Original Article

Epidemiology of Mucormycosis in a Thai Tertiary-Care Hospital, King Chulalongkorn Memorial Hospital, Bangkok, during 2006 to 2016

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Background: Mucormycosis is an invasive fungal infection from non-septate fungi with high morbidity and mortality especially in uncontrolled diabetic patients and immunocompromised patients.

Objective: To review the epidemiology of mucormycosis between 2006 and 2016 to understand the trend of this infection and treatment success rate.

Materials and Methods: The present study collected inpatient records from the principal diagnosis of mucormycosis from medical records at a tertiary-care hospital, King Chulalongkorn Memorial Hospital, between 2006 and 2016. Thirty-six patients with mucormycosis were classified as proven, probable, and possible mucormycosis according to EORTC/MSG criteria.

Results: From the present study, 34 patients (94.4%) were classified as proven mucormycosis using histopathological evidence (27 patients, 79.4%) or both histopathological and microbiological evidence (7 patients, 20.6%). The rest were diagnosed as possible mucormycosis using clinical characteristics alone. The most common underlying disease for this infection was diabetes (61.1%). Paranasal sinuses (75%) were the main sites of infection. The most common causative agent was *Rhizopus* spp. (four out of seven isolates). The effective treatment of this infection, especially inside paranasal sinuses and lungs, was surgery (endoscopic sinuscope with debridement or lobectomy) with antifungal therapy (amphotericin B). Conventional amphotericin B was used mostly in the present study when some patients had to switch into liposomal amphotericin B (25%) or posaconazole (16.67%) because of side effects of amphotericin B. Acute kidney injury, the most common form of amphotericin B side effects, was observed in about 55.9% of the patients using conventional amphotericin B. The overall mortality rate was 30.6%.

Conclusion: The incidence of mucormycosis infection has not changed much during these ten years. The reason behind this may come from the under-diagnosis of this serious infection. The conventional culture method does not seem to be very helpful in this infection while histopathological evidence is still crucial for the diagnosis. For the early detection of this infection, the molecular approach, e.g., ITS-sequencing, could be implemented. The mortality rate of this infection in the present study is mostly depending on the response to conventional amphotericin B treatment. Therefore, liposomal amphotericin B and posaconazole are the alternative better options for invasive mucormycosis.

Keywords: Mucormycosis, Epidemiology, Thailand, King Chulalongkorn Memorial Hospital

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Mucormycosis is an emerging fungal infection that causes high morbidity and mortality[1-3]. Mucormycosis is caused by fungi in the order Mucorales, subphylum Mucoromycotina, e.g., *Rhizopus* spp., *Mucor* spp., *Rhizomucor* spp., *Lichtheimia* spp.[4]. Important risk factors for mucormycosis are patients with uncontrolled diabetes mellitus, patients with diabetic ketoacidosis, prolonged use of steroid, neutropenic patients, desferoxamine use, hematological malignancies, intravenous drug user, prophylactic use of voriconazole and echinocandins, and patients with burn injuries[5]. The signs and symptoms usually start at the paranasal sinuses. If the diagnosis and treatment

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are delayed, the infection will progress to the eyes and brain. The unique clinical features of this infection are the necrosis of the tissue and the black eschar as a result. This infection can occur in the disseminated form from lungs to other organs including skin[6].

The incidence of mucormycosis is increasing especially in India[1-3,7-9]. During the last five years, there were 129 patients with this mucormycosis in India[8,9]. However, within the past 18 months, there were about 75 patients with mucormycosis[8,9]. These studies support that the infection rate is increasing. The increase of this infection comes along with the increase of diabetic patients and climate change[3]. Thailand is one of those tropical countries that the number of diabetic patients is increasing. Therefore, soon, the mucormycosis infection rate will be rising in Thailand. It is possible that the incidence of this infection in Thailand is underestimated because this study is limited.

In India, the most common clinical manifestation of this infection is the rhino-orbito-cerebral mucormycosis[3]. Furthermore, the mortality rate of this infection is about 50 percent and the main cause of this high mortality rate is the delayed diagnosis and treatment[3]. The main treatment of this infection is controlling the blood sugar or treating the ketoacidosis condition including surgery to remove the source of infection and using effective antifungal agents, e.g., amphotericin B[3].

In European countries, there were fewer cases of mucormycosis and most of them were pulmonary mucormycosis with hematological disorders or malignancies[2]. The most common causative agent is *Rhizopus* spp. together with *Aspergillus* spp. co-infection in around 44 percent[2]. The overall mortality rate is about 67 percent[2]. In the U.S., most mucormycosis-infected patients were bone marrow transplant patients using immunosuppressive agents[7,10,11]. Another factor that needs to be considered is the voriconazole prophylaxis in patients with hematological malignancies[12-14]. Voriconazole would prevent *Aspergillus* spp. infection but not Mucorales[12-14].

The main difference of this infection in European or U.S. countries and India is the location of infections and the underlying diseases. Patients with hematological malignancies commonly have pulmonary mucormycosis while patients with diabetic ketoacidosis or poorly controlled blood sugar have rhino-orbito-cerebral mucormycosis[2,3,10].

Therefore, the main objective of the present study was to understand the incidence of this mucormycosis in Thailand to raise the concerns of this severe infection together with early diagnosis and prompt treatment.

Materials and Methods

Surveillance

The present study was retrospectively collected from the inpatient medical records using the principal diagnosis of mucormycosis (ICD-10: B46 Mucormycosis) at a tertiary-care hospital, King Chulalongkorn Memorial Hospital (KCMH, around 1,500-bed medical school hospital), from 2006 to 2016.

Inclusion criteria

These patients with mucormycosis were classified as proven, probable, and possible mucormycosis according to the European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) criteria[15]. Proven mucormycosis was defined as a positive finding in the histopathologic or cytopathologic or direct microscopic examination with Gomori's methenamine silver stain of the tissue or a positive culture from sterile sites. Probable mucormycosis required at least a host factor and a microbiological criterion and a clinical criterion according to EORTC/MSG criteria[15]. Possible mucormycosis could be categorized by the proper host factors together with clinical criteria supporting the infection without the mycological support according to EORTC/MSG criteria[15].

Fungal identification

All specimens were examined using the direct examination, i.e., 10% potassium hydroxide (KOH) wet mount, Gram stain, Wright stain, and Gomori's methenamine silver stain (GMS), and they were cultured for at least four weeks in Sabouraud dextrose agar (BD Difco™, Sparks, MD, USA), Sabouraud dextrose agar with chloramphenicol (Acumedia™, Lansing, MI, USA), Mycosel™ or Sabouraud dextrose agar with chloramphenicol, and cycloheximide (BD BBL™, Sparks, MD, USA), and sheep blood agar (BD BBL™, Sparks, MD, USA) at both 37°C and 25°C. All positive colonies were observed under light microscopes with lactophenol cotton blue staining[16]. Polymerase chain reaction (PCR) for fungal detection from tissue or Formalin-fixed paraffin-embedded (FFPE) tissue was performed as previously described[17].

Galactomannan enzyme immunoassay test

The Platelia *Aspergillus* Ag (Bio-Rad, Redmond,

WA) was used for measuring galactomannan (GM) levels[16]. Bronchoalveolar lavage (BAL) and/or sera samples from patients were processed and mixed well with reagents following the manufacturer's instructions. Optical densities (ODs) at 450/620 nm were read in each well by a microplate reader (BioTek Instruments, Winooski, VT). Negative controls, cut-off controls, and positive controls were read at the same time in each assay. Results were determined as an index relative to the OD of the mean cut-off control (GM index = OD sample / mean cut-off control OD). GM index 0.5 was indicated as a positive result.

Histopathology

Tissue or sample sections were stained with hematoxylin and eosin (H&E) and Gomori's methenamine silver stain for fungal diagnosis[17]. The morphologic characteristics were utilized to identify fungal infections. Mucorales were reported by using the morphology of broad, non-pigmented, pauci-septated hyphae with random right angle branching as previously described[17,18].

Statistical analysis

Data in the present study were analyzed by mean, standard deviation, and the percentage using GraphPad Prism version 7.0 (GraphPad Software, La Jolla California USA).

Ethical approval

The present study was approved by the Institutional Review Board (IRB No.053/61) at the Faculty of Medicine, Chulalongkorn University.

Results

Demographic data of patients with mucormycosis (Table 1)

Between 2006 and 2016, 36 were patients diagnosed as mucormycosis, categorized into 94.4% (34 patients) of proven mucormycosis and 5.6% (2 patients) of possible mucormycosis. Patients with mucormycosis are shown in Figure 1. The age range of patient was between 3 and 81 years old (average 48.6±20.9 years old). Twenty-three patients were male (63.89%) while 13 patients were female (36.11%). The most common underlying disease was diabetes mellitus (61.11%, 22 patients). The following underlying diseases were hematological malignancies (leukemia, lymphoma) 25%, other immunodeficiency diseases (Acquired Immune Deficiency Syndrome [AIDS]; unspecified cell-mediated immunity defects) 11.1%, and structural defects (lung cavity) 2.8%.

Table 1. Demographic data of 36 patients with mucormycosis at King Chulalongkorn Memorial Hospital from 2006-2016

Characteristics	No. of patients n (%)
Age (years), Mean±SD (range)	48.6±20.9 (3 to 81)
Sex	
Male	23 (63.89)
Female	13 (36.11)
Underlying diseases	
Diabetes mellitus	22 (61.11)
Hematological malignancies	9 (25.00)
Immunodeficiency	4 (11.11)
Others	1 (2.78)
Area of infection	
Sinuses, eyes, brain	27 (75.00)
Lungs	8 (22.22)
Abdomen	1 (2.78)
Length of stay (days), Mean (range)	61.05 (1 to 733)
Wards	
Medicine, general	19 (50.00)
Medicine, ICU	4 (10.53)
Pediatrics	2 (5.26)
Pediatrics, ICU	1 (2.63)
ENT	11 (28.95)
Surgery	1 (2.63)

SD=standard deviation; ENT=eye-nose-throat clinic; ICU= intensive care unit

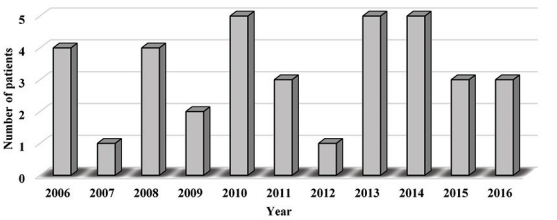

Figure 1. Number of patients with mucormycosis at King Chulalongkorn Memorial Hospital by year (2006-2016).

Paranasal sinuses, eyes, and brain (75%) were the most common sites of infections followed by lungs (22.2%) and abdomen (1%). Medicine (50%) and ear, nose, throat ward (ENT) (28.95%) are the common wards of patients with mucormycosis. Pediatrics (2.63%) and surgery wards (2.63%) are less common. The mean

Table 2. Medical characteristics of each patient with invasive mucormycosis at King Chulalongkorn Memorial Hospital during 2006-2016

Case No.	Year	Age (years)	Sex	Ward	Underlying diseases	Site of infection	Direct examination for fungi	Fungal culture result	PCR for detection	Pathology	Treatment	Outcome	Note
1	2006	67	Female	Medicine	Diabetes mellitus and hypertension	Sinuses	Negative	No growth	N/A	Invasive mucormycosis	Sphenoidectomy and ESS with amphotericin B	Survive	
2	2006	22	Female	ENT	AML	Sinuses	N/A	N/A	N/A	Invasive mucormycosis	ESS with amphotericin B and caspofungin	Survive	
3	2006	9	Male	ICU Pediatrics	Combined immunodeficiency	Sinuses	Negative	No growth	N/A	Invasive mucormycosis	ESS with amphotericin B	Dead	
4	2006	66	Male	Medicine	Diabetes mellitus	Sinuses	GMS stain from tissue: positive	No growth	N/A	Invasive mucormycosis	Ethmoidectomy and sphenoidectomy with amphotericin B	Dead	
5	2007	64	Female	Medicine	Diabetes mellitus and hypertension	Lungs	Negative	N/A	N/A	Invasive mucormycosis	Amphotericin B with itraconazole	Survive	
6	2008	56	Male	Medicine	Mantle cell lymphoma	Sinuses	Negative	No growth	N/A	Invasive mucormycosis	ESS with amphotericin B	Survive	
7	2008	60	Female	Medicine	Diabetes mellitus and hypertension	Eyes and brain	Negative	No growth	N/A	Invasive mucormycosis	ESS with orbital exenteration with amphotericin B and itraconazole	Dead	
8	2008	63	Female	Medicine	Diabetes mellitus, hypertension, and ESRD	Sinuses	KOH wet mount from tissue: positive	No growth	N/A	Invasive mucormycosis	Amphotericin B and caspofungin	Survive	
9	2008	15	Male	ENT	Diabetes mellitus	Eyes and sinuses	N/A	No growth	N/A	Invasive mucormycosis	ESS with orbital exenteration	Survive	
10	2009	62	Male	Medicine	Diabetes mellitus and hypertension	Sinuses	Negative	No growth	N/A	Invasive mucormycosis and aspergillosis	ESS with amphotericin B	Survive	GM from serum positive
11	2009	55	Female	Surgery	Diabetes mellitus and hypertension	Sinuses and brain	Negative	*Rhizopus* spp.; *Aspergillus flavus* complex	N/A	Invasive mucormycosis	ESS with craniectomy and amphotericin B	Dead	
12	2010	62	Male	ENT	Diabetes mellitus, hypertension, dyslipidemia, and atherosclerotic heart disease	Sinuses	KOH wet mount from tissue: positive	No growth	N/A	Invasive mucormycosis	Turbinectomy with amphotericin B	Survive	
13	2010	47	Female	Medicine	Diabetes mellitus	Sinuses	Negative	No growth	N/A	Invasive mucormycosis	ESS with amphotericin B	Survive	
14	2010	53	Male	Medicine	HIV (CD4 26)	Sinuses	N/A	No growth	N/A	Invasive mucormycosis	Itraconazole	Survive	
15	2010	58	Male	Medicine	Diabetes mellitus, hypertension, gout, and asthma	Sinuses	Negative	No growth	N/A	Invasive mucormycosis	ESS with amphotericin B	Survive	
16	2010	56	Male	ENT	Diabetes mellitus and cirrhosis	Sinuses	Negative	No growth	N/A	Invasive mucormycosis	ESS with orbital exenteration with amphotericin B	Survive	
17	2011	21	Male	Medicine	ALL	Lungs	KOH wet mount and Wright stain from BAL: positive	*Mucor* spp.	N/A	Invasive mucormycosis	Lobectomy with amphotericin B and voriconazole with acute renal failure; switching to liposomal amphotericin B with itraconazole	Survive	GM from BAL positive
18	2011	59	Male	Medicine	DLBCL, diabetes mellitus	Sinuses	Negative	No growth	N/A	Invasive mucormycosis	ESS with amphotericin B	Survive	
19	2011	19	Male	Medicine	Beta-thalassemia with common variable immunodeficiency with predominant immunoregulatory T-cell disorders	Sinuses	Negative	No growth	N/A	Negative finding	ESS with amphotericin B	Dead	

ENT=eye-nose-throat clinic; ICU=intensive care unit; ALL=acute lymphoblastic leukemia; AML=acute myeloblastic leukemia; BAL=bronchoalveolar lavage; DLBCL=diffuse large B-cell lymphoma; ESRD=end-stage renal disease; ESS=endoscopic sinuscope surgery; FFPE=formalin-fixed paraffin-embedded tissue; GM=galactomannan; GMS=Gomori's methenamine silver; KOH=potassium hydroxide; N/A=not available

Table 2. (continued)

Case No.	Year	Age (years)	Sex	Ward	Underlying diseases	Site of infection	Direct examination for fungi	Fungal culture result	PCR for detection	Pathology	Treatment	Outcome	Note
20	2012	69	Female	Medicine	Diabetes mellitus and hypertension	Lungs	KOH wet mount from sputum: positive	No growth	N/A	Invasive mucormycosis	Lobectomy with amphotericin B	Survive	
21	2013	54	Male	ENT	Diabetes mellitus and chronic kidney disease	Sinuses	GMS stain from tissue: positive	*Rhizopus* spp.	N/A	Invasive mucormycosis	ESS with amphotericin B with acute renal failure; switching to liposomal amphotericin B with posaconazole	Survive	
22	2013	41	Female	ICU Medicine	Diabetes mellitus and hypertension	Sinuses	Negative	No growth	N/A	Invasive mucormycosis	ESS with amphotericin B with posaconazole	Dead	
23	2013	59	Female	ENT	Diabetes mellitus	Sinuses	Negative	No growth	N/A	Invasive mucormycosis	ESS with amphotericin B	Survive	
24	2013	49	Male	ICU Medicine	Diabetes mellitus	Abdomen	Negative	No growth	N/A	Invasive mucormycosis	Biopsy with amphotericin B	Dead	
25	2013	74	Female	ENT, ICU Medicine	Diabetes mellitus, hypertension, and dyslipidemia	Sinuses	KOH wet mount from tissue: positive	*Rhizopus* spp.	N/A	Invasive mucormycosis	ESS with amphotericin B and posaconazole with acute renal failure; switching to liposomal amphotericin B and posaconazole	Survive	
26	2014	38	Male	Medicine	HIV infection with HCV infection	Sinuses, soft, and hard palate	Negative	No growth	N/A	Invasive mucormycosis	Amphotericin B	Dead	
27	2014	77	Female	ENT	Follicular lymphoma	Sinuses	Wright stain from tissue: positive	*Rhizopus* spp.	Positive from tissue (*Rhizopus* spp.)	Invasive mucormycosis	ESS with amphotericin B	Dead	
28	2014	42	Male	ENT	Diabetes mellitus with HCV infection	Sinuses	KOH wet mount: positive	No growth	Negative from tissue	Invasive mucormycosis	ESS with amphotericin B with acute renal failure; switching to liposomal amphotericin B	Survive	
29	2014	16	Male	Medicine	ALL	Sinuses	KOH wet mount: positive	*Mucor* spp.	Negative from tissue	Invasive mucormycosis and invasive aspergillosis	ESS with amphotericin B with posaconazole	Survive	GM from serum positive
30	2014	67	Male	Medicine	AML, Sjogren syndrome	Lungs	Negative	No growth	N/A	N/A	Voriconazole; switching to ampohotericin B	Dead	
31	2015	81	Male	ENT, ICU Medicine	Diabetes mellitus, hypertension, and dyslipidemia	Sinuses	KOH wet mount and Wright stain from tissue: positive	Sterile non-septate hyphae	N/A	Invasive mucormycosis	ESS with orbital exenteration with amphotericin B with acute renal failure; switching to liposomal amphotericin B	Dead	
32	2015	58	Male	Medicine	Previous TB lung infection	Lungs	Negative	No growth	Negative from tissue and FFPE	Invasive mucormycosis	Amphotericin B; switching to posaconazole	Survive	
33	2015	13	Male	Pediatrics	AML	Lungs	Negative	No growth	Negative from FFPE	Invasive mucormycosis	Lobectomy with voriconazole	Survive	
34	2016	55	Male	Medicine	Diabetes mellitus and hypertension	Lungs	Negative	No growth	N/A	Invasive mucormycosis	Amphotericin B with acute renal failure; switching to liposomal amphotericin B with posaconazole	Survive	
35	2016	38	Male	ENT	Diabetes mellitus, hypertension, and dyslipidemia	Nasopharynx and hard palate	KOH wet mount from tissue: positive	No growth	Negative from tissue	Invasive mucormycosis	Debridement, turbinectomy, and sphenoidectomy with amphotericin B with acute renal failure; switching to liposomal amphotericin B with posaconazole	Survive	
36	2016	3	Female	Pediatrics	Aplastic anemia	Lungs	Negative	No growth	Positive from BAL (*Mucor* spp.)	Invasive mucormycosis	Liposomal amphotericin B with posaconazole	Survive	

ENT=eye-nose-throat clinic; ICU=intensive care unit; ALL=acute lymphoblastic leukemia; AML=acute myeloblastic leukemia; BAL=bronchoalveolar lavage; DLBCL=diffuse large B-cell lymphoma; ESRD=end-stage renal disease; ESS=endoscopic sinuscope surgery; FFPE=formalin-fixed paraffin-embedded tissue; GM=galactomannan; GMS=Gomori's methenamine silver; KOH=potassium hydroxide; N/A=not available

length of stay in the hospital was around two months. Additionally, the medical summary of each patient with invasive mucormycosis is described in Table 2.

Diagnosis of patients with mucormycosis

Most diagnoses of this infection in the present study were from histopathological evidence (79.4%); however, the diagnosis from microbiological evidence or positive fungal cultures was found in seven cases with six specimens from paranasal sinuses and one specimen from lungs (20.6% of positive fungal cultures from total 34 cultures). *Rhizopus* species were recovered from four specimens when two *Mucor* species were isolated. In addition, one sterile non-septate mold was isolated from BAL.

Nevertheless, about 35.3% (12 positives from 34 samples) of the direct examination, e.g., KOH wet mount, Wright stain, and GMS stain, could detect non-septate fungal elements in the specimens. For PCR from tissue and FFPE tissue, there were two PCR positive from tissue and BAL (28.6% of seven PCR results).

Furthermore, some *Aspergillus* spp. co-infections were observed. There were two cases that histopathological evidence and serological (galactomannan) evidence supported *Aspergillus* spp. co-infections, one case from only serological (galactomannan) evidence, and one case from only microbiological (culture) evidence.

Treatment and mortality rate of patients with mucormycosis

The standard treatment of this infection inside paranasal sinuses and lungs is surgery (endoscopic sinuscope with debridement or lobectomy) with antifungal treatment (amphotericin B or combined antifungal agents). In the present study, about 80% of the treatment was surgery together with antifungal therapy while the proportion of patients with antifungal therapy alone or surgery alone was 17% and 3%, respectively. Considering the standard treatment, surgery with conventional amphotericin B (58.6%) was used mostly in the present study. Moreover, acute kidney injury, the most common form of amphotericin B side effects, was found about 55.9% in patients treated with conventional amphotericin B.

The mortality rate from patients treated with surgery and conventional amphotericin B alone was 41.2% (from 24 patients) while from patients treated with surgery and combination of conventional amphotericin B and other antifungal agents, e.g., voriconazole, posaconazole, itraconazole, and caspofungin, was 40% (from seven patients). The mortality rate from patients switching from conventional amphotericin B to liposomal amphotericin B alone was 25% (from five patients) while from patients switching from conventional amphotericin B to liposomal amphotericin B with other antifungal agents, e.g., posaconazole, voriconazole, was none (from three patients) (Figure 2). The overall mortality rate was 30.6%.

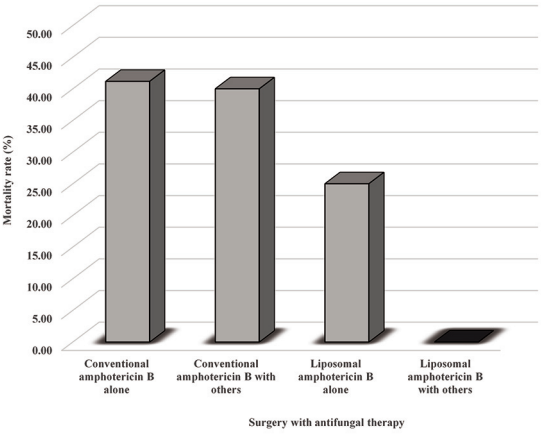

Figure 2. Mortality rate of mucormycosis patients with surgery and antifungal therapy. Antifungal therapy is categorized into four groups, which are conventional amphotericin B alone, conventional amphotericin B with other antifungal agents, liposomal amphotericin B alone, and liposomal amphotericin B with other antifungal agents.

Discussion

In this decade, to the best of our knowledge, the present study is the largest retrospective study of patients with invasive mucormycosis in Thailand. There have been few reports of invasive mucormycosis in Thailand since 1978[19]. Previous studies in Thailand showed that most Mucorales infections were rhinocerebral form followed by pulmonary, cutaneous, and gastrointestinal forms, respectively[19]. Most patients with invasive mucormycosis had diabetes mellitus (52.2%)[19]. Most of the diagnosis were from clinical suspicion with direct examination, and then confirmed by histopathology[20]. From a report of rhinocerebral mucormycosis in 1991 by Chetchotisakd et al[21], only two cases from eleven cases had positive cultures for *Rhizopus* spp. and *Cunninghamella* spp. However, other reports failed to recover the Mucorales from the cultures[19,20].

In the present study, there were 36 inpatients with invasive mucormycosis between 2006 and 2016 at

King Chulalongkorn Memorial Hospital, Bangkok, Thailand, a 1,500-bed tertiary-care university hospital. Nevertheless, the under-diagnosis and under-recognition of the infection may be one of the factors affecting the number of patients. The diagnosis of invasive mucormycosis depends on the suspicion of clinical manifestations and host factors[22]. Even though the characteristic of this infection is the tissue necrosis, the clinical manifestations alone are still not specific and accurate for the diagnosis[22]. Therefore, the upward trend of mucormycosis is still unclear in the present study when compared to reports from India[23].

The current study revealed that the underlying diseases of mucormycosis in Thailand was still mainly the diabetes mellitus (61.11%), and the most common sites of infection were at the paranasal sinuses, eyes, and brain (75%) (Table 1). According to many reports from India, poorly controlled diabetes mellitus and diabetic ketoacidosis are the major risk factors of the rhino-orbito-cerebral mucormycosis[24]. The present study showed the same trend for the risk factor of mucormycosis among Asian countries. Interestingly, the authors did not observe any diabetic ketoacidosis conditions in the study group. While diabetes mellitus and trauma are the main predisposing factors of the rhino-orbito-cerebral mucormycosis in developing countries in Asia, the hematological malignancies are the major risk factors of the pulmonary mucormycosis in developed countries, i.e., Europe and the United States of America[1,22,24,25].

In addition to clinical manifestations and host factors, the diagnosis of mucormycosis is also depended on the microscopic examination including direct examination, fungal culture, and histopathology[22]. Direct examination from clinical specimens, e.g., potassium hydroxide wet mount or calcofluor white stain, could give quick preliminary information of this infection for physicians to choose the empirical treatment[26]. The Mucorales have a 6-25-µm-width non-septate or pauci-septate hyphae showing in the direct examination[26,27]. Badiee et al showed the sensitivity of direct examination for Mucorales infections, e.g., KOH wet mount, GMS stain, was around 25%[28]. In the present study, the sensitivity of direct examination for invasive mucormycosis from KOH wet mount, Wright stain, and GMS stain was about 35.3%, which is similar to the study of Badiee et al[28].

For the conventional culture method, the sensitivity is about 30%[29]. Even though most of Mucorales grow fast (three to five days) on the most fungal media, e.g., Sabouraud dextrose agar, potato dextrose agar, at 25°C to 30°C[30], these fungal non-septate hyphae are very easy to get damaged through the mechanical force during the specimen processing, i.e., grinding. Therefore, mincing the tissue is recommended for the culture method of Mucorales[10,29]. Furthermore, some Mucorales, i.e., *Apophysomyces elegans*, *Saksenaea vasiformis*, need special media (water agar with 0.1% yeast extract) to grow and sporulate[31]. The characteristic features of most Mucorales for identification are the presence of sporangia, sporangiola, or merosporangia, including the presence or shape of columella, apophysis, rhizoid, and zygospores[29]. However, during the use of antifungals or other conditions, some clinical isolates could not sporulate, so it would be difficult for the identification[29]. In the present study, the sensitivity of the culture was 20.6%, and the most common Mucorales were *Rhizopus* spp., followed by *Mucor* spp., which are similar to other reports[1,2,23,25,32].

Besides the direct examination and the culture, the histopathology is the method to establish the proven diagnosis for invasive mucormycosis[10]. The characteristics of invasive mucormycosis from histopathology are tissue infarction, angioinvasion, and perineural invasion. The presence of broad, hyaline, wide-angled branching, non-septate hyphae with tissue invasion would confirm the diagnosis of invasive mucormycosis. Gomori's methenamine silver stain would enhance the fungal elements for better observation[10]. In the present study, the proven diagnosis was mainly from histopathology (94.4%), similar to the other reports[1,2,23,25,32]. Nevertheless, the culture to discover the causative organisms would be essential for species identification and further antifungal susceptibility study[10].

Since the sensitivity of direct examination and the culture is lower than 50% and the histopathology would take time to report the results and could not identify the causative organisms, the molecular approach for mucormycosis diagnosis may be necessary to overcome this limitation[29]. Molecular methods that are common for the diagnosis of invasive mucormycosis are the conventional PCR, DNA sequencing, and real-time PCR[29]. Conventional PCR with internal transcribed spacer (ITS) sequencing is the most commonly used to identify mucormycosis[29,33,34]. This method could differentiate Mucorales from cultures and tissue samples into *Rhizopus*, *Lichtheimia*, and *Mucor* species with sensitivity and specificity of more than 90%[29,33,34]. In addition, real-time PCR with high-resolution melt curve analysis (HRM) had higher sensitivity than normal PCR and was able to identify

into the genus *Apophysomyces*, *Cunninghamella*, *Lichtheimia*, *Mucor*, *Rhizopus*, and *Saksenaea*[35]. This real-time PCR method using 18S rRNA and ITS as a target had both sensitivity and specificity of 100% to detect Mucorales in cultures and tissue samples while the sensitivity and specificity to detect from FFPE tissue were about 60% and 100%, respectively[34-37]. Nonetheless, the main limitation is that these molecular methods are still expensive to apply to clinical use and, for some clinical use, there are still lacking significant data in terms of the lower limit of detection, sensitivity, specificity, and cross-reactivity. The main reason behind these limitations may partially come from a limited number of patients with invasive mucormycosis[29]. In the present study, the authors used the PCR-sequencing method at the ITS regions and it had the sensitivity around 28.6% (seven cases), which was lower than other reports[29,33,34]. This could be from the lower number of cases submitting for PCR, the type of tissue (FFPE), which may be more difficult to extract DNA, and the amount of fungal elements in tissue samples as mentioned in other reports[29,33,34]. Therefore, the combination of all available methods, i.e., direct examination, conventional culture methods, histopathology, and molecular methods, would enhance the sensitivity and specificity of the diagnosis of invasive mucormycosis.

For the treatment of invasive mucormycosis, multiple approaches, i.e., disposal of risk factors, early and prompt surgical treatment with antifungal therapy, are necessary[22]. The most common risk factor is diabetes mellitus and diabetic ketoacidosis, so control of blood sugar and correction of metabolic ketoacidosis including adjusting immunosuppressive agent doses are crucial[22,38-40]. Early diagnosis and prompt treatment with surgery or debridement with antifungal therapy are very important for the survival of patients with invasive mucormycosis. The mortality rate would increase about two folds for the delay in antifungal therapy, which was from 48.6% with the immediate treatment to 82.9% with more than a 5-day delay in treatment[41].

Mucorales group is resistant to echinocandins due to a lack of β-glucan on its cell wall[22]. Voriconazole is resistant in vitro while amphotericin B is an active drug against this group of fungi except for *Apophysomyces* and *Cunninghamella* species[1]. Posaconazole and isavuconazole show some activity against Mucorales[22,42]. The 2016 recommendations from the European Conference on Infections in Leukemia (ECIL-6) and the European Society for Clinical Microbiology and Infectious Diseases/European Confederation of Medical Mycology (ESCMID/ECMM) guidelines suggested the lipid formulation of amphotericin B as the first-line treatment for invasive mucormycosis (5 mg/kg/day to 10 mg/kg/day)[38,39]. In addition, ECIL-6 recommended posaconazole as maintenance therapy while the ESCMID/ECMM suggested posaconazole as an alternative first-line therapy[38,39].

In the present study, the authors observed that liposomal amphotericin B with other antifungal agents, e.g., posaconazole, had a higher survival rate even though the number of patients was small. Further investigation is necessary to study the combination of the treatment for invasive mucormycosis. The side effects and drug-drug interaction of antifungal agents may play an important role in the survival rate of patients with invasive mucormycosis. Therefore, it is very important to choose the early empirical treatment wisely. In the context of our country, using liposomal amphotericin B as the first-line antifungal therapy is very difficult due to the cost of the drug. Using conventional amphotericin B with closely monitoring the creatinine clearance may be an alternative option. However, the present study has some limitations, i.e., a small number of patients with invasive mucormycosis and lack of well-controlled designs, which are important to determine the effectiveness of these treatments. Therefore, further investigations in the treatment options need to be done.

The mortality rate of mucormycosis depends on the type of mucormycosis and host factors. The range of invasive mucormycosis is between 20% and 80%[2,10]. In the present study, the authors observed about 30.6% of the overall mortality rate, similar to the previous reports[2,10]. The participants in the present study mainly were poorly controlled diabetes without ketoacidosis and the main type of invasive mucormycosis was rhinocerebral mucormycosis without cerebral involvement. This may explain the lower mortality rate in the present study.

Conclusion

From the present study, there are still gaps in the diagnosis and treatment of mucormycosis. This severe infection may be underdiagnosed, affecting the incidence over the past ten years. The main underlying diseases of this infection are diabetes and hematological malignancies, similar to the previous studies. The diagnosis of this infection is very important for the early treatment and survival of patients. The conventional culture method of these

fungi causing mucormycosis is not very useful, while the direct examination and the histopathological evidence are essential. For the early detection of this infection in the future, the molecular technique, i.e., ITS-sequencing, would be very helpful. The standard treatment is still the surgery with antifungal therapy. The present study suggests that the surgery with liposomal amphotericin B or liposomal amphotericin B with other antifungal agents, e.g., posaconazole, would lead to better survival for patients with invasive mucormycosis.

What is already known on this topic?

It has been reported in many Asian countries of the underlying disease and the incidence of patients with mucormycosis. Uncontrolled diabetes mellitus is the most common underlying disease in these Asian countries while, in developed countries, the hematological malignancies are more common. This would lead to a difference in clinical manifestations. In developing countries, they found more rhino-orbito-cerebral mucormycosis. In contrast, pulmonary mucormycosis was more common in developed countries. Nevertheless, the most common causative agent is still the same, which is *Rhizopus* species. Furthermore, amphotericin B is still an effective antifungal agent against mucormycosis.

What this study adds?

In this study, the authors demonstrated the largest retrospective study of invasive mucormycosis in ten years at King Chulalongkorn Memorial Hospital. The authors observed in their hospital that the demographic data of patients with invasive mucormycosis were the same as other Asian countries. Diabetes mellitus was the most common underlying disease in patients with rhino-orbito-cerebral mucormycosis. *Rhizopus* species were still common isolates recovered from patients with mucormycosis. However, the recovery rate of causative agents using conventional culture method was not very effective. In this study, the authors found that direct examination and histopathology were very helpful in the diagnosis of this infection. Furthermore, the molecular technique may play an important role in the diagnosis of this infection in the near future. For the treatment, the present study supports treating with liposomal amphotericin B and posaconazole may be alternative effective treatments for patients with invasive mucormycosis.

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Conflicts of interest

The authors declare no conflict of interest.

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