MMTN
MEDICAL MYCOLOGY TRAINING NETWORK

Identification of zygomycetes

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ASIA FUNGAL WORKING GROUP
an ISHAM working group

IDENTIFICATION OF MUCOROMYCOTINA (ZYGOMYCETES)

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Mucormycoses

- Emerging fungal infection caused by a group fungi called mucormycetes (zygomycetes)
- A life threatening infection
- An aggressive & highly destructive invasive fungal infection in immunocompromised patients

Ibrahim et al., CID 2012;54(S1):S16–22

Incidence: France 1997-2006

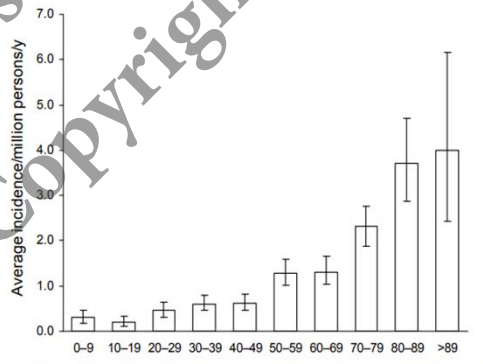


Figure 2. Average annual incidence rate of zygomycosis, by age group, France, 1997–2006. Error bars indicate 95% confidence intervals.

- 828 hospital, 531 incident cases were identified
- 283 males and 248 females (ratio 1:1);
- mean age: 57.1 years (median 60 years, range: <1 month–96 years).
- The annual incidence rate (AIR) increased from 0.7 cases/million persons in 1997 to 1.2/million persons in 2006
- yearly increase was +7.4% (p<0.001).

Bitar et al. Emerg Infect Dis. 2009; 5: 1395-1401

Five patients with sinusitis (only) & dissemination to adjacent tissue were diagnosed as mucormycoses.

Diabetes (adults), one patient with leukemia (pediatric) was infected with rhizopus

Jakarta, January – November , 2018

Sent from one hospital only

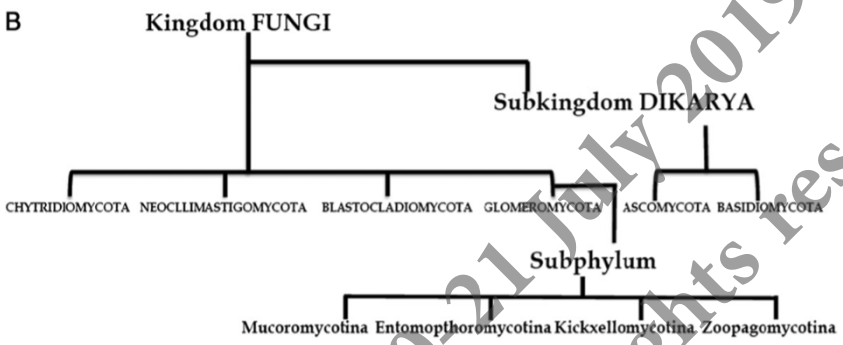
Data Dept. Parasitology, FKUI, 2018

Epidemiology: India

- a steady increase in the number of patients:
  - 129 cases over 10 years (13 cases/year during 1990–1999),
  - 178 cases over next 5 years (36 cases/year during 2000– 2004),
  - 75 cases over 18 months (50 cases / year during July 2006 - December 2007)
- Diabetes mellitus as major risk factors (65.1 million), ca. 70% are uncontrolled DM.
- Environmental factors: the tropical & sub-tropical humid climate & high environmental temperature accommodates the survival of the fungi,

Chakrabarti & Dhaliwal. Curr Fungal Infect Rep (2013) 7:287–92
Chakrabarti & Singh. Mycoses, 2014, 57 (Suppl. 3), 1–6

Taxonomy



Kyung J. Kwon-Chung CID 2012;54(S1):S8–1

The causes

- **Consisting of two important genera:**
  - **Entomophthoromycotina, a natural insect pathogens i.e.**
    - **Conidiobolus & Basidiobolus,**
    - **are found in tropical and subtropical regions**
    - **cause chronic subcutaneous infections in immunocompetent host**
  - **The Mucoromycotina: found worldwide as common saprobe in soil, recycling of organic materials, e.g. leaves, compost, rotten wood**
    - **invasive infection in immunocompromised host**

Hoffmann et al., Persoonia 2013; 30: 57–76

TABLE I. Classification of clinically relevant fungi formerly regarded as 'zygomycetes' [9,13]

Subphylum	Genus	Species most frequently isolated from patients
Mucormycotina	*Apophysomyces*	*A. variabilis*
	Cunninghamella	*C. bertholletiae*
	Lichtheimia (Absidia)	*L. corymbifera*
		L. ramosa
	Mucor	*M. circinelloides*
	Rhizopus	*R. arrhizus (oryzae)*
		R. microsporus
	Rhizomucor	*R. pusillus*
	Saksenaea	*S. vasiformis*
Entomophthoromycotina	*Basidiobolus*	*B. ranarum*
	Conidiobolus	*C. coronatus*

Binder et al. Clin Microbiol Infect 2014; 20 (Suppl. 6): 60–66

Mucormycoses: portal of entry

- **Inhalation of spores to the respiratory tract,**
- **injured skin or percutaneous route: inoculation of spores by contaminated needles or catheters**
- **ingestion of contaminated food.**

Binder et al. Clin Microbiol Infect 2014; 20 (Suppl. 6): 60–66

Classification of mucormyocses

Anatomic location	disease
Sinus & adjacent tissue	Rhino – orbito- cerebral
Lung	Pulmonary
Skin	Cutneous/subcutaneous
Gastrointestinal	Ingestion of contaminated food
Diseminated form	Dissemination from primary site
others	Bones, kidney, etc

Spellberg et al Clin Microbiol Rev 2005; 18: 556–69.
Marpaung et al.; J Penyakit Dalam Indonesia; 2018; 5.

Major risk factors

- uncontrolled diabetes mellitus (ketoacidosis)
- other forms of metabolic acidosis,
- Corticosteroids treatment
- organ & bone marrow transplantation
- neutropenia
- trauma & burns,
- malignant hematologic disorders,
- deferoxamine therapy in patients receiving hemodialysis

Chakrabarti & Dhaliwal Curr Fungal Infect Rep (2013) 7:287–92
Binder et al. Clin Microbiol Infect 2014; 20 (Suppl. 6): 60–66

TABLE 1. Relationship between predisposing condition and site of infection

Predisposing condition	Predominant site of infection
Diabetic ketoacidosis	Rhinocerebral
Neutropenia	Pulmonary and disseminated
Corticosteroids	Pulmonary, disseminated, or rhinocerebral
Deferoxamine	Disseminated
Malnutrition	Gastrointestinal
Trauma, catheter/injection site, skin maceration	Cutaneous/subcutaneous

Spellberg et al Clin Microbiol Rev 2005; 18: 556–69

Clinical presentation

- **Based on vascular invasion that causes thrombosis & tissue infarction/necrosis**
- **black eschar**
- **occurs in patients:**
  - **With defects in immune defense &/or with increased available serum iron,**
  - **Changes in their metabolism (DM- ketoacidosis)**
  - **Very rare in normal hosts**
- **most cases, are progressive infection & lethal, unless identified early & treated promptly**

Spellberg et al Clin Microbiol Rev 2005; 18: 556–69

DIAGNOSIS OF MUCORMYCOSIS

TABLE 3. Recommendations on diagnosis of mucormycosis: laboratory diagnosis using conventional, serological and molecular methods

Population	Intention	Method/Finding	SoR	QoE	References	Comment
Any	To diagnose mucormycosis	Direct microscopy preferably using optical brighteners	A	IIu	30,31	Allows rapid presumptive diagnosis; non-septate or pauci-septate, irregular, ribbon-like hyphae, angle of branching 45–90°, identification to genus and species level not possible, hyphal diameter in aspergillosis 2–3 μm, in mucormycosis 6 to >16 μm
Any	To diagnose	Culture	A	IIIr	32,35	Avoid grinding, preferred temperature 37°C
Any	To diagnose	Histopathology	A	IIu	7,26,36–38	Features as in direct microscopy, does not allow for genus or species differentiation; perineural invasion commonly seen, if nerves sampled
Any	To diagnose	Immunohistochemistry	C	IIu	39	No commercial assay available Monoclonal antibodies commercially available
Any	To diagnose	Galactomannan in blood or bronchoalveolar lavage	B	III	41 43 192	$n = 2$ $n = 1$ $n = 2/8$ missed mucormycoses: consider mucormycosis, if galactomannan test negative, but radiology positive
Any	To diagnose	1,3-β-D-glucan in blood	D	III	44,45	Not a reliable marker
Haematological malignancy	To monitor treatment	ELISPOT	C	IIu	46	No commercial assay available
Any	To diagnose	Molecular based tests on fresh clinical material	B	IIu	30,47,193,194	No commercial assay available; fresh material preferred over paraffin-embedded
Any	To diagnose	Molecular based tests on paraffin slides	B	IIu	48,49, 51	No commercial assay available

Cornely et al., Clin Microbiol Infect. 2014; 20 (suppl.3: 5-26

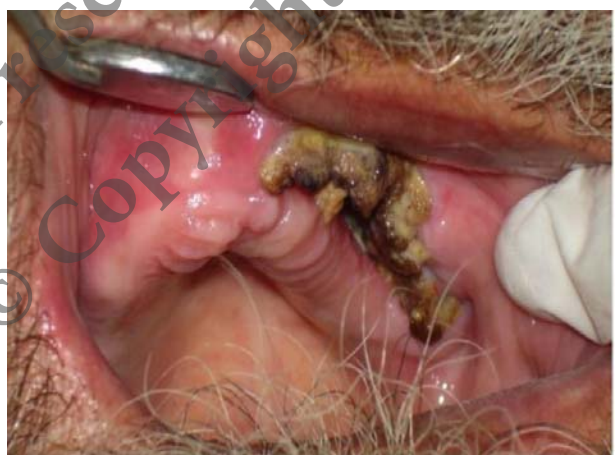
rhino cerebral mucormycosis in diabetic patient

Day - 1

Day - 2

Singh et al., BMJ Case Rep 2013.

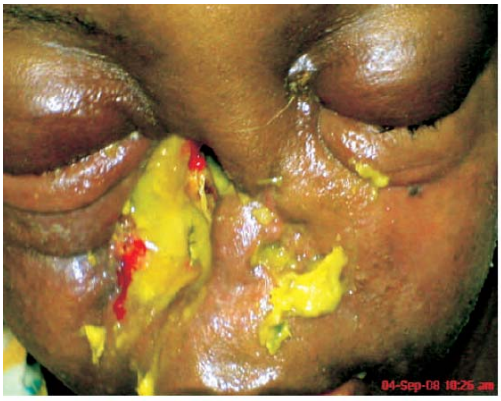
Rhinocerebral mucormycosis in diabetic patient

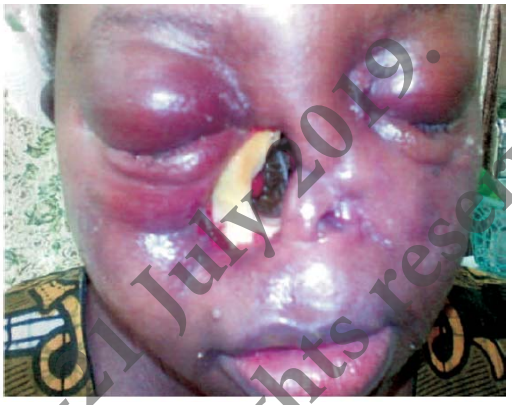


Intraoral photograph of rhinocerebral mucormycosis. Started by left maxilary region discoloration

Sahota et al. Ethiop J Health Sci. 2017; 27

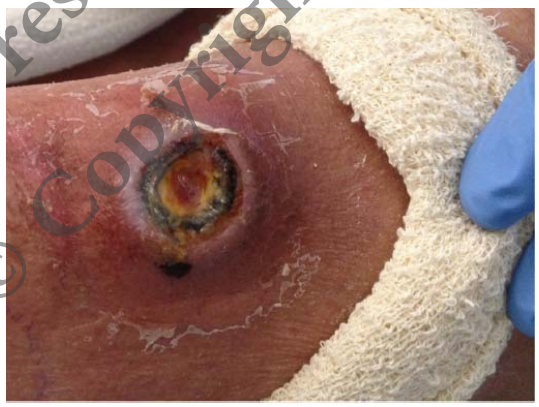
Rhino-cerebro-orbital mucormycosis in DM

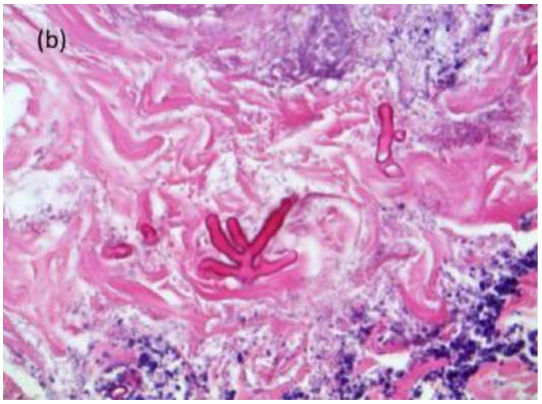




Oladeji et al. J West African Coll Surge 2013; 3: 93-100

Cutaneous mucormycosis





Gardiner et al., Med Mycol Case Reports 7(2015) 8–11

Mucormycoses in human tissue

- The amount of fungi that cause mucormycosis is very large but in human tissues they grow as coenocytic hyphae (septum is quite rare) that are similar to one another and rarely spores production
- The mucormycetes hyphae generally do not have septa, excessive manipulation of clinical material will cause leakage of the cells which results in fungal death & does not grow on culture
- Direct examination (KOH) is quite important in the identification of the disease

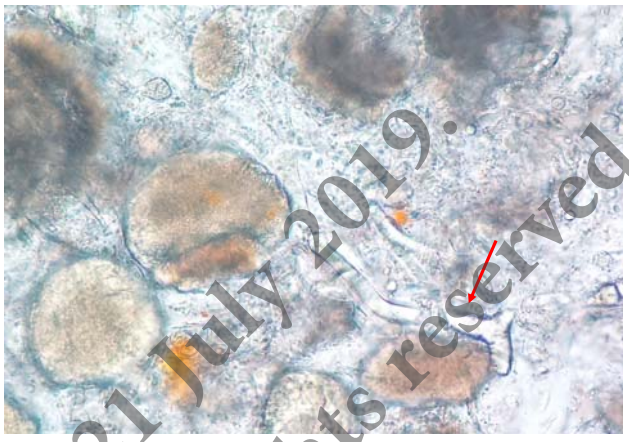
Direct examination: KOH wet slide

- Brain tissue, of a girl with tubular acidosis
- a coenocytic hyphae, no septum
- thick walled, refractile
- 400 ×magnification

Pic. Wahyuningsih, Dept. Parasitology FKUI; Case of Dr. Satari, Dept of Pediatric FKUI

KOH wet slide

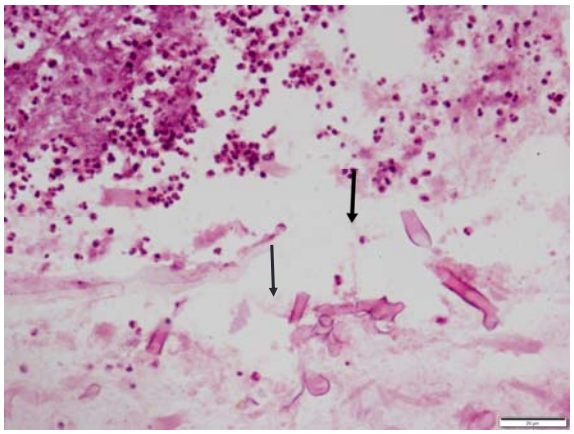
- Orbital tissue from a patient with rhino-orbito-cerebral mucormycoses
- Branched coenocytic (aseptate) hyphae among eye tissue
- 400 ×magnification



Pic. Wahyuningsih, Dept. Parasitology FKUI; Case of Dr. Satari, Dept of Pediatric FKUI

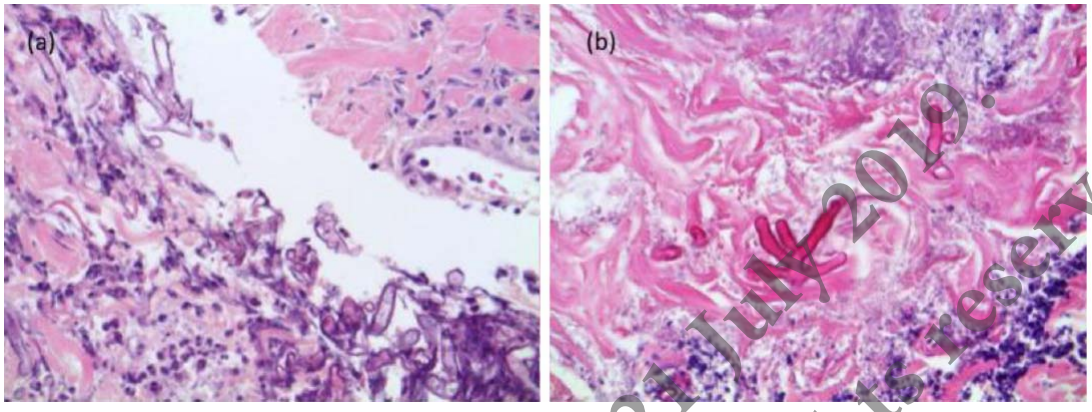
Histopathology

- Rhinocerebral mucormycoses
- HE staining
- Coenocytic hyphae (aseptate)
- Inflammatory cells



Pic. Wahyuningsih Dept. of Parasitology FKUI

Histopathology



HE staining (left) and PAS staining (right)

Gardiner et al., Med Mycol Case Reports7(2015)8–11

Calcofluor white

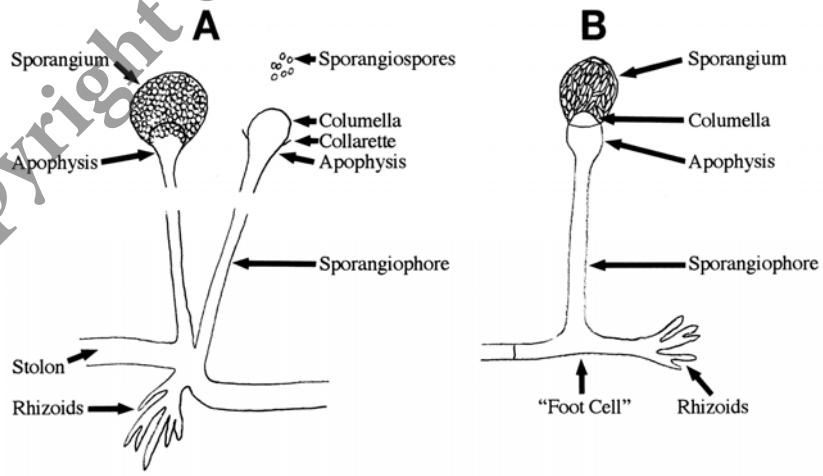
Direct microscopy using calcofluor white, a clear large hyphae

Lass-Florl. CMI. 2009; 15 (Suppl. 5), 60–6

CULTURE & PHENOTYPIC

Phenotypic identifications

Schematic diagram labeling the morphologic structures seen in the sporangium-producing Mucorales (not drawn to scale).

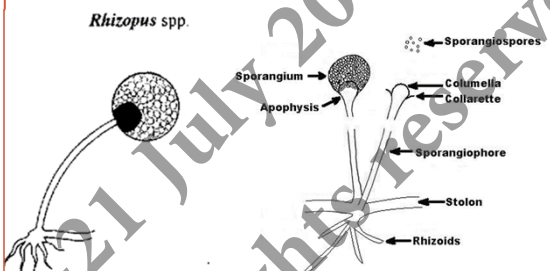


Julie A. Ribes et al. Clin. Microbiol. Rev. 2000; doi:10.1128/CMR.13.2.236

Rhizopus oryzae (most common cause)

Culture & microscopy

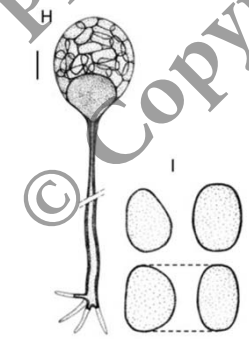
Schematic diagram

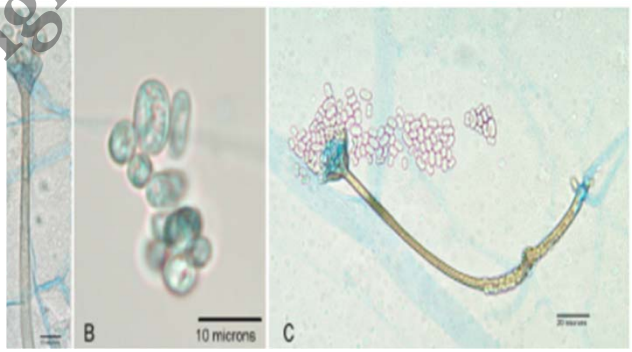


Bonifaz et al Clin Dermatol (2012; 30: 413-9
Thomas PA. CMR; 2003; 16: 730-97.

Apophysomyces variabilis

H. Sporangiphore
I. Sporangospore





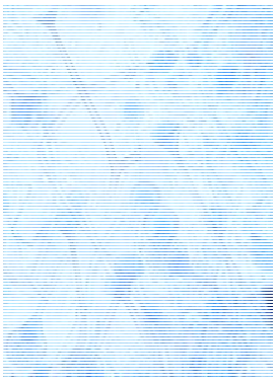


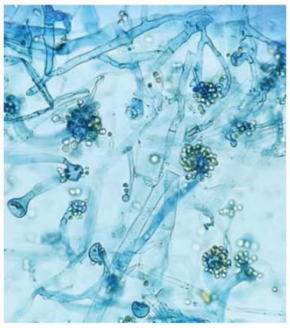
Diagram modified from Alvarez et al. Rev Iberoam Micol. 2010;27(2):80–89

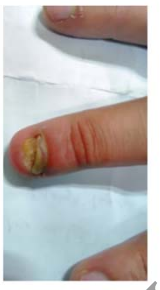
Chander et al. Rev Iberoam Micol. 2015;32(2):93–98
dela Cruz et al. JCM. 2012; 50: 2814 –2817

Cunninghamella bertholletiae

Microscopy LPCB mounts:

- **branching sporangiospores, vesicles,**
- **Sporangiolum & sporangiospores & denticles**
- **hyalinous broad hyphae & septae**





eft middle finger nail showing onycholysis and ony-
y.

FIGURE 3: Growth on SDA within 48 hours at 30°C.

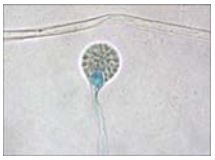
FIGURE 4: Mature growth on SDA after 72 hours.

Tadepalli et al., Case Rep Infect Dis. 2015, Article ID 703240
Mycology online

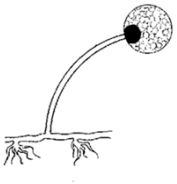
Lichtheimia corymbifera = Mucor corymbifera = Absidia corymbifera

A common human pathogen, pulmonary, rhino-cerebral, disseminated, & cutaneous mucormycosis.

world-wide distribution, can be found in soil & decaying plant debris.



A. corymbifera



Mycology online; Thomas PA. CMR; 2003; 16: 730-9
Vyas & Shah. Indian J Otol. 2011; 17: 33-6 |

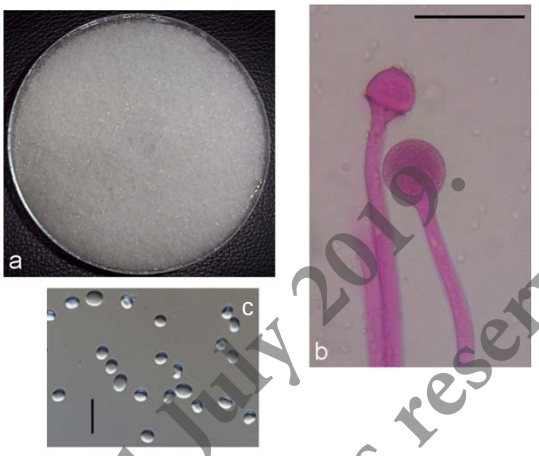
Lichtheimia ramosa
syn. *Mycocladus ramosus, Absidia ramosa*

In nature: soil, decaying plant debris & foodstuffs.

immunocompromised hosts,

becoming increasingly common in individuals without predisposing factors (e.g. in traumatic injuries).

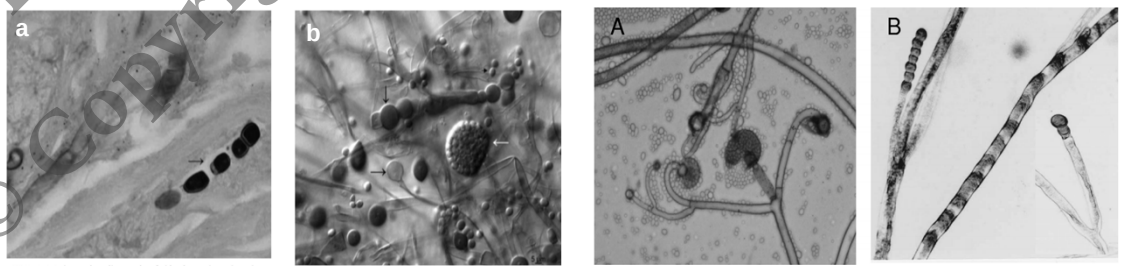
Associated with cutaneous, pulmonary, rhinocerebral, CNS & disseminated form



a. Culture of 3 days (30˚)
b. Sporangiophore, intact sporangium & ruptured sporangium with columella
c. sporangiospore

LIFE 2018;
Bibashi et al., Med Mycol Case Rep 2013; 2:

Mucor circinelloides

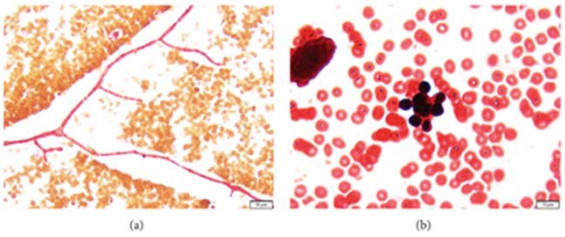


GMS of skin tissue: nonseptate hyphae & intercalary oval to subglobose chlamydospores (arrow, a). Culture on PDA, 6 days, 30°C, sporangium (white arrow), sporangiospores (black arrowhead), & chlamydospores produced singly & short (b)

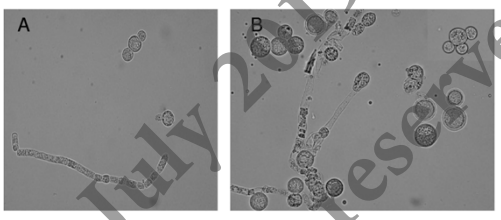
(A) Branched circinate sporangiophores, sporangia, & collumellae (B) chlamydospores formed successively in chains. 400×

Iwen et al., JCM. 2007; 45: 636–40
Khan et al., JCM. 2009, 47:1244–8

Dimorphic stage of M. *M.circinelloides*



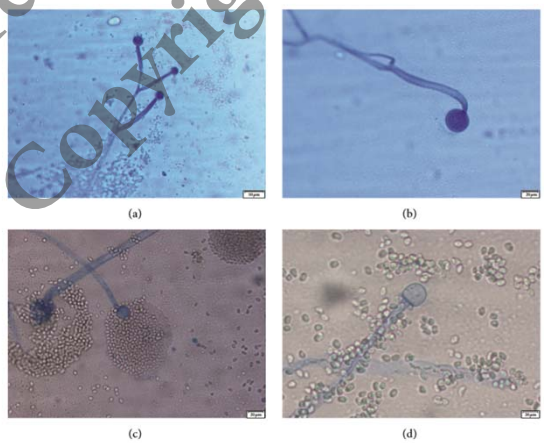
Bactec blood culture: gram stain showed branching hyphae (a) & yeast phase resembling *P. brasieliensis*



(A and B) BHI agar, 37°C, hyphae & arthroconidium & yeast forms with single, bipolar, and multipolar buds. 600×

Khan et al., JCM. 2009, 47:1244–8
Arroyo et al., Case Rep Infect Dis 2016; ID 3720549

M.circinelloides culture RT

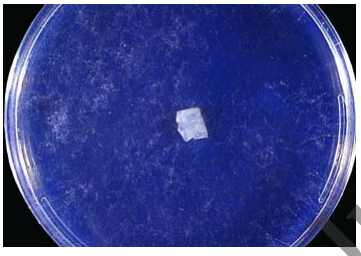


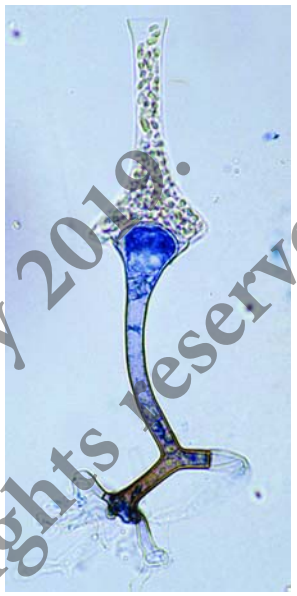
Microscopic from PDA "tape prep", LPCB
(a) Sympodially branched sporangiophores (100x);
(b) circinate sporangiophores (200 ×);
(c) deliquescent sporangia (100×);
(d) columella with collarette (200×).

Arroyo et al., Case Rep Infect Dis 2016; ID 3720549

Saksenea vasiformis

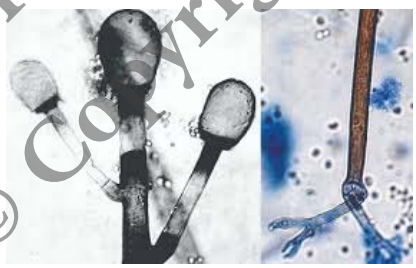
A special method to stimulate sporulation:
A small block of agar is cut from a well established culture grown on PDA & is placed in the center of petri dish containing 1% agar in distilled water.
After 21 days at 26˚C sporangium formation can be seen at the periphery of the petri dish.

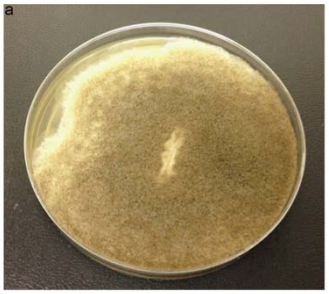


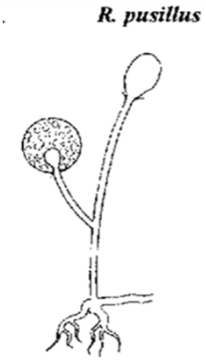


Mycology online
Padhye & Ajello . JCM 1988; 26: 1861–1863

Rhizomucor pusillus

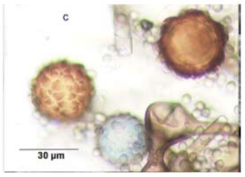






Bard et al., Med Mycol Case Rep. 2014; 5: 20-23
Thomas PA. CMR; 2003; 16: 730-97; Mycology Online

Rhizomucor microsporus



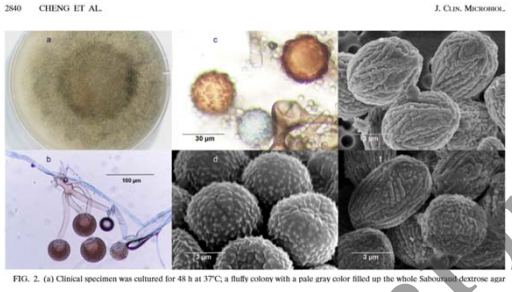


FIG. 2. (a) Clinical specimen was cultured for 48 h at 37°C; a fluffy colony with a pale gray color filled up the whole Sabouraud dextrose agar

SDA, 48 hour culture, globose sporangia
3 weeks-old culture, azygospores was produced

R. microsporus var. rhizopodiformis

Cheng et al. JCM 2009; 47: 2834–43;
http://www.pf.chiba-u.ac.jp/gallery/fungi/r/Rhizomucor_microsporus_var_rhizopodiformis.htm

MOLECULAR BASED

Diagnosis of mucormycosis

- mucormycosis remains difficult to diagnose,
- Direct methods investigation is the “gold standard” for diagnosis, but requires expertise & does not allow species identification.
- Culture of clinical specimens often fail to grow (ca. 50%).
- Require other technique: **molecular based identification**

Roden et al., 2005. Clin Infect Dis. 41:634–653.

Molecular based method

- A retrospective study using tissue blocks, semi nested PCR continued by sequencing
- primers developed from 18S ribosomal DNA, the V4 and V5 variable regions
- The outer primers ZM1 (5-ATT ACC ATG AGC AAA TCA GA-3) and ZM2
- (5-TCC GTC AAT TCC TTT AAG TTT C-3)
- Products of the seminested reaction using primers ZM1 and ZM3 (5-CAA TCC AAG AAT TTC ACC TCT AG-3) are 175 to 177 bp long
- Able to distinguish variability to identify genera but not to species level.
- 12 positive culture (10 PCR pos & 2 PCR neg); 15 negative culture (12 PCR pos, 3 PCR neg)

Hammond et al., JCM 2011; 49: 2151–3

Species identification of culture using universal fungal primers (ITS regions)

TABLE I. Available loci and techniques used for species identification of Zygomycetes from cultures

Species	Target region	Method	References
Several species	28S	PCR + sequencing	Voigt *et al.* 1999 [4]
Several species	18S	PCR + RFLP	Machouart *et al.* 2006 [11]
Several species	28S	MicroSeq®[a]	Hall *et al.* 2004 [12]
Several species	ITS	PCR + sequencing	Schwarz *et al.* 2006 [13]
Several species	Cyt b	Real-time PCR	Hata *et al.* 2008 [18]
Several species	ITS	PCR + sequencing	Kontoyiannis *et al.* 2005 [19]
Rhizopus species	ITS	Multiplex PCR	Nagao *et al.* 2005 [15]
Rhizopus oryzae	ITS	PCR + sequencing	Abe *et al.* 2003 [14]
Rhizopus species	FTR1	PCR + sequencing	Nyilasi *et al.* 2008 [17]
Apophysomyces elegans	ITS	PCR + RFLP	Chakrabarti *et al.* 2003 [16]

[a]D2 large subunit ribosomal DNA sequencing kit.
28S, large subunit ribosomal DNA; 18S, small subunit ribosomal DNA; ITS, ribosomal DNA internal transcribed spacer; Cyt b, cytochrome b gene; FTR1, high-affinity iron permease 1 gene; PCR, polymerase chain reaction; RFLP, restricted fragment length polymorphism.

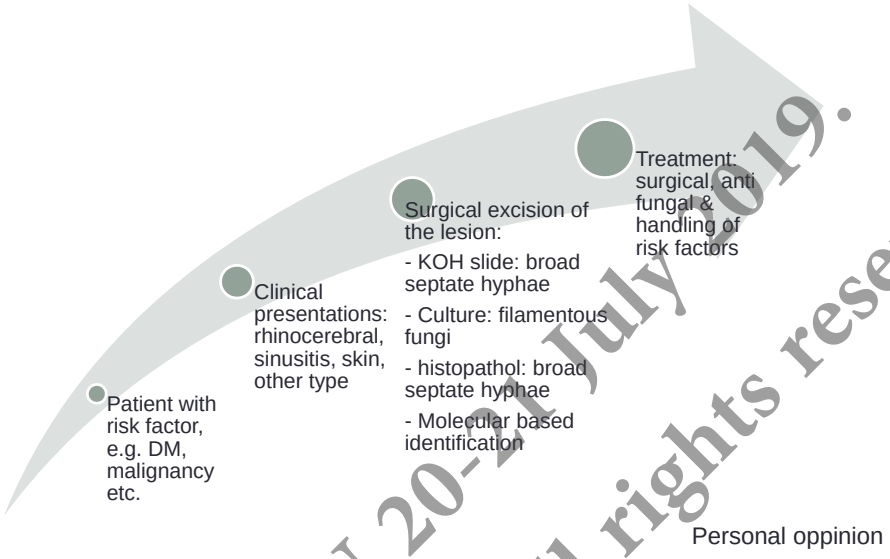
Dannaoui E. Clin Microbiol Infect 2009; 15 (Suppl. 5): 66–7

Species identification

- Sequencing of the ribosomal genes:
  - Universal fungal primers - ITS (primers ITS1 & ITS 4)
  - D1/D2 ribosomal DNA (primers NL-1 & NL-4)
- Beta tubulin
- Calmodulin

Romanelli et al., JCM. 2010, 48: 741–52
Atkins & Clark. J Appl Genet. 2004;1:3–15. 2.
Balajee et al., JCM. 2009; 47:877–84.

Algorithm for diagnosis



Conclusion

- Suspicion of mucormycoses should be started when we recognize underlying condition (patient at risk)
- Clinical presentation & its relation with underlying condition
- The importance of direct microscopic investigations: **KOH wet slide** & histopathology. Fast result, facilitate fast treatment for the patient
- Species identification is important which can be done based on phenotypic identification (culture) or molecular based method

COLLABORATION BETWEEN DOCTORS FROM MYCOLOGY lab., BETTER RESULT IN PATIENT

Take home message

THANK YOU

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