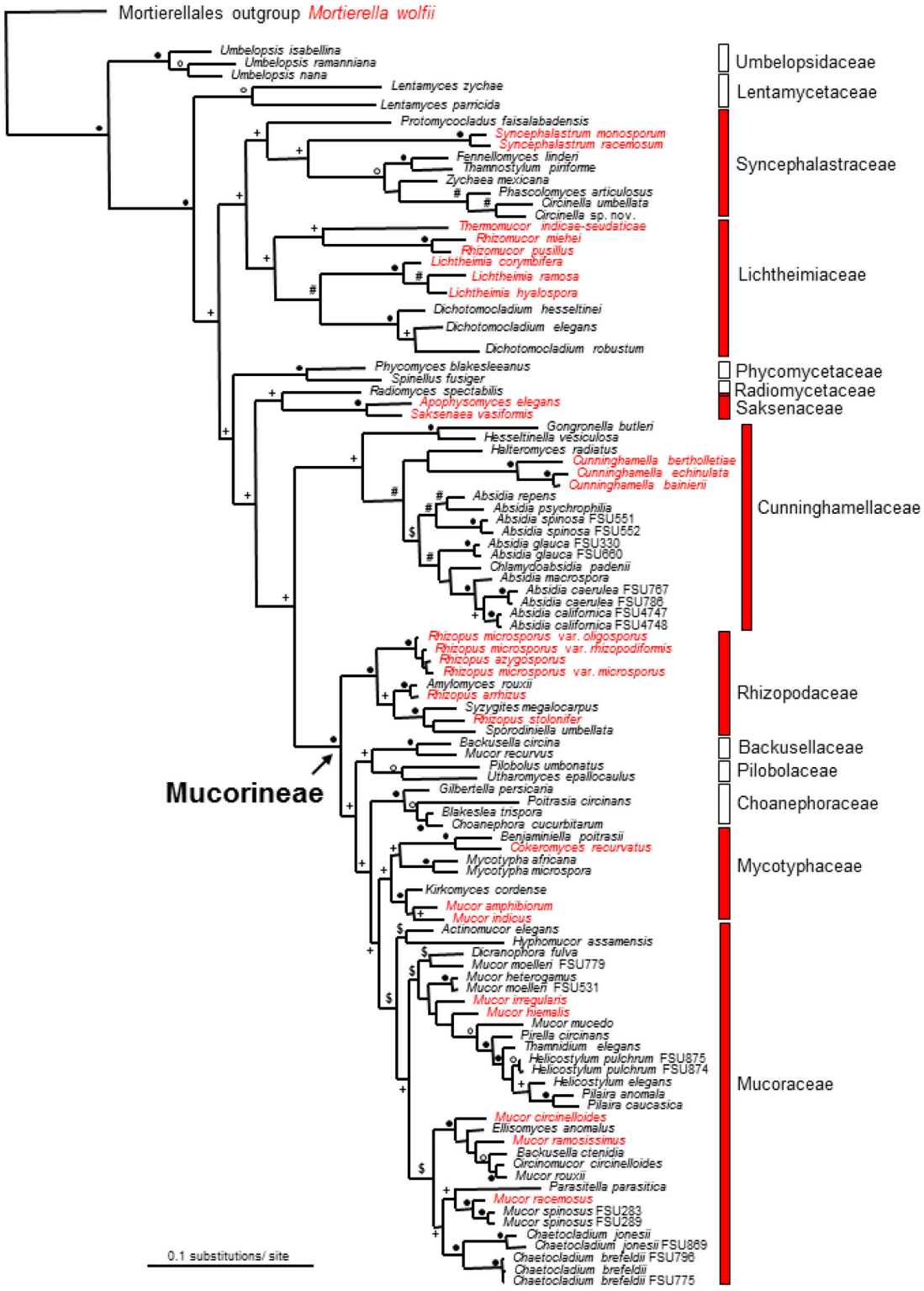

Figure 3. Evolution of 27 human pathogenic species within the order Mucorales based on Bayesian inferred phylogenetic analysis of four-locus phylogeny comprising 807 and 1,092 characters of exonic genes from actin and translation elongation factor EF1 alpha as well as 1215 and 389 characters of the nuclear small (18S) and the large (28S) subunit rRNA, respectively. Clade stability values obtained by Bayesian, distance and Maximum Parsimony analysis, are given above the branches with the following meaning: Full black dots: Clade support equal or greater than 95%; White dots: Clade support equal or greater than 90%; #: Clade support equal or greater than 75%; +: Clade support equal or greater than 95% but only in Bayesian analysis; $: Clade support equal or greater than 75%, but only in Bayesian and Neighbor-Joining analysis. The tree is rooted in three species of the Mortierellales including the animal pathogenic *Mortierella wolfii* used as an outgroup. The family structure is indicated in accordance with Voigt et al. (2009)[119] and Hoffmann et al. (2012).[120] – Adopted from Voigt (2012, 138–139).[121] The pathogenic potential of the species was evaluated based on Hoog et al. (2009).[18] This Figure is reproduced in color in the online version of *Medical Mycology*.

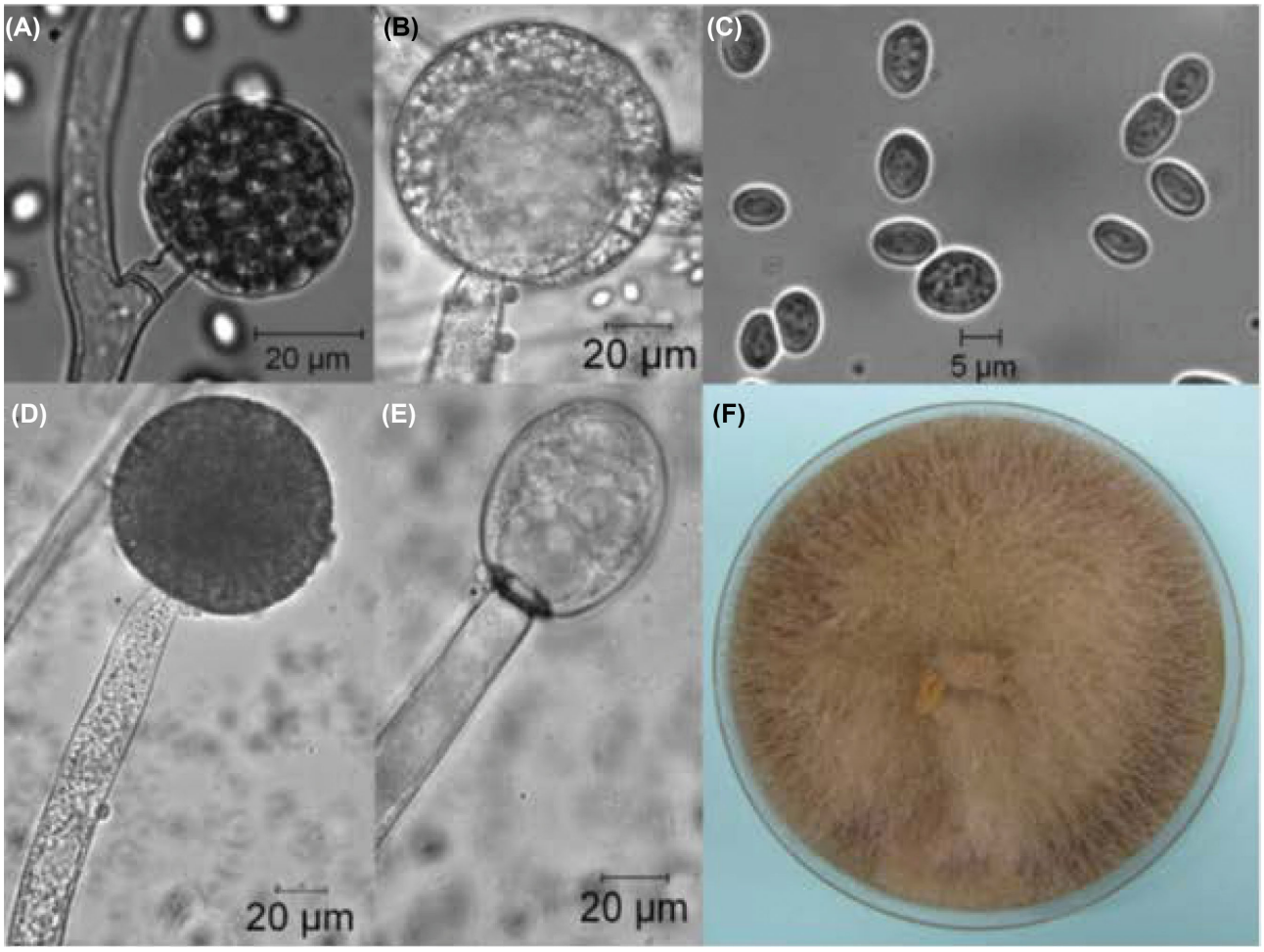

Figure 4. Morphology of a typical member species (exemplarily: *Mucor flavus*) of the genus *Mucor* representing the type of the order Mucorales. A: Mature sporangium. B: Mature sporangium with prominently visible columella, a sterile and bulbous vesicle on the sporangiophore apex. C: Sporangiospores. D: Immature sporangium. E: Egg-shaped columella. F: Typical appearance of mycelial lawn consisting of well-developed hyphae after 10 days growth on 3% malt extract agar at room temperature. (Photo: Orig., courtesy by C. Kesselboth, June 2009). This Figure is reproduced in color in the online version of *Medical Mycology*.

Cokeromyces, *Cunninghamella*, *Mycotypha*, and *Syncephalastrum* all genera form multi-spored sporangia (Fig. 1B), while *Syncephalastrum* develops merosporangia that represent a special type of sporangiola (Fig. 1C) by having the spores arranged in a single row (Fig. 1D). The shapes and size of spores and sporocarps in conjunction with the colony morphology are traditionally used to determine species designation (exemplarily: *Mucor flavus*; Fig. 4).

From an ecological point of view, the Mucorales represents a heterogeneous group of filamentous fungal saprobionts or facultative parasites, usually found in soil, compost, animal feces, decaying vegetables, agricultural debris, or other organic matter and in association with plants, fungi, animals, and humans as opportunistic pathogens.[1,3] Many are cosmopolitan components of the biosphere, ubiquitously occurring in all climatic zones of the Earth. A recent study investigated soil samples from various geographical areas in France found *Rhizopus arrhizus* (synonym: *Rhizopus oryzae*), *Mucor circinelloides*, *Lichtheimia corymbifera*, *Rhizopus microsporus*, and *Cunninghamella bertholletiae* to be the most common among the mucoralean fungi.[3,21] Spores are dispersed by air and taken up by humans via inhalation or ingestion of contaminated food, even though detection on the nasal mucus is aggravated by mucociliary transport-mediated removal.[3] Once the predisposing risk factors become favorable for infection, mucoralean fungi are dreaded to cause fatal disease in a broader range of human and animal hosts compared with other opportunistic fungi.[3]

Epidemiology and clinical presentation of mucormycosis

Besides aspergillosis and candidiasis mucormycosis counts as a third most important disease in Europe in hematological patients.[22] The infection mainly threatens immunocompromised patients in particular, those suffering from the consequences of

uncontrolled diabetes, bone marrow or solid organ transplantation, corticosteroids treatment, hematological malignancy, and traumata. Mucormycosis can cause the following types of disease: (1) Rhinocerebral mucormycosis that can infect the sinuses and the brain leading to fever, swelling of one side of the facial organ, black lesions inside the mouth/outside the face, headache, and sinus congestion; (2) pulmonary mucormycosis that mainly infects the lung leading to chest pain, disturbance in breathing, fever, and cough; (3) cutaneous mucormycosis that causes local skin infections leading to ulcers or blisters, redness, and swelling of the infected skin region; (4) gastrointestinal mucormycosis which is uncommon in adults, but more often in premature neonates leading to nausea, vomiting, gastrointestinal bleeding, and abdominal pain; (5) disseminated mucormycosis, which occurs in patients suffering from multiple medical complications impeding the symptomatic discrimination of mucormycosis from other infectious diseases; (6) uncommon presentation as renal infection.[22–25] The rate of mortality and morbidity of mucormycosis varies depending on the organ affected by the infection, the causative fungal species and the medical status of the patient; for example, 46% mortality was observed among patients suffering from sinus infections, and a mortality rate of 76% and 96% was reported for pulmonary and disseminated mucormycosis infections, respectively.[26] Among patients undergoing stem cell and solid organ transplantation, the chances of survival are better as accounted by mortality rates of 8% and 2%, respectively.[27,28] Diabetes is the most common clinical risk factor that distinguishes mucormycosis from Pseudallescheriasis or Fusariosis, and other uncommon mould diseases.[29] Diabetes appears markedly in rhino-orbital and rhinorbital-cerebral mucormycosis and associates with mucormycosis infecting sinuses and brain but does not play role in pulmonary mucormycosis.[29]

Most cases of mucormycosis are occasional, although mucormycosis outbreaks were recently reported in community and healthcare-association (e.g., tornado, hospital linens, adhesive bandages, building construction, and wooden tongue depressors).[30–32] The lack of surveillance all over the world hampers the estimation of absolute case numbers of mucormycosis[33] and necessitates the establishment of a strong connection between surveilling public health agencies and physicians diagnosing mucormycoses.[34] In the United States, the rate of the disease increased from 0.12 to 0.16 per 10 000 patients[35] between January 2005 and June 2014. In India, the incidence of mucormycosis is 70 times higher than the total rate of mucormycosis worldwide with about 0.14 cases/1000 population from January 2010 to December 2014.[36] Males were more susceptible to mucormycosis than females.[36] Rhino-orbito-cerebral, cutaneous, pulmonary, oral cavity, and gastrointestinal mucormycosis were the most common clinical manifestation in the investigated cases.[36]

In Egypt, *Rhizomucor* spp. were reported in children suffering from cancer, which was fatal in all cases.[37] Uncontrolled diabetic ketoacidosis was ascribed to be the main precursor for mucormycosis disease with a prevalence for male patients.[38] Pulmonary and rhinocerebral infections were referred to be the most common mucormycosis caused by *Lichtheimia* spp. (40%) followed by *Rhizopus* spp. (30%), *Syncephalastrum* spp., and *Rhizomucor* spp. (20%, and 10%, respectively)[39] from January 2010 to December 2010. In Spain, *Lichtheimia* spp. were the most prominent species with 42% followed by *Rhizopus* spp. and *C. bertholletiae* with 21% and 16%, respectively, in patients suffering from hematological malignancies as well as from complications connected with trauma or surgical wounds[40] from 2007 to 2015.

The clinical state of mucormycosis for other countries is summarized in Table 1.[41–51]

Table 1. Incidence of mucormycosis in several countries all over the world.

Continent	Country	No. of mucormycosis cases per 100 000*	Reference	Year
Africa	Cameroon	0.2	[41]	2014–2017
	Senegal	0.2	[42]	2012–2014
Asia	China	0.1	[43]	2011–2012
	Jordan	0.2	[43]	2011–2013
	Nepal	0.2	[44]	2012–2013
	Qatar	1.23	[45]	2011
	Saudi Arabia	0.034	[43]	2011–2012
	Vietnam	1.2	[46]	2009–2012
Australia	Australia	0.1	[43]	2013–2017
Europe	France	0.1	[47]	2005–2007
	Norway	0.1	[48]	2015–2016
	Romania	0.04	[49]	2017
Latin America	Argentina	0.17	[50]	2014–2017
	Colombia	0.2	[51]	2005–2017
North America	Canada	0.1	[43]	1997–2006
	Mexico	0.1	[43]	2010–2013

*Refers to total number of patients.

Diagnosis and management of mucormycosis

Besides diagnostic tools on the bedside like computerized tomography (CT) scan, the positron emission tomography-computed tomography (PET/CT) with [18F]-fluorodeoxyglucose (FDG), common tools on the bench side like microscopy (direct and histopathology) and culture of various clinical specimens, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and serological tests (enzyme-linked immunosorbent [ELISA] assays, immunoblots, and immunodiffusion tests), polymerase chain reaction (PCR), DNA sequencing of targeted gene regions, restriction fragment length polymorphism (RFLP), and melt curve analysis of PCR products are considered to be the most convenient, easily accessible, and reliable tools in the diagnostic laboratory.[52] Quantitative PCR (qPCR) was proven as a valuable diagnostic tool for detecting mucoralean fungi in pulmonary mucormycosis caused by *Lichtheimia*, *Mucor*, *Rhizopus*, and *Rhizomucor* spp.[53] Large subunit (LSU) was applied rather than small subunit (SSU) to discriminate Zygomycetes species. While *in vitro*

molecular detection is solved, it remains still difficult and tedious in patient samples.[8,54] Genes encoding spore coat proteins (CotH), that are species representative and specific for Mucorales, were used as a diagnostic targets for the molecular detection of mucormycosis.[55] The *cotH* genes were successfully amplified from biological fluids like urine, plasma, and bronchoalveolar lavage retrieved from mice infected with mucoralean species, while mock-infected mice or mice infected with other pathogens (e.g., *Aspergillus fumigatus*) remained true negative.[55] Amplification of *cotH* by PCR from urine samples was accurate compared to PCR from other biological fluids and is a reliable, easy, and rapid tool for detecting mucormycosis in patients.[55] Extracellular vesicles (ERs) comprising exosomes and microvesicles, which contain various biological materials like proteins and lipids, are considered as well as targets for diagnosis and treatment.[56] Recently, *R. delemar* was found to secrete ERs with small RNAs displaying various pathways important for growth and metabolic biosynthesis.[57] This new discovery has the potential to be applied as a novel method for detecting mucormycosis. Apart from infectious diseases, mucoralean fungi are able to cause noninfectious disease that is commonly known as farmer lung disease (FLD).[58] During FLD, the production of interleukin (IL)-8 as marker regulator of the acute inflammatory response is upregulated.[58] FLD originates from persistent inhalation of spores from agricultural products (like hay, straw, chaff, etc.) that causes hypersensitivity pneumonitis. *L. corymbifera* is the most prominent cause of FLD.[58] Confrontation (blotting) of *L. corymbifera* proteins with the serum from patients having FLD and healthy exposed control revealed that dihydrolipoyl dehydrogenase was the most effective recombinant antigen to diagnose FLD with ELISA resulting to sensitivity and specificity rates of 81.4% and 77.3%, respectively.[60] The Western blotting technique was the method of choice to detect the FLD in laboratories without sensitive instruments with a reasonable proportion of accuracy.[60]

Due to multiple sites of infection in the host, various species causing the disease, the different duration required for therapy, the treatment of mucormycosis is difficult and implements surgery as the central method for the control of mucormycosis.[61,62] Management depends desperately on multiple strategies like removing the infected tissue or partially infected organs, the administration of a suitable dose of antifungal agents, and the utilization of different adjunctive therapies.[52] The measurement of *in vitro* susceptibilities of 66 strains to various antifungal agents (caspofungin, amphotericin B, terbinafine, posaconazole, voriconazole, itraconazole, micafungin, and 5-fluorocytosine) revealed a taxon-specific pattern, which is in concordance to the phylogenetic divergence among the Mucorales.[52] Liposomal Amphotericin B was the first choice for the management of mucormycosis because it possessed the highest effect for combating most of the species causing mucormycosis but appeared to be less effective against *Cunninghamella* and *Rhizopus* spp. Posaconazole was found to be the second effective antifungal agent against mucormycosis; however, it did not influence the activity of *Cunninghamella* and *Mucor* spp. Novel members of the azole group like posaconazole, itraconazole, and terbinafine were accounted for key players in the eradication of *Lichtheimia* and *Syncephalastrum* spp., while *Cunninghamella* spp. remained resistant to posaconazole and itraconazole but susceptible to terbinafine. *R. arrhizus* and *M. circinelloides* were resistant to the ergosterol synthesis inhibiting azoles and all mucoralean species had the resistance against caspofungin, 5fluorocytosine, and micafungin in common.[63] The *CYP51* gene encoding the lanosterol 14-alpha-demethylase was attributed to the intrinsic voriconazole and fluconazole resistance in *R. oryzae*.[64] Natural products specifically from plants count for promising alternatives to reduce the side effects of chemotherapeutic compounds. In particular, oil (Calli oil) extracted from the Fire Bush *Calligonum comosum* had significant antimicrobial activity against *Rhizopus* spp. at 250 μg /ml and converted the color of the dark spores to irreversible white.[65]

Interaction of the Mucorales with the immune system

Several studies investigated the interaction between the most abundant species causing mucormycosis and immune cells. We will summarize these interactions as follows:

The bronchial alveolar macrophages

The bronchial alveolar macrophages are one of the most important attributes of the innate immune system by providing the first line of defense.[66] *R. oryzae* caused higher mortality rate in diabetic mice than *A. fumigatus* but did not have any effect on virulence in non-diabetic, healthy mice.[67] In healthy mice, the germination of spores of *R. oryzae* was inhibited within bronchial alveolar macrophages, while the spore germination was enhanced in diabetic mice.[67] In comparison, *A. fumigatus* was killed in the early stage of phagocytosis by alveolar macrophages.[68] In immune suppressive mice, alveolar macrophages could neither inhibit the germination nor kill the spores of both, *R. oryzae* and *A. fumigatus*.[68] The oxidation of L-arginine to nitrite was found to be the key player for the inhibition of the germination of *R. oryzae* spores in murine alveolar macrophages, whereas gamma interferon and endotoxin were essential for preventing the germination of *R. oryzae* spores in human macrophages.[69] Opsonization of spores enhanced the phagocytosis ratio of *L. corymbifera* spores by cell culture murine alveolar macrophages compared with resting and swollen spores.[70] Alveolar macrophages were postulated to serve as a shuttle to transfer spores of *L. corymbifera* to other organs.[70] The composition of the spore wall appears to determine the recognition and the intracellular killing by phagocytes. *Rhizopus* spp. inhibit the phagosome maturation of mice alveolar

macrophages by the presence of melanin on the spore surface and by iron metabolism playing a key role in regulating the immune defense.[71]

Epithelial cells

Epithelial cells surround the outer surface of the skin and alveoli and provide therefore the first line of contact with the fungal pathogens.[72] Mucoralean fungi typically damage epithelial cells with equal ratios without any significant differences.[73] Transcriptomic analysis of the interaction of *L. corymbifera, R. oryzae, R. delemar,* and *C. bertholletiae* with cell culture human epithelial cells (A-549) showed that platelet-derived growth factor receptor B (PDGFRB) signaling was the main response of A-549 during infection with Mucorales. Blocking of PDGFRB reduced the damage of A-549 caused by mucoralean fungi.[73] *In vitro* studies on polymeric material revealed that mucoralean spores equally attached to extracellular matrices—like laminin or type IV collagen—under depletion and supplementation of carbohydrates, but the attachment failed under addition of fibronectin or glycosaminoglycans.[74] This result proposed that lectins do not play a key role in the interaction of mucoralean fungi with immune cells and suggested adherence of the spores to the epithelial basement membrane.[74] Transcriptomic analysis revealed that the epidermal growth factor receptor (EGFR) is upregulated during the interaction of *R. arrhizus* var. *delemar* with epithelial cells of the lung.[75] Upon infection with the spores, EGFR was phosphorylated on the surface of epithelial cells and was found to be co-localized with the spore surface. Moreover, the EGFR inhibitors cetuximab or gefitinib diminished *in vitro* the capability to invade and to cause damage to epithelial cells, abolished the fungal burden, and attenuated the virulence of *R. arrhizus in vivo*.[75]

Polymorphonuclear leukocytes (PMNs) or neutrophil granulocytes

Polymorphonuclear leukocytes (PMNs) or neutrophil granulocytes are an important part of the innate immune system and participate in the regulation of adaptive immune system.[76] Neutrophils play a key role in combating the pathogen by the production of cytokines and the formation of neutrophil extracellular traps (NETs).[77] Chemotactic factors may have an effect on the inflammatory response of neutrophils to Mucorales pathogens.[78] These factors were decreased during ketoacidosis and hyperglycemia leading to reduced killing of fungal hyphae of *R. oryzae* by human neutrophils'.[78] Swollen spores enhance the neutrophils to produce chemotaxis but not resting (dormant) spores and the hyphae reduced the production of neutrophils chemotaxis.[79] The total neutrophils intracellular and extracellular production of superoxide anion ($O2^-$) was increased upon contact with hyphae of mucoralean species but was less compared to *A. fumigatus*.[80] The hyphae of *Rhizopus* spp. activate the Toll-like receptor TLR2 and proinflammatory genes as IL-1B and tumor necrosis factor-alpha (TNFα or TNFA) in neutrophils.[80] The rate of the mortality of neutropenic patient's suffering from mucormycosis increased and only voriconazole is available as prophylaxis until now.[81] Investigating the role of TLRs in combination with liposomal amphotericin B (L-AmB; AmBisome) in the interaction of neutrophils with spores showed that neutrophils reduced of the proinflammatory response by turning TLR signaling pathways from TLR-2 to TLR-4 which highlights the capability of TLR to increase the microbicidal activity of neutrophils without cytotoxicity effect.[82] Interferon-gamma (IFNγ) and granulocyte-macrophage-colony-stimulating factor (GM-CSF) alone or in combination stimulated the neutrophils to release the TNFα leading to hyphal damage and eradication of mucormycosis.[83] IFNγ reduced the release of IL-8 by neutrophils during their contact with mucoralean fungi.[83]

T cells

T cells are part of the adaptive immune system. Antigen-specific T cells count for promising diagnostic tools to control infectious diseases, especially mucormycosis.[84] Mucorales-specific T-cells were found only in patients who suffered from mucormycosis but not in other patients that produced interleukins (IL- 4, IL-10, and IL-17) and IFNγ.[84] The cytokines prompt hyphal damage of Mucorales fungi.[84] Treating T- inactivated cells with IL-2, IL-7, or both cytokines enhances the production of Mucorales-specific T cells and their cytokines IL-5, IL-10, IL-13, and also stimulates the production of CD4+ T cells that are a specific Mucorales antigens.[85] This new immune therapeutic method is a promising way to increase the response of T cells against mucormycosis.[85]

Natural killer (NK) cells

Natural killer (NK) cells are lymphocyte cells contributing to the immune defense against infected pathogens by reducing dissemination.[86] NK cells express various receptors that can recognize infected cells and inhibit major histocompatibility complex (MHC) class I molecules that inhibit the activation of the receptors.[87] These cells have shared interfaces with various immune cells in the innate immune system like macrophages and dendritic cells.[86] The hyphae of mucoralean fungi were damaged by stimulated or unstimulated NK cells, but the NK cells did not cause any damage to resting (dormant) spores.[88,89] The protein perforin that is produced by NK cells has similar structure and function to complement system, contributes to hyphal damage of mucoralean species.[88,89] The hyphae reduced the release of various immunomodulatory molecules as RANTES (regulated upon activation, normal T -cell expressed and secreted) and IFNγ secreted by NK cells.[88,89] This damage is fungal growth dependent and appears to be more effective in the early stage of infection.[89]

Platelets

Platelets play a key role not only in hemostasis but also in recognition and killing of pathogens.[90] Platelets adhere to spores and hyphae of mucoralean fungi but cause damage only to the hyphal structure.[91] Moreover, the hyphal growth was diminished in response to contact with platelets.[91]

Endothelial cells

Endothelial cells make up the internal layer of blood vessels and possess various important roles in pathogen recognition and maintaining physiological functions.[92] Endothelial cells are able to phagocytose and to damage the spores of mucoralean fungi.[93] The glucose-regulated protein 78 (GRP78) is a receptor present on the surface of endothelial cells and can specifically recognize *Mucor* spp. but not other fungal pathogens like *A. fumigatus*.[93] In addition, GRP78 mediates invasion and damage of endothelial cells by mucoralean fungi.[93] Increasing the concentration of iron and glucose in diabetic ketoacidosis mice resulted in enhancement of the expression of GRP78 on the surface of endothelial cells especially in brain, lung, and sinus compared with normal mice.[93] Immunochemistry investigation of the ethmoidal sinus tissue infected with mucoralean fungi revealed that GRP78 was highly expressed on endothelial cells and was significantly accessible on the surface of macrophages on necrotic tissue.[94]

Dendritic cells (DCs)

Dendritic cells (DCs) are considered as a linker between the innate immunity and the adaptive immunity as they can be found between the epithelium and the interstitium.[95] DCs usually move to the site of infection in response to the release of microbial antigens that enhance the immune response.[95] The resting (dormant) spores and germ tubes of mucoralean fungi stimulate the maturation of DCs; in contrast, resting spores of *A. fumigatus* do not influence the maturation of DCs.[96]

Virulence of the Mucorales in infection models

A wide variety of murine infection models are available for the study of mucormycoses.[97] However, due to increasing conflicts with the animal protection legacies, there is an increasing demand for alternative infection models.[98,99] Several studies inspected the virulence of *Mucor* species in various infection models (invertebrate and vertebrate) that we will briefly discuss in the following paragraph.

The fruit fly (*Drosophila melanogaster)* is applicable for genetic modification and has its innate immune system that is similar to a human immune system, in particular with respect to the Toll pathway.[100] Injection of flies of *D. melanogaster* with various strains of the Mucorales revealed that Toll knock-out genes flies were more vulnerable to be killed by mucoralean fungi than the wild-type of *D. melanogaster*.[100] Overexpression of Dromycin in *D. melanogaster*—which is considered to be an important weapon of *D. melanogaster* for killing pathogens—diminished the rate of mortality caused by Mucorales.[100] Treating *D. melanogaster* with the iron chelator deferasirox reduced significantly the virulence of the Mucorales in *D. melanogaster* by induction of iron starvation.[100] Transcriptomics analysis of *D. melanogaster* flies infected with Mucorales revealed downregulation of the genes responsible for immune response, pathogen recognition and stress response compared with uninfected flies.[100]

The greater wax moth or honeycomb moth (*Galleria mellonella*) was proven to be a promising model for testing the virulence of various species because it is easier to implement in the lab without prerequisites to expert, special instrument or ethical permission for carrying on the experiment.[101] Additionally, its innate immune system resembles the innate immune system of vertebrates; however, it lacks an adaptive immune system.[101] The most important and clinically relevant species of the genera *Rhizopus*, *Rhizomucor*, *Lichtheimia*, and *Mucor* were tested in *G. mellonella* with various doses, different incubation times, and temperature.[102] The difference in the virulence potentials correlates with spore, infection dose, iron availability, and the oxidative stress for the fungus.[102] Studying the efficacy of various antifungal agents in *G. mellonella* toward successful management of mucormycosis demonstrated that Nystatin-intralipid had a strong influence on the performance of mucormycosis in contrast to posaconazole.[102] Clinically relevant strains of *Rhizopus* spp. that are thermophilic are more virulent than nonclinical strains that are known as mesophilic species; nonetheless, nutritional and stress factor did not have an impact on the virulence of *Rhizopus* spp. in *G. mellonella*.[103]

The zebrafish *(Danio rerio)* counts as alternative infection model because it takes advantage of the availability of its full genome information, of several commercial kits for gene microarrays and a genetic manipulation system.[104] Injecting larval zebrafish with *M. circinelloides* showed that the site of infection (hindbrain ventricle or swim bladder) is one of the major players in the susceptibility of zebrafish to *M. circinelloides*.[98] Two important phagocytes of the innate immune system (macrophages and neutrophils) are directed toward the site of infection explaining the distribution of spores in the whole body of the zebrafish.[98] Additionally, the spores of *M. circinelloides* were not successful in enhancing the proinflammatory response.[98] Recently, adult zebrafish could clarify the relationship between the size of sporangiospores and virulence in *Mucor circinelloides*. Live spores were able to germinate and to invade the tissue resulting in enhanced inflammatory response in adult zebrafish. On the contrary, the UV-killed spores did not cause any action in zebrafish. Transcriptomic analysis addressed that a total of 857 genes were modulated in adult zebrafish during the interaction with *Mucor circinelloides* as genes encoding peptidoglycan

recognition proteins, cytokines, complement factors, and iron acquisition.[105]

The embryonated hen egg *(Gallus gallus)* is promising for an infection model that is at the interface between the insect and mammalian models.[99] The embryonated hen egg model contributed to monitor the virulence among clinical and environmental strains and species in *Lichtheimia*.[99,106] Out of a total of six species of the genus *Lichtheimia*, three were found to be clinically relevant; *L. corymbifera, L. ramosa, L. ornata*. Among those species, 55% of the investigated strains turned out to be attenuated.

Virulence factors of the Mucorales

Human pathogens cause disease in the host based on two steps: (1) capability of infecting microorganisms to evade the immune system and surviving inside the host, and (2) perturbation of the immune system and causing damage to the host cells.[107] Virulence factors of the pathogens play a key role to accomplish the damage process.[107] In our review, we will summarize the previous studies that addressed several virulence factors of the Mucorales that are well known and contribute to the immune invasion. While tolerance to physiological stressors (high temperature, osmolarity, hypoxia) and the ability for dimorphic growth resemble among the general virulence factors equally valid for fungal infections. We highlight in the followings virulence factors which are specific for the Mucorales:

High-affinity iron permease *(FTR1)* has a role in iron uptake and transport in Mucorales fungi especially during the lack of iron in the environment.[108] The *FTR1* gene is highly expressed during the infection of immunodeficient mice with *R. oryzae*.[110]Moreover, knock-down of *FTR1* reduces virulence and iron uptake in *R. oryzae*.[108] Passive immunization of infected mice with an antibody against *FTR1* protein diminishes the rate of mortality and enhances the survival rate.[108] Fob1 and Fob2 proteins are receptors for iron uptake on the surface of the Mucorales are highly expressed in presence of ferrioxamine that mediates iron transfer through the reductase/permease system without interfering of siderophore-iron complex into the fungal cells.[109] These receptors have a key role in the pathogenesis of *R. oryzae* in mice treated with the iron chelator deferoxamine.[109] *M. circinelloides* harbors ferroxidases that are important for iron acquisition, virulence and fungal dimorphism.[110]

Spore coat (CotH) protein is another virulence factor that detected universally on the spore surface of all Mucorales but not on any other species like *A. fumigatus*.[111] CotH plays a key role as invasins in the pathogenesis of mucormycosis, disrupts and damages immune cells.[111] CotH was found to be a Mucorales-specific ligand of the GRP78 receptor.[111]

Alkaline *Rhizopus* protease enzyme (Arp) was detected in the culture filtrate of a clinical isolate of *R. microsporus* var. *rhizopodiformis* that had a role in enhancing the coagulation process in patients suffering from mucormycosis.[112] Genome expansion and phenotypic studies for virulent and avirulent strains of *M. circinelloides* (CBS277.49 and NRRL3631, respectively) found several absent, discontiguous and truncated genes in the avirulent strain compared with the virulent strain; however, tolerance to heat and to cell wall stress (calcofluor white and sodium dodecyl sulphate) was lower in the avirulent compared with the virulent strain. These observations address the importance of cell wall components as virulence factors.[113] Knock-out of gene ID112092 coding an extracellular protein reduced the pathogenesis of *M. circinelloides in vivo* confirming the importance of surface proteins as virulence factors of the Mucorales.[113]

ADP-ribosylation factor (Arf) is a virulence factor that is necessary for growth, fungal dimorphism and virulence in *M. circinelloides*.[114] A recent study confirmed the lack of a layer composed by rodlet hydrophobins (which is an important virulence factor in *A. fumigatus*) on the spore surface of Mucorales, which excludes a rodlet-like layer as a virulence factor for the Mucorales.[96]

Dihydrolipoyl dehydrogenase was identified as the most abundant antigen in the serum of patients suffered from FLD compared to healthy donors.[59]

Calcineurin (CaN) is a calcium and a calmodulin-dependent serine/threonine protein phosphatase and serves as a critical factor in the virulence of Mucorales, because it has a tangible role in the transition from yeast to hyphae in *M. circinelloides*.[115,116] Phagosomal maturation was impaired when spores were phagocytosed by macrophages, whereas yeast cells were not able to inhibit phagolysosomal maturation. Calcineurin is closely linked to protein kinase A activity (PKA). Both are necessary for the pathogenesis of *M. circinelloides*.[115,116] Disturbance of the Calcineurin-encoding gene resulted in the production of larger spores compared with the wild-type by expressing a spore size-dependent role in the virulence. Genome mining in *L. corymbifera* revealed that about 3.3% of the whole genome was predicted to encode secreted proteases that are indispensable for virulence.[117]

Serine and aspartate proteases (SAPs) were the most common candidates of the secreted proteases in the genome of *L. corymbifera* with 55% and 36% of the total predicted proteins, respectively.[117] Moreover, expansion of the genome of *L. corymbifera* leads to 768 transcription factors that constitute 6.2% of the total genome. 4.8% of the transcription factors are specific only to *L. corymbifera* and were not detected in other fungi before.[117] Heat shock transcription factors are predominant in the genome of *L.corymbifera* compared with other fungal pathogens, a fact that might explain the thermotolerance of Mucorales species.[117] Interestingly, genome mining in *Apophysomyces variabilis* predicted about 6% of the total genes to be related to known virulence factors, such as CotH proteins, hydrolytic enzymes like serine proteases and components of iron uptake pathway.[118] Additionally, the carbohydrate active enzymes (CAZymes)

represented the majority of the secreted proteins which may possibly play role in the interaction with the environment and the host.[118]

Mucormycosis is one of the most invasive mycoses caused by filamentous fungi in several organs in patients undergoing weakness in the arsenal of immune defense. In our review, we addressed the underestimation, the severity, and the emergence of mucormycosis. Progress was partially achieved in the detection of ligands and receptors of host-pathogen interaction, but monitoring, reliable diagnosis, and therapy remain still unsatisfactory. The present mini-review attempts to increase the awareness of these difficult-to-manage fungal infections.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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