Published in final edited form as:

Mycoses. 2014 December ; 57(0 3): 2–7. doi:10.1111/myc.12249.

Development of new strategies for early diagnosis of mucormycosis from bench to bedside

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Summary

Early diagnosis and initiation of amphotericin B (AmB) for treatment of mucormycosis increases survival from approximately 40% to 80%. The central objective of a new study of the European

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Presented in part at 'Emerging Zygomycetes, a New Problem in the Clinical Lab, a Workshop of the ECMM/ISHAM Working Group on Zygomycetes'. Utrecht, the Netherlands, April 8, 2013.

Disclosures

None

Confederation of Medical Mycology (ECMM) and the International Society for Human and Animal Mycology (ISHAM) Zygomycosis Working Group is to improve the clinical and laboratory diagnosis of mucormycosis. The diagnostic tools generated from this study may help to significantly improve survival from mucormycosis worldwide. The study has three major objectives: to conduct a prospective international registration of patients with mucormycosis using a well-established global network of centres; to construct a predictive risk model for patients at risk for mucormycosis; and to establish an international archive of specimens of tissues, fluids, and organisms linked from the patients enrolled into the registry that will be used for development of leading edge molecular, proteomic, metabolic and antigenic systems for mucormycosis.

Keywords

mucormycosis; zygomycosis; diagnosis; network; epidemiology

Introduction

Mucormycosis is an important emerging fungal infection, associated with high morbidity and mortality.¹⁻⁴ The recent Schueler Foundation Symposium conducted in Chicago, Illinois in the United States underscored the suffering, tragedy and challenges of mucormycosis through a comprehensive series of papers on its epidemiology, pathogenesis, clinical manifestations, diagnosis and treatment.⁵ The symposium underscored the need for new advances in diagnosis, treatment and prevention as the key to improving survival.

The Working Group on Zygomycosis (ZWG) of the European Confederation of Medical Mycology (ECMM) successfully completed its first study, to analyse prospectively collected cases of proven and probable zygomycosis in 13 European countries occurring between 2005 and 2007. During the study period, 230 cases fulfilled preset criteria for eligibility.⁶ The median age of the patients was 50 years (range, 1 month to 87 years); 60% were men. Underlying conditions included haematological malignancies (44%), trauma (15%), hematopoietic stem cell transplantation (HSCT) (9%) and diabetes mellitus (9%). The most common manifestations of zygomycosis were pulmonary (30%), rhinocerebral (27%), soft tissue (26%) and disseminated disease (15%). Diagnosis was made by both histology and culture in 108 cases (44%). Among 172 cases with cultures, *Rhizopus* spp. (34%), *Mucor* spp. (19%) and *Lichtheimia corymbifera* (19%) were most commonly identified. Thirty-nine per cent of patients received AmB formulations, 7% posaconazole and 21% received both agents; 15% of patients received no antifungal therapy. Total mortality in the entire cohort was 47%. On multivariate analysis, factors associated with survival were trauma as an underlying condition ($P = 0.019$), treatment with AmB ($P = 0.006$) and surgery ($P < 0.001$); factors associated with death were higher age ($P = 0.005$) and the administration of caspofungin prior to diagnosis ($P = 0.011$). The study concluded that zygomycosis is a highly lethal disease but that administration of AmB and surgery, where feasible, significantly improved survival. Unfortunately, mortality and morbidity remain devastatingly high from zygomycosis.

Consistent with the importance of early diagnosis, as with all well designed studies, the completion of the first ZWG study led to new questions that are important for the outcome of patients suffering from mucormycosis. How can we improve early clinical diagnosis of mucormycosis? How can we improve the rapid laboratory diagnosis of mucormycosis? What is the incidence of mucormycosis in selected populations? These questions then led to formulation of the objectives for the second protocol of the Zygomycosis Working Group.

Objectives for the Second ZWG Protocol (ZWG2)

Amphotericin B remains the gold standard of therapy of mucormycosis. Posaconazole also has some activity against the agents of mucormycosis. However, overall outcome of mucormycosis remains poor despite the availability of these agents.

In the absence of a major conceptual breakthrough of therapeutic intervention, early diagnosis will likely have the greatest impact in improving survival and outcome. The most effective means by which to improve early diagnosis followed by prompt initiation of antifungal therapy is through (i) early clinical recognition and (ii) development of advanced laboratory diagnostic tools.⁷

Early diagnosis and rapid initiation of antifungal therapy is a cornerstone of successful treatment of invasive fungal infections. Early treatment of invasive mucormycosis may attenuate angioinvasion and prevent direct tissue injury of the respiratory tract. Early intervention may prevent direct extension from lung into great vessels and reduce the probability of dissemination. Early initiation of antifungal therapy also may reduce the need or extent of debilitating and disfiguring surgical resection. Early diagnosis and initiation of antifungal therapy ultimately improves outcome and survival.

Underscoring this key principle of the importance of early diagnosis and initiation of antifungal therapy, Chamilos *et al.* [8] demonstrated that early initiation of AmB in patients with mucormycosis and haematological malignancies improved survival by nearly 70%. In studying the impact of delaying effective AmB-based therapy on outcome among 70 consecutive patients with haematologic malignancy who had mucormycosis at the MD Anderson Cancer Center during the period 1989–2006, Chamilos *et al.* used classification and regression tree analysis to identify the mortality break-point between early and delayed treatment. They found that delaying AmB-based therapy by initiating treatment 6 days after diagnosis resulted in a twofold increase in mortality rate at 12 weeks after diagnosis, compared with early treatment (82.9% vs. 48.6%). This benefit remained constant across the years of the study and was an independent predictor of poor outcome (odds ratio, 8.1; 95% confidence interval, 1.7–38.2; $P = 0.008$) in multivariate analysis.

The new ZWG2 protocol will build upon the well-established registration format that is successfully utilised in the first study but will modify the database to include more greatly detailed information to address the new study objectives.⁶ Formulation and implementation of these objectives will position ZWG2 to be the definitive, leading edge, international, prospective, observational study of mucormycosis that will provide key advances: (i) most advanced known registry for studying mucormycosis; (ii) predictive risk-based bedside

model; and (iii) development of rapid diagnostic assays through a critical central archive of human specimens.

Objective I. Establish an advanced global registry of mucormycosis for cases and contemporaneous controls

The registry builds upon the existing database of the ECMM/ISHAM Working Group. The Working Group (WG) was initially formed under the auspices of ECMM, in 2004. The aims of the WG were to form a European registry, collecting cases of mucormycosis from various European countries. During the period 2005–2007, 230 cases were submitted from 13 countries.⁶ While this study and others studies have characterised risk factors for mortality in mucormycosis, there is no reported contemporary, international, case–controlled study of the epidemiological, metabolic and immunological risk factors for mucormycosis that would facilitate early clinical diagnosis.

The newly configured ZWG2 markedly expands the number of participating centres and countries and is now known as the ECMM/ISHAM WG. The database will be migrated to the auspices of the Infection Control Program at ELPIDA in Athens, Greece. The portal for remote data entry will remain <http://www.zygomycosis.net/>. For the first time, infected patients and two contemporaneous case–controls will be included prospectively. Prognostic variables will also be built into the new database for infected patients and non-infected controls. The database will now include multiple expanded and risk variables with high levels of quantitative refinements summarised in Table 1.

The new database will establish for the first time an international profile for the epidemiology, clinical manifestations, risk factors and outcome of mucormycosis. Denominators will be established for select groups of underlying conditions, particularly leukaemia and allogeneic HSCT in order to provide a marker for incidence. These two populations are most readily tracked in institutions.

All participating investigators will enrol infected patients and two contemporaneous controls who will be followed through the duration of treatment and for 6 month follow-up for a total duration of 1 year, whichever is shorter.

All cases of mucormycosis entered through Fungiscope (<http://www.fungiquiest.net/>) will be shared with the ZWG2 study. Concurrent untreated controls will be identified for these cases by the investigator enrolling the patient with mucormycosis.

Objective II. Development of a bedside risk-based model for development of mucormycosis

Recognition of possible host factors

Early identification of host factors is an important strategy for assessment of the Bayesian prior probability of a patient's risk for invasive mucormycosis. The classic host factors for mucormycosis are diabetic ketoacidosis and profound and persistent neutropenia. However, not all patients with diabetic ketoacidosis or profound and persistent neutropenia develop

mucormycosis. Additional data are required to understand risk factors in these populations. Moreover, other host groups, including those with allogeneic HSCT, type 2 diabetes, low birth weight infants, burns and trauma, solid organ transplantation, autoimmune disorders and illicit intravenous drug use are also at risk (Table 2).

Recognition of clinical manifestations

Identification of certain clinical manifestations in association with risk factors may further refine early diagnostic accuracy and predictive power. There are several examples of early clinical manifestations of invasive mucormycosis that carry a potentially high predictive value in appropriate host.⁹ A necrotic eschar in maxillary, facial, or sino-orbital mucosal surfaces in an immunocompromised host may be an early sentinel marker of invasive mucormycosis. Pleuritic pain in a neutropenic host also may signify an angioinvasive filamentous fungus. Pleuritic pain in a neutropenic or HSCT patient receiving voriconazole prophylaxis has a high probability of being invasive mucormycosis instead of aspergillosis.

Diplopia is an early manifestation of sino-orbital mucormycosis in a diabetic patient that usually signifies involvement of the extraocular muscles or their innervating nerves.¹⁰ Hyperglycaemia in diabetic patients may produce blurring of vision, but does not produce diplopia. During sino-orbital mucormycosis, hyphae involving the ethmoid sinus breach the lamina papyracea to invade the medial rectus muscle creating dysconjugate vision. The organism may extend along the emissary veins to the ethmoid sinus to the cavernous sinus and encroach upon the critical cranial nerves involve III, IV, V (1, 2) and VI. Diplopia in a diabetic patient or other compromised host with ethmoidal sinusitis should be assessed aggressively for sino-orbital mucormycosis.

Necrotic cutaneous lesions in immunocompromised patients may also be caused by mucormycosis. The differential diagnosis includes other angioinvasive pathogens including *Aspergillus*, *Fusarium*, *Pseudallescheria*, *Scedosporium* species. *Pseudomonas aeruginosa* and occasionally members of Enterobacteriaceae in the same host also cause ecthyma gangrenosum. The preponderance of cases of cutaneous mucormycosis is associated with direct inoculation rather than haematogenous dissemination.¹ Characteristic hyphal structures are seen on biopsy and wet mount of tissue.

Recognition of diagnostic imaging

Earlier recognition of sinus and pulmonary lesions by CT scanning is an important advance over conventional sinus and chest radiographs. Early CT findings may reveal pulmonary or sinus lesions before localising symptoms in immunocompromised patients who are at high risk for invasive sino-pulmonary mucormycosis.

Among the lesions associated with angioinvasive filamentous fungi are nodules, halo signs, reverse halo signs, cavities, wedge-shaped infiltrates and pleural effusions associated with pleuritic pain.¹¹ Among these lesions, the reverse halo sign in the neutropenic patient has high predictive value for mucormycosis.¹² Early recognition of risk factors, clinical manifestations and diagnostic imaging findings may increase the probability of an early

recognition and lead logically to a definitive diagnosis by culture and biopsy of tissue or the use of novel molecular and antigenic assays.

A key objective of the prospectively collected observational data of patients with mucormycosis is to develop a predictive risk model that can be used for rapid bedside application and that will also increase the Bayesian positive predictive value of the newly developed rapid laboratory assays. The model will be robustly developed from a large database of multiple host factors, clinical manifestations, diagnostic imaging and antifungal agents. A risk scorecard will be developed that will allow physicians worldwide to identify patients who are at greatest risk for development of mucormycosis.

Categorical variables will be analysed by Fisher's exact test, and continuous variables by Mann–Whitney *U*-test. Logistic regression will be used to identify variables independently associated with development of mucormycosis. Survival will be plotted by Kaplan–Meier analysis and analysed by Mantel–Haenszel chi-square (log rank test). A separate logistic regression model will be developed for mortality. All variables associated in the bivariate analysis will be included in the model at a threshold of $P < 0.1$. A stepwise logistic approach will be used to identify independent predictors of mortality. The final models will contain variables at the threshold of $P < 0.05$.

This prospective cohort study will use propensity-matched analyses to control for underlying comorbidities and prognostic imbalances in the determination of attributable mortality, length of stay and hospital charges associated with mucormycosis. Control patients will be matched 2 : 1 against diagnostic cases with control for age, gender and underlying disease process.

Objective III. Development of the International Mucormycosis Archive of Specimens (IMAS) for development of diagnostic assays and characterisation of organisms

Culture of the Mucorales

Identification of the Mucorales to the level of genus and species depends upon colonial morphology, microscopic morphology and growth temperature. Most medically important Mucorales are thermotolerant and grow rapidly at temperatures $> 37^{\circ}\text{C}$. Microscopic characterisation of non-septate hyphae, rhizoids, columellae, sporangia and sporangiospores help to define genus and species within the order Mucorales.¹³ *Rhizopus oryzae* is the most commonly reported single species.¹ Less common *Rhizopus* species include *Rhizopus rhizopodiformis* and *Rhizopus microsporus*. The genus *Mucor* is the second most commonly reported with *Mucor circinelloides* being the most common species. Less common species include *Cunninghamella bertholletiae*, *Apophysomyces elegans* and *L. corymbifera*.

Since identification of the Mucorales to the genus or species level carries important epidemiological, therapeutic and prognostic significance, accurate identification of the Mucorales is important. While *R. oryzae* is the most common organism among the Mucorales recovered from clinical specimens, it tends to have relatively high minimum

inhibitory concentrations (MICs) of posaconazole; whereas, *M. circinelloides* is less commonly isolated but more susceptible to posaconazole. *Cunninghamella* tends to have higher AmB MICs, relatively low posaconazole MICs, and a higher associated overall mortality compared to other species.

Microbiology—Essential to the study of the relationship between species and outcome is accurate identification of infecting organisms.

In order to optimise recovery of the Mucorales, clinical specimens should be inoculated onto appropriate media such as Sabouraud glucose agar and incubated at room temperature and at 37 °C. Tissue suspected of being infected with Mucorales should be minced into small pieces with a scalpel or single edge razor blade before inoculation onto media; grinding or homogenisation of tissue specimens may destroy the delicate hyphae rendering cultures negative. Colonies of Mucorales usually appear within 24–48 h unless residual antifungal agents, which can suppress growth. Most species demonstrate a greyish white, aerial mycelium with a wooly texture and fill a culture dish within 3–5 days.

This study will therefore utilise morphological, physiological and molecular methods for identification of organisms in culture and, where feasible, in paraffin-embedded tissue. Development of an archive of organisms recovered from patients with documented mucormycosis is essential to achieving objective III.

Rapid laboratory diagnostic assays—There are now several molecular and antigenic assays that detect the presence of Mucorales in laboratory animal models of mucormycosis.^{14,15} Other systems have not been studied in animal model systems but also exhibit analytical sensitivity and specificity for the Mucorales.^{16–19} Although one report describes the analytical performance of a three quantitative polymerase chain reaction assays using hydrolysis probes in 10 patients, the small number of cases and complexity of the molecular diagnostic platform limit regulatory review or extrapolation to other laboratories.²⁰

To enable candidate assays to become widely available for early diagnosis of mucormycosis and to improve patient outcome, an archive of specimens for mucormycosis is critically required. As these assays must be validated in human specimens of mucormycosis for scientific, clinical and regulatory acceptance, the development of this archive (IMAS) is critical.

This specimen archive will consist of the clinical samples (Table 3), where feasible and applicable, from each patient enrolled into ZWG2. Each investigator will store the specimens at his or her centre. At a designated time, specimens will be divided in equal amounts by the investigator and shipped to two central facilities under the care of Dr. Olivier Lortholary at the ZWG Archive Center in Paris and Dr. Thomas Walsh at the ZWG Archive Center in New York City. Storage in two geographically distinct locations assures preservation of specimens in the event of natural or human-made disasters. Following review of candidate assays, specimens will then be shipped to investigators conducting laboratory diagnostic projects approved by the ZWG Steering Committee.

Anticipated projects derived from the IMAS will consist of but are not limited to development of molecular, antigenic and other diagnostic assays that have been first validated in animal models, molecular identification of organisms recovered from clinical specimens, characterisation of the genetic diversity of species within the Mucorales, and correlation between molecular signal from BAL or serum with overall outcome, rate of therapeutic response, type of antifungal therapy and underlying host risk for development of mucormycosis.

Protecting patients from research risks

This study involves minimal to no risk to patients. All patient records would be anonymised. As all clinical specimens would be collected from samples that were drawn for standard clinical indications, no extra blood will be drawn. Since the study is not designed to assess for genetic risks, patient DNA will not be extracted.

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Table 1

Critical variables for expanded assessment of risk factors for development of mucormycosis.

Cycles and types of cytotoxic chemotherapy
Durations and depth of neutropenia
Dosages and duration of corticosteroids
Dosages and duration of immunomodulators
Type of solid organ transplantation
Dosages and duration of agents used to prevent or treat allograft rejection
Type of hematopoietic stem cell transplantation
Dosages and duration of agents used for conditioning regimen
Stages of graft versus host disease (GVHD)
Dosages and duration of agents used for treatment of GVHD
Parameters of glycaemic control over time
Parameters of iron metabolism

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Table 2

Categories for assessment of possible predictive risk factors.

Gender
Primary and secondary sites of infection at the time of diagnosis and whether the infection remains localised or disseminated
Disseminated infection
Cutaneous infection
Infection confined to the cutaneous or subcutaneous tissue (localised disease)
Invasion into muscle, tendon or bone (deep extension)
Patients with cutaneous disease involving another non-contiguous site (disseminated infection)
Pulmonary infection
Disease confined to the lungs (localised infection) and single vs. multiple lesions
Pulmonary disease that extended to the chest wall, pulmonary artery, aorta or heart
Sino-orbital involvement
Sino-pulmonary disease
Primary underlying condition
Types of immunosuppression or immune impairment
Diabetes mellitus (type and presence of ketoacidosis)
Neutropenic status
Genus, species of organism
Type of antifungal therapy
Timing of initiation of antifungal therapy
Previous antifungal therapy
Surgery
Hyperbaric oxygen therapy
Immunomodulatory agents

Table 3

Clinical specimens to be collected and stored for the International Mucormycosis Archive of Specimens for development of diagnostic assays and characterisation of organisms.¹

Organism recovered by culture
Tissues containing the organism
Serum obtained near the time of initial diagnosis
Bronchoalveolar lavage fluid containing the organism

¹ All human specimens will be excess from those obtained during routine clinical care. All specimens will be anonymised to patient identity. A central registry will be maintained in the same database through Zygomycos.net.