Int J Clin Exp Pathol 2015;8(11):14840-14852
www.ijcep.com /ISSN:1936-2625/IJCEP0015340

Original Article

Invasive fungal disease in university hospital: a PCR-based study of autopsy cases

Komkrit Ruangritchankul[1], Ariya Chindamporn[2], Navaporn Worasilchai[3], Ubon Poumsuk[1], Somboon Keelawat[4], Andrey Bychkov[4]

[1]Department of Pathology, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok, Thailand; [2]Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; [3]Interdisciplinary Program of Medical Microbiology, Graduate School, Chulalongkorn University, Bangkok, Thailand; [4]Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Received August 31, 2015; Accepted October 19, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Invasive fungal disease (IFD) has high mortality rate, especially in the growing population of immunocompromised patients. In spite of introduction of novel diagnostic approaches, the intravital recognition of IFD is challenging. Autopsy studies remain a key tool for assessment of epidemiology of visceral mycoses. We aimed to determine species distribution and trends of IFD over the last 10 years in unselected autopsy series from a large university hospital. Forty-five cases of visceral mycoses, confirmed by histopathology and panfungal PCR, were found in 587 consecutive autopsies. Major underlying diseases were diabetes mellitus (20%), hematologic malignancies (15.6%) and systemic lupus erythematosus (15.6%). There was a high risk for disseminated IFD in immunocompromised patients stayed in the hospital over 1 month with a fever longer than 3 weeks. The most common fungi were *Aspergillus* spp. (58%), *Candida* spp. (16%), *Mucorales* (14%) and *Fusarium* spp. (10%). We found significant increase in *Aspergillus flavus* ($P = 0.04$) and *Mucorales* ($P < 0.01$) infections over the last 5 years. Concordance rate between histopathology and panfungal PCR was 89.5% to the genus level. All 6 cases of fusariomycosis were misinterpreted as aspergillosis by histology alone. The precise species identification, necessary for targeted antifungal treatment, was rendered only by the molecular technique. Panfungal PCR showed high performance on formalin-fixed paraffin-embedded specimens, providing important epidemiological data in retrospective autopsy series. Rapid detection of fungi by panfungal PCR assay has high potential for intravital diagnostics of IFD in surgical and biopsy specimens.

Keywords: Invasive fungal disease, autopsy, panfungal PCR, FFPE

Introduction

Invasive fungal disease (IFD) continues to be a major cause of mortality in either community settings or general hospitals. Up to one third of immunocompromised patients die of visceral mycoses [1, 2]. The most vulnerable populations are those with hematological malignancies, AIDS and transplant recipients [3-5].

A study of epidemiologic trends provides reasonable hints to direct empirical antifungal treatment and chemoprophylaxis. However, an analysis of clinical data alone cannot efficiently estimate a rate of invasive fungal infections, since most of them remain undetected antemortem due to difficulty in diagnostics. The diagnosis of proven IFD essentially requires a culture or histopathology [6]. Complimentary biochemical markers (galactomannan, D-arabinitol) and molecular assays (FISH, panfungal PCR) have been introduced to improve the detection of fungal infections [7]. At the same time, newly emerging mycoses caused by *Mucorales* and *Fusarium* spp. bring additional obstacles to IFD diagnostics. In such circumstances, autopsy series remain a principal tool for the epidemiological study of IFD [4, 8].

Epidemiological heterogeneity of invasive and endemic mycoses is partially contributed by geography [9]. Available Asian reports are widely represented by periodic Japanese autopsy series [10-12]. Postmortem [13, 14] and ante-

mortem [15] epidemiological data from South Asia are very limited. More studies on regular basis from this rapidly developing and densely populated region are highly anticipated to monitor dynamic changes in IFD. In this study, we used panfungal PCR applied to archived autopsy specimens to identify genus and species distribution and trends of IFD in our hospital over the last 10 years.

Materials and methods

Study settings

The study was performed at the King Chulalongkorn Memorial Hospital (Bangkok, Thailand), the third largest university teaching hospital in the country with a bed capacity of 1,479 that covering all major medical departments. We retrospectively analyzed all autopsy records of patients who died at the hospital between January 1, 2005, and December 31, 2014. Medico-legal cases were not included in the study. 587 autopsies were performed during 10-year period. Selected clinical records and autopsy reports from the Department of Pathology were reviewed. Relevant demographic and clinical data were recorded. All academic autopsy cases were seronegative for HIV. The histopathological diagnosis of IFD was reviewed by two pathologists (KR and SK). The study was approved by the Chulalongkorn University Institutional Review Board (IRB No. 267/56).

Autopsy procedure

Complete autopsies involving removal and examination of all visceral organs and the central nervous system were performed in all cases. After gross examination, all major organs and grossly suspicious tissues were systematically sampled (30-40 tissue specimens per case) following standard protocol.

Tissue sections were stained with hematoxylin and eosin. Gomori's methenamine silver stain and Mucicarmine stain were additionally employed for diagnosis of fungal infections. Cultures from fresh tissues were not routinely performed.

Diagnostic criteria and definitions

IFD was defined as the presence of fungal elements in tissues with necrosis and etiologic classification was made on the basis of morphologic criteria. Fungal infections localized to the mucosa of the upper gastrointestinal and upper respiratory tracts to the tracheal bifurcation were not considered. The morphologic features used for identification of fungi in tissue were as follows [16]:

Candida spp. - oval budding yeasts with pseudohyphae; *Aspergillus* spp. - non-pigmented, septated hyphae with acute angle branching; *Cryptococcus* spp. - narrow-based budding yeasts without hyphae and with mucicarmine-positive capsule; *Mucorales* genera - broad, non-pigmented, pauciseptated hyphae with random right angle branching; *Histoplasma* spp. - small yeasts with narrow-based budding, grouping in clusters inside macrophages; *Pneumocystis jirovecii* - intraalveolar foamy eosinophilic exudate, containing thin-wall spheres with intracystic focus.

A diagnosis of disseminated IFD required the involvement of two or more non-contiguous organs by the same fungus. Mixed IFD was defined by the presence of several fungal species into one organ or different fungi into different organs. IFD was considered as a primary cause of death by pathologists if the infection was responsible for the death of a patient due to significant involvement of vital organ(s). Discrepancies between antemortem and postmortem diagnosis were analyzed to measure the impact of the missed or incorrect clinical diagnosis on the patient's management and survival. Severe neutropenia was defined as a neutrophil count < 500 mm^{-3} for more than 10 days. Persistent fever was defined as a continuous fever for more than 10 days.

Molecular study

After reviewing the slides, one formalin-fixed paraffin-embedded (FFPE) block containing the largest amount of fungal elements was selected for further PCR and sequencing. If organs were involved by different fungi, several representative blocks with the largest amount of fungal elements were selected for molecular study.

For each tissue sample, 10 FFPE sections (5-10 μm thick) were cut using a sterile microtome blade. The sections were deparaffinized in 1 ml xylene and washed with absolute ethanol. Samples were treated with a urea buffer (8 M

Table 1. Trends in the annual number of autopsies and invasive fungal disease (IFD) cases in King Chulalongkorn Memorial Hospital, Bangkok, Thailand

Year	Number of deaths	Number of autopsies	Autopsy rate	Cases of IFD	IFD rate
2005	1,437	76	5.29%	5	6.6%
2006	1,456	70	4.81%	3	4.3%
2007	1,346	70	5.20%	7	10%
2008	1,399	96	6.86%	8	8.3%
2009	1,313	86	6.55%	2	2.3%
2010	1,188	40	3.37%	6	15%
2011	1,260	40	3.17%	3	7.5%
2012	1,324	28	2.11%	3	10.7%
2013	1,279	37	2.89%	2	5.4%
2014	1,315	44	3.35%	6	13.6%
2005-2009	6,951	398	5.74%	25	6.3%
2010-2014	6,366	189	2.98%	20	10.4%
Total	13,317	587	4.40%	45	7.7%

urea, 0.5 M NaCl, 20 mM Tris, 20 mM EDTA, 2% SDS, pH 8.0) and a lysis buffer (0.5% w/v SDS in TE buffer: 10 mM Tris, pH 8.0; 1 mM EDTA, pH 7.5) with glass beads and centrifuged at 13,200 g for 20 min. For protein precipitation the aqueous phase was mixed with phenol : chloroform : isoamyl alcohol (25:24:1, v/v) followed by proteinase K (10 mg/ml) treatment. DNA precipitation was performed using cold isopropanol and the DNA pellet was obtained by centrifuging at 13,200 g for 10 min. The quantity and quality of DNA were measured by NanoDrop (NanoDrop 1000 Spectrophotometer, Thermo Scientific, USA).

DNA was amplified in the region of internal transcribed spacers (ITS1 and ITS4) following conventional protocol [17] by universal fungal primers, ITS1 and ITS4 using i-TaqTM DNA polymerase (iNtRON Biotechnology, South Korea). Sterile distilled water was used as a negative control. PCR products were purified using the PCR Clean-up QIAquick PCR Purification kit (Qiagen, USA) and sequenced at outside facility (1st BASE Laboratory, Malaysia). Sequences were identified using GenBank BLAST searching tool.

Statistical analysis

All data were entered into an Excel spreadsheet (Microsoft, CA), and statistical analysis was performed using SPSS 17.0 (SPSS, Chicago, IL). Trends in the prevalence over two 5-year periods (2005-2009, 2010-2014) were analyzed by chi-square test for trend. The comparison of continuous data was performed using Mann-Whitney U test or Student's t test, and categorical data were analyzed by chi-square test. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Clinical characteristics

During the study period 13,317 deaths were recorded by the hospital. A total of 587 autopsies were performed, and 45 cases of IFD were diagnosed postmortem, including 2 pediatric and 43 adult cases. The total number of autopsies performed in the hospital has declined over the last 10 years, from around 80 per year from 2005-2009 to around 40 per year after 2010 (**Table 1**). Autopsy rates dropped respectively from 5.74% over first 5-year period to 2.98% more recently.

The median age of the patients was 49 years old with a male-female ratio of 0.96. The average number of days in a hospital was 30, ranging from 1 day (3 patients) to 7 months. Most of the patients were presented with a persistent fever (62.2%). Other remarkable chief complains listed in order of frequency were dyspnea, cough, alteration of consciousness, seizures, headache, nausea/vomiting and chest pain.

Major underlying diseases were malignant tumors, including leukemia/lymphoma (15.6%) and carcinoma (6.7%). Five patients (11.1%) had non-malignant hematological diseases such as pancytopenia and thalassemia. Every fifth patient had a history of long-standing diabetes mellitus. The other significant diseases were systemic lupus erythematosus (SLE), gout, liver cirrhosis and hepatitis (**Table 2**). Past medical history was remarked by heart surgery and organ transplantation in 2 and 3 patients, respectively. IFD in three post-transplant patients was noted at intervals of 3 years, 4

Table 2. Demographic and clinical characteristics of 45 patients with IFD

	Number	Percentage
Baseline characteristics		
Age, mean (range), years	48.5 (6 days-79 years)	
Sex		
Male	22	48.9%
Female	23	51.1%
Clinical characteristics		
Persistent fever	28	62.2%
Neutropenia	14	31.1%
Days in hospital, mean (range)	30 (1-202)	
Underlying condition*		
Hematologic malignancy	7	15.6%
Hematologic non-malignant	5	11.1%
Solid cancer	3	6.7%
Diabetes mellitus	9	20.0%
Chronic liver disease	4	8.9%
SLE	7	15.6%
Gout	3	6.7%
Organ transplantation	3	6.7%
Other somatic disease	4	8.9%
Prolonged treatment:		
Corticosteroid	26	57.8%
Chemotherapy	8	17.8%
Immunosuppressive	13	28.9%
No evident predisposing factor	4	8.9%
Antifungal therapy	22	48.9%
IFD evident only at autopsy	27	60.0%
IFD as a cause of death	35	77.8%

*Patients could have more than one underlying condition and could be on prolonged combined pharmacotherapy.

months and 5 days, following transplantation. The prevalence of neutropenia as a predisposing risk factor for IFD was confirmed in 31.1% cases.

Prolonged drug treatment for major diseases prior to IFD was recorded in most of cases: 26 patients (57.8%) were on systemic corticosteroids, 13 (28.9%) received immunosuppressive drugs and 8 patients (17.8%) were on chemotherapy. There were 4 cases without underlying predisposing conditions.

Eighteen patients (40%) had an antemortem proven or probable IFD by EORTC/MSG criteria [6]. All of these and 4 additional patients received antifungal treatment (48.9%). There were 9 cases with fungi detected in sputum, BAL or pleural fluid, but no specific treatment was administered.

Terminal IFD was considered by pathologist as a primary cause of death in 35 cases. Definite antemortem diagnosis of IFD was made in 18 patients, whereas suspicious fungal infection was traced in clinical records of 13 patients (28.9%), and not suspected in 14 cases (31.1%).

Organ involvement

Eighty major visceral organs were affected by IFDs in 45 patients (**Figure 1**). The most common systems involved were respiratory (50%), gastrointestinal (18%), cardiovascular (10%) and CNS (9%). The lung was the most common organ involved followed by large intestine, heart, brain and kidney.

Patterns of organ involvement were disseminated with up to 5 organs affected by the same fungus, and non-disseminated (**Table 3**). Disseminated IFD was found in 13 patients (29%) and basically involved 2 systems/organs. Non-disseminated cases were represented by localized disease with a single organ affected by one fungus (26 cases, 58%) or two independent visceral organs invaded by different fungi (mixed IFD - 5 cases, 11%). Localized mixed infection with 2 fungi in the same organ was identified in 1 case.

Histopathology

Tissue reaction in the visceral organs infected by *Aspergillus* and *Fusarium* species was marked by neutrophilic infiltration and necrosis. Fungal hyphae invaded the walls of blood vessels and grew within vascular lumen producing related infarction and hemorrhage. Tissue reaction to *Mucorales* genera was similar to the above species, even with more profound angioinvasion (**Figure 2E** and **2F**). *Candida* spp. formed microabscesses with minimal neutrophilic and lymphocytic infiltration and without angioinvasion.

Angioinvasive aspergillosis with fungal balls (**Figure 2A** and **2B**), diffuse necrotizing pneumonia and pulmonary hemorrhage were the most common pathological manifestation of respiratory *Aspergillus* spp. infection. *Fusarium* species caused similar necrotizing pneumonia

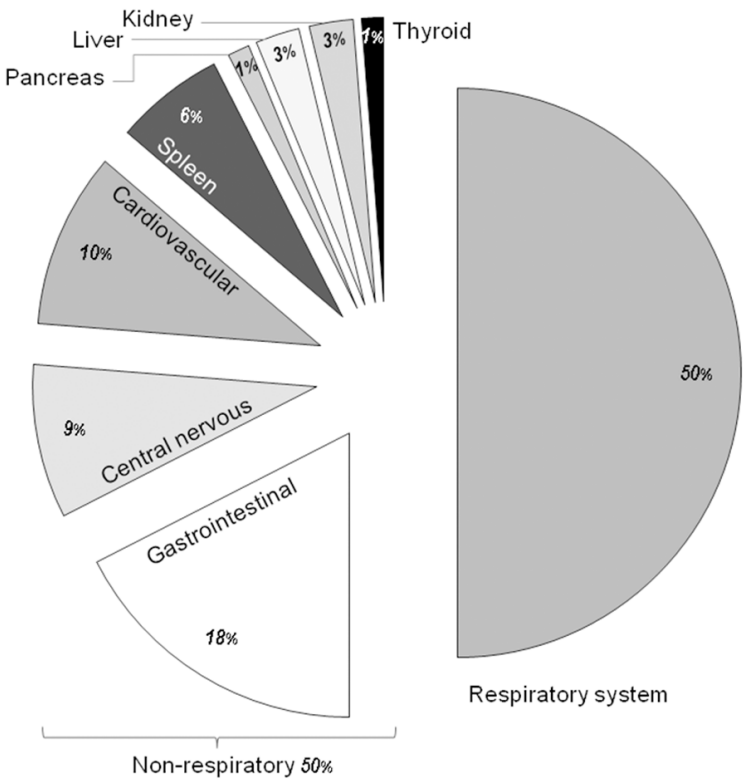

Figure 1. Distribution of systems and organs involved by invasive fungal disease (IFD). Totally 80 visceral organs were affected in 45 patients.

(**Figure 2C** and **2D**). All pulmonary mucormycosis cases revealed angioinvasion with hemorrhage, necrotizing pneumonia, and further pleural extension. Lung involvement by *Candida* spp. was marked by multiple microabscesses. Brain infected by *Aspergillus* spp. and *Mucorales* genera mostly showed angioinvasion with surrounding hemorrhage and necrosis. One case of cerebral aspergillosis produced granulomatous inflammation with central necrosis. Meningeal extension was uncommon. Multiple myocardial abscesses and angioinvasion by fungus were observed in the hearts infected by *Mucorales* genera (**Figure 2G** and **2H**), *Aspergillus* spp. and *Fusarium* spp. All cases of gastrointestinal candidiasis showed mucosal abscesses with mild acute and chronic inflammation.

Molecular study

Fungal DNA was successfully amplified from all specimens histologically proven as IFD. All sequences showed 98-100% identity to sequences deposited in the GenBank database. The most common genera identified by panfungal PCR were *Aspergillus* spp. (44 samples, 58%), followed by *Candida* spp. (12 samples, 16%), *Mucorales* (11 specimens, 14%) and *Fusarium* spp. (8 samples, 10%). The most common sequenced species were *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus* not specified, *Candida tropicalis* and *Rhizopus oryzae* (**Table 4**).

Histopathological diagnosis was mainly limited by fungi genus without designation of species. Correlation rate between histological diagnosis and panfungal PCR was 89.5%, including complete concordance in diagnosing of *Candida* spp., *Mucorales* genera, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Pneumocystis jirovecii*. All 8 samples infected by *Fusarium* spp. were misinterpreted as *Aspergillus* spp. by histopathology (**Table 4**). Two cases of rare IFD caused by *Lichtheimia corymbifera* and *Basidiobolus ranarum*, both of *Zygomycota* phylum, were correctly identified as *Mucorales* by histopathology.

All fungi, except *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Pneumocystis jirovecii* were found in multiple visceral organs (**Table 5**). Twelve different fungal species were identified by panfungal PCR in respiratory system. *Aspergillus* spp. were the most common pathogens affecting lungs (*Aspergillus fumigatus*), brain and heart (both-*Aspergillus flavus*). The most frequent cause of gastrointestinal IFD was *Candida tropicalis*.

Average number of organs invaded by fungus was 1.8 for *Aspergillus* spp. and *Mucorales* (**Table 6**). The main contributors of multiple organ involvement were *Rhizopus oryzae* with the average 3.5 organs affected and *Aspergillus flavus* - 1.9 organs. Mixed IFDs were primarily represented by visceral candidiasis combined with aspergillosis or fusariomycosis (5/6).

Time trends

Postmortem prevalence of IFD increased from 6.3% in 2005-2009 to 10.4% in 2010-2014.

Table 3. Pattern of organ involvement by IFD

Pattern	System/ Fungus	Cases	Period	
			2005-2009, 25 cases	2010-2014, 20 cases
Localized		27	17 (68%)	10 (50%)
Localized isolated	1S/1F		16	10
Localized mixed	1S/2F		1	0
Mixed		6		
Single organ (see Localized mixed)				
Different organs	2S/2F		2	4
Disseminated		13	6 (24%)	7 (35%)
	2S/1F		3	4
	3S/1F		1	0
	4S/1F		0	2
	5S/1F		2	1
Non-disseminated		32	19 (76%)	13 (65%)

We found an increase of disseminated disease from 24% to 35%, and shift from respiratory to non-respiratory (rise from 42.5% to 57.5%) systems involvement. A rate of isolated pulmonary IFD dropped from 68% to 50% over the last 5 years of the study. Disseminated IFD often originated from the intestine and tended to spread via blood into kidney and heart. However, none of these trends were statistically significant, most likely due to a small sample size.

There was a notable re-distribution of causative agents over two 5-year periods. A rate of aspergillosis decreased from 60% to 40%, mainly contributed by lower incidence of *Aspergillus fumigates*. However *Aspergillus flavus* demonstrated statistically significant raising trend in 2010-2014 (12 vs. 7 samples, $P = 0.04$). A prevalence of candidiasis and *Fusarium* IFDs did not change considerably over the time. There was statistically significant increase in rate of mucormycoses in 2010-2014 compared to 2005-2009 ($P < 0.01$).

Discussion

The autopsy remains an established tool for obtaining epidemiological information about diseases and producing vital statistics [18]. It serves as an important measure in monitoring the quality of care by comparing antemortem with postmortem findings. The recommended autopsy rate of 30% is proposed to achieve the above goals [8]. However, over the past recent decades, autopsy rates have steadily declined all over the world. The rates ranging 2-5% were reported from USA, UK and Germany [8, 19, 20]. Statistics from South Asian countries is scarce and also shows very low autopsy rate [14]. Postmortem examinations in our hospital are uncommon (4.4%) and are generally conducted on difficult diagnostic cases. We observed twice the reduction in the number of autopsies during the 10-year observation period with the constant yearly mortality rates. Interestingly, a study published 10 years ago from another local university hospital claimed a 15-20% autopsy rate, probably supplied with forensic cases [13]. There are various reasons contributing the fall in autopsy rates, such as advances in imaging and laboratory diagnostics, difficulties in obtaining consent, religious issues, high costs, and the shift from autopsy to surgical pathology [3, 18].

Current diagnostic approaches offer limited utility for IFD [21]. Autopsy continues to serve as an important tool for evaluating epidemiological trends of IFD [4]. Nationwide, multi-institutional and single-center autopsy series with a focus on visceral mycoses are systematically reported from Japan, Germany and USA [5, 10, 12, 21-23]. Studies in immunocompromised population showed 20-30% rate of terminal IFD [1, 2, 5, 24]. Unselected large-scale series demonstrated lower rates of visceral mycoses in general population ranged 1-5% [8, 11, 21, 25]. The rate of 7.7% found in our study is similar to the ones reported from India (8.7%) and Ramathibodi Hospital, Bangkok (9.4%) [13, 14]. We found rising trend in prevalence of IFD in our series over last 5 years. Partially, it could be influenced by selection bias, due to the drop in autopsy rate and increased proportion of difficult diagnostic cases including visceral mycoses. We were able to conclude that the IFD rate is not decreasing and keeps a level higher than in developed countries.

Compromised immune system is a major predisposing factor to IFD. Most studies from multispecialty hospitals found that hematologic

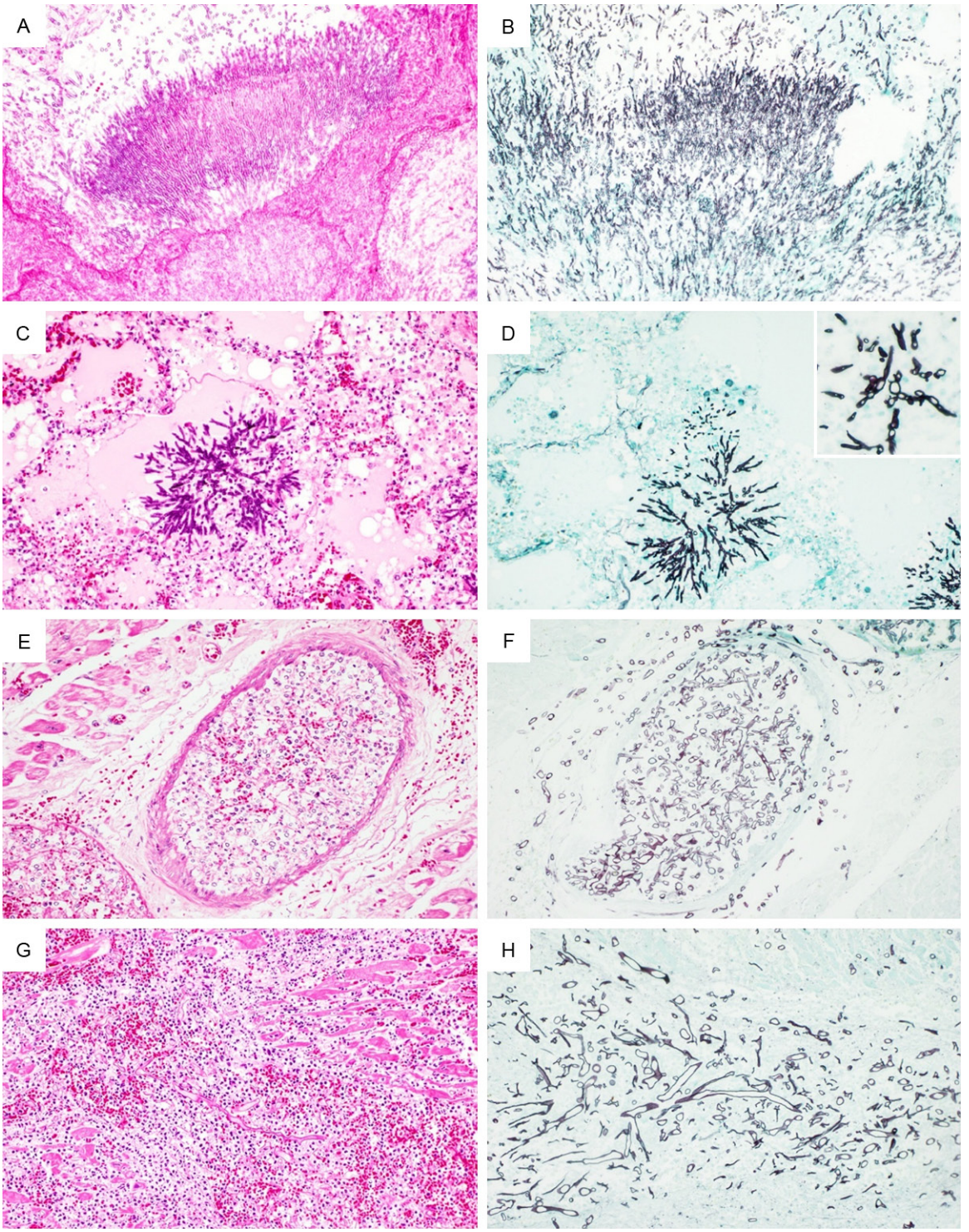

Figure 2. Histopathology of IFD. A, B. *Aspergillus fumigates* forming fungal ball in lung parenchyma. C, D. Intra-alveolar hyphae of *Fusarium equiseti*. Inset shows high power appearance of intercalated chlamydoconidia (i.e. vesicular swellings), which are useful in differential diagnostics between fusariomycosis and aspergillosis. E, F. Cardiac mucormycosis with invasion and obstruction of blood vessel. G, H. Myocardial microabscess caused by *Rhizopus oryzae*. A, C, E, G. Hematoxylin & eosin; B, D, F, H. Gomori's methenamine silver; × 200, inset × 600. All fungi species were classified by panfungal PCR.

malignancy was the most common underlying disease with a frequency 25-45% among all cases of IFD, followed by solid cancer and diabetes mellitus [11-13, 26]. Top three underlying

Table 4. Correlation between fungi species identified by histopathology and panfungal PCR

Histopathological diagnosis	Samples (cases*)	Species by PCR	Samples (cases*)
Aspergillus spp.	48 (30)	*Aspergillus flavus*	19
		Aspergillus fumigatus	13
		Aspergillus niger	2
		Aspergillus not specified	10
		Aspergillus spp.	44 (24)
		Fusarium equiseti	3
		Fusarium not specified	5
		Fusarium spp.	8 (6)
Candida spp.	12 (10)	*Candida albicans*	4
		Candida tropicalis	8
		Candida spp.	12 (10)
Mucorales	11 (6)	*Lichtheimia corymbifera*	1
		Basidiobolus ranarum	2
		Mucor circinelloides	2
		Rhizopus oryzae	6
		Mucorales	11 (6)
Cryptococcus	1 (1)	*Cryptococcus neoformans*	1 (1)
Histoplasma	1 (1)	*Histoplasma capsulatum*	1 (1)
Pneumocystis	3 (3)	*Pneumocystis jirovecii*	3 (3)

*Total number of cases exceeds 45 because of 6 patients with mixed IFD.

diseases in our study were diabetes mellitus, hematologic malignancies and SLE. There are several mechanisms predisposing diabetic patients to fungal infection, which include glycation of complement C3 with impaired attachment to the bacterial surface and further interruption of phagocytosis of fungal element [27], hyperglycemia leading to defective intracellular killing associated with both oxidative and non-oxidative processes [28], and hyperglycemia with low pH condition causing impairment of neutrophilic chemotaxis [29]. Immunosuppression in hematologic malignancy is driven by impaired function of leucocytes and effects of chemotherapy [30]. Hypogammaglobulinemia, hypocomplementemia and steroid therapy contribute to immunosuppression in SLE [31]. High proportion of SLE is concordant with the data from South and Southeast Asia [13-15], however it has not been reported in American and European series. Moreover SLE cases had 3-times higher preponderance of brain involvement, which is in line with earlier Indian series [14]. One more finding that has not been reported previously is an extremely low prevalence of aspergillosis in patients with diabetes mellitus (1 case out of 9). At the same time a prevalence of mucormycoses in diabetic patients was typically high (3/9) [7]. We suppose that our findings regarding SLE and diabetes in IFD are of practical importance and need further validation in a larger cohort. No evident risk factor was found only in 4 patients (2 of them were staying less than 1 day in the hospital), which prompts to suspect IFD in high-risk group subjects.

Intravital diagnostics of IFD is a challenging task due to many reasons including non-specific manifestations, non-specific imaging, lack of sensitive diagnostic tools, loss of serologic response in immunocompromised patients, limitations to use invasive procedures in coagulopative hematologic patients [21]. Fungal infections were diagnosed or suspected antemortem in 68.9% of our cases, similar to the data from another local general hospital [13]. However final clinical diagnosis included IFD just as a part of more complex list of diseases without particular accent in majority of the cases. None of patients that died during first week of stay in the hospital were diagnosed with IFD. Conversely, all the patients that stayed over 1 month had suspected or evident antemortem diagnosis of visceral mycosis. Antemortem culture was done in 24/45 cases, almost exclusively in the cases with respiratory manifestations (BAL, sputum, galactomannan, pleural fluid). Galactomannan assay was positive in 8 patients, 6 of them were confirmed with invasive aspergillosis after autopsy, whereas remaining 2 cases turned out to be *Mucorales* and *Pneumocystis jirovecii* infections. The only case of cryptococcosis was diagnosed by cerebrospinal culture. The correlation between results of antemortem cultures and panfungal PCR was 10/24 (42%) to the genus level.

The most common administered antifungals were amphotericin B (11/22), caspofungin (5/22) and voriconazole (6/22). Four out of these patients received a combination of 2 antimycotics. The mean stay in the hospital for treated patients was 48.1 days. Antifungal

Table 5. Organ involvement by fungi identified by panfungal PCR

	Lung	Bronchus	Stomach	Intestine	Liver	Pancreas	Brain	Heart	Kidney	Spleen	Thyroid	Total
Aspergillus flavus	8	1	1		1	1	3	3	1			19
Aspergillus fumigatus	9	1	1	2								13
Aspergillus niger	2											2
Aspergillus not specified	2			1			1	2	1	2	1	10
Aspergillus spp.	21	2	2	3	1	1	4	5	2	2	1	44
Fusarium equiseti	2								1			3
Fusarium not specified	4							1				5
Fusarium spp.	6							1	1			8
Candida albicans	2			2								4
Candida tropicalis	2			5					1			8
Candida spp.	4			7					1			12
Lichtheimia corymbifera		1										1
Basidiobolus ranarum				1		1						2
Mucor circinelloides	1							1				2
Rhizopus oryzae	1		1				2	1	1			6
Mucorales	2	1	1	1		1	2	2	1			11
Cryptococcus neoformans							1					1
Histoplasma capsulatum	1											1
Pneumocystis jirovecii	3											3
	37	3	3	11	1	2	7	8	5	2	1	80

therapy was never administered to the patients staying less than 1 week. Overall, antifungal treatment, being mainly empirical, could not prevent a lethal outcome. It was demonstrated in the cohort of bone marrow transplant recipients that chemoprevention did not affect significantly rates of autopsy-proven IFD other than candidiasis [32].

We revealed an interesting pattern regarding hospital stay, which showed association of mixed IFD with short stay (median - 6 days) and disseminated IFD with long stay (median - 38 days). Furthermore, only 2 out of 6 patients with mixed IFD were treated with antimycotics, whereas majority of disseminated cases were on antifungal therapy (9/13). Duration of the fever was much longer in disseminated IFD (mean 26.1 vs. 14.3 days, P = 0.03), which may be attributed to the longer stay. Neutropenia was surprisingly rare finding in disseminated cases (15.4% vs. 37.5%). Summarizing the above facts brings awareness to a high risk for disseminated IFD in all immunocompromised patients, who are staying in a hospital over 1 month with a fever over 3 weeks.

We found that women stayed in the hospital significantly longer than men (40.1 vs. 19.2 days, P = 0.02). This issue has not been addressed in previous publications and needs further validation before drawing conclusions on its significance.

Aspergillosis was the most common IFD in our institution followed by candidiasis, fusariomycosis and zygomycosis. The prevalence of aspergillosis outnumbered a sum of three above mycoses. Similar high rates of *Aspergillus* IFD was reported in majority of autopsy studies [1, 5, 10, 12-14, 21, 22, 26]. Visceral candidiasis occupied a second position, however a prevalence of 22% was lower than in most of the published series [5, 10, 12, 13, 21, 26]. The sum of two most common IFDs reached 75%, which is also in a line with the available data [5, 10, 12, 21]. Rare mycoses caused by *Fusarium* spp. and *Mucorales* genera were found in substantial proportion of the cases (25%). Mucormycoses are emerging worldwide, currently being the third most prevalent group of visceral mycoses [4]. Intravital diagnostics of these IFDs is difficult and can be considerably improved by molecular approaches [7].

The most common systems affected by IFD were respiratory and gastrointestinal, which is consistent with the previous reports [11, 13]. This distribution pattern clearly reflects a route of infection. Thus, only 1 case of aspergillosis (out of 24) was found without lung involvement,

Table 6. Average number of organs/systems involved by fungus

	Aspergillus spp.	*Candida* spp.	*Fusarium* spp.	*Mucorales*
Single organ	16	7	3	4
Respiratory	16	3	3	2
Other		4		2
Multiple organs	9	3	2	2
2 organs	4	3	2	1
3 organs	1			
4 organs	2			
5 organs	2			1
Total	25	10	5	6
Organs: Fungus ratio	1.8	1.3	1.4	1.8

and 3 cases of visceral candidiasis (out of 10) were without gastrointestinal lesions. A study of organ involvement in disseminated IFD is important because some new antifungal agents have very low concentration in particular visceral organs [5].

We found some notable differences in epidemiology of IFD when compared our findings with the results from another local university hospital [13]. The rate of aspergillosis was much higher in our study (53% vs. 39%), and the rate of visceral candidiasis was substantially lower (22% vs. 34%). We also found a predominance of *Candida tropicalis* over *Candida albicans* which is opposite to Ramathibodi Hospital, and different *Aspergillus flavus* : *Aspergillus fumigates* ratio (1.5:1 vs. 1:3). All these findings may be partially resulting from time trend, because of the previous study that was done 10 years ago [13]. In addition, a considerable proportion of the *Aspergillus* species were identified as "*Aspergillus* spp." without further stratification (10/44 in our series, 5/34 in Ramathibodi Hospital series), which could affect final numbers. However we strongly believe that there is a real diversity between fungal infections in different hospitals within one city, and this speculation highlights again the role of autopsy in understanding epidemiology of IFD.

As we expected, the epidemiological data from autopsy studies are significantly different from intravital series. A recent study from Northeast Thailand showed that the most common causative agents of IFD were *Cryptococcus* (35%), *Candida* (25%) and *Aspergillus* (12%) [15]. Both antemortem and postmortem approaches significantly complement each other, and are able to provide complete epidemiological profile of IFD in a hospital.

Histological examination of infected tissue is an effective and rapid diagnostic tool for IFD [33, 34]. The major limitation is inability to differentiate between species within a genus, and sometimes between genera within a family. We encountered 6 cases microscopically diagnosed as invasive aspergillosis, but molecular studies identified a fusarial infection. Histopathology of *Aspergillus* and *Fusarium* species shares non-pigmented, septated hyphae with acute angle branching, which makes these genera almost indistinguishable by light microscopy. There are some minor features specific for *Fusarium* spp., including intercalated chlamydoconidia, irregular arrangement of hyphae, constriction of branching point, and scattered right angle branching [16]. After retrospective reviewing of the cases we could find intercalated chlamydoconidia only in 2 cases out of 6 (**Figure 2D**, inset). It suggests that routine culturing and pan-fungal PCR have a critical role to distinguish fusariosis from aspergillosis. Distinction between the genera is important because of different response to antifungal drugs. First line treatment for aspergillosis is voriconazole monotherapy, whereas fusarial infection requires combination of amphotericin B and voriconazole [35].

Overall high histopathology concordance rate to the genus level found in our series (89.5%) did not allow precise species identification, which is essentially today for targeted treatment. Routine culturing is time consuming and cannot reliably recognize some currently emerging fungi like *Mucorales* [36]. New molecular diagnostic approaches for IFD are largely based on DNA sequencing. The most acclaimed method is panfungal PCR, which can identify a wide range of species via targeting of ITS regions of the fungal genome. ITS regions combine both highly conserved and variable sequences, making them universal fungal barcodes. Efficiency of panfungal PCR has been shown in different specimens, including cultures, fresh tissue, blood and FFPE samples [37]. Fungal DNA extraction is a crucial step of

the procedure, and routine histopathology of tissue sections can substantially facilitate identification of a target area containing higher fungal load. DNA degradation is a common drawback of long-stored FFPE samples. Nevertheless we could amplify DNA and reach 98-100% sequence identity in all the specimens, even 10 years old samples. Babouee Flury et al. found similar results and speculated that fungal cell wall may protect against the DNA-degrading effect of formalin [38]. It is important to note that extracted fungal DNA can further be screened for genes of antifungal drug resistance [7]. Total turnaround time in our case, dependent on abroad outsource facility, was 2 weeks. It is still much shorter than the standard 4 weeks for the cultures. In-house facility offers 3-5 days rapid turnaround time [39].

There are several limitations that have to be noted while considering results of the study. First, fungal culture is not routinely performed for postmortem examination in our hospital. Diagnostic yield of postmortem culture is generally low, ranging 30-40% [14, 38, 40]. Low recovery can be attributed to effects of tissue necrosis and antifungal treatment. Being the gold standard for many years, a role of culture in molecular era is re-estimated. Sequence analysis of fungal culture is already proposed as a new "gold standard" for identification of fungi [7]. Panfungal PCR substantially improves diagnosis of mycoses on FFPE samples and reaches 62.5% detection rate, with a high concordance between species identified by culture and molecular technique [38, 39]. Besides postmortem diagnostics necessary for epidemiological and quality control needs, this approach applied to surgery and biopsy specimens can significantly contribute to intravital diagnostics of IFD [37].

Other limitations are relatively low number of cases and short study period. Trends in IFD epidemiology are often recorded over 10 years period, however 5-year intervals for trends were also reported [5, 15, 21, 26]. We could find significant increase in *Aspergillus flavus* and *Mucorales* infections over last 5 years, probably attributed to epidemiological shift within particular hospitals or local population. Our study can be a solid starting point for the future follow up, especially keeping in mind extreme rarity of published autopsy series from Southeast Asia. Additionally, some clinically relevant findings, such as low prevalence of aspergillosis in diabetics, high rate of brain IFD in SLE patients, longer hospital stay for women than men, and association of disseminated IFD with long stay should be further explored in larger series.

In summary, our study showed that IFD is not a rare postmortem finding in unselected Asian population, outnumbering the rates from Western series. The majority of the patients had an immunocompromised background. The most common IFDs were aspergillosis and candidiasis, with emerging rare mycoses caused by *Mucorales* genera and *Fusarium* spp. over the last years. Antemortem diagnostics of visceral fungal infections is challenging, and high missing rates emphasize a role of autopsy study as an essential epidemiological and quality control tool for IFD in the settings of a hospital. Histopathology remains reliable screening approach able to identify fungi to the genus level. Panfungal PCR shows high performance on FFPE samples, allowing rapid detection of IFD causative species. It can be applied retrospectively to archived specimens for epidemiological needs.

Acknowledgements

This study was supported by the Rachadapisek Sompot Endowment Fund of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (KR, SK) and Rachadapisek Sompot Fund for Postdoctoral Fellowship, Chulalongkorn University, Bangkok, Thailand (AB).

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Andrey Bychkov and Somboon Keelawat, Department of Pathology, Faculty of Medicine, Chulalongkorn University, Rama IV Rd, Patumwan, Bangkok 10330, Thailand. Tel: (+66) 2652-4208; Fax: (+66) 2652-4208; E-mail: andrey.bychkov@live.com (AB); Tel: (+66) 2256-4235; Fax: (+66) 2652-4208; E-mail: trcskl@gmail.com (SK)

References

[1] Sinko J, Csomor J, Nikolova R, Lueff S, Krivan G, Remenyi P, Batai A and Masszi T. Invasive fungal disease in allogeneic hematopoietic stem cell transplant recipients: an autopsy-driven survey. Transpl Infect Dis 2008; 10: 106-109.

[2] Antinori S, Nebuloni M, Magni C, Fasan M, Adorni F, Viola A, Corbellino M, Galli M, Vago G, Parravicini C and Ridolfo AL. Trends in the postmortem diagnosis of opportunistic invasive fungal infections in patients with AIDS: a retrospective study of 1,630 autopsies performed between 1984 and 2002. Am J Clin Pathol 2009; 132: 221-227.

[3] Bychkov A, Yamashita S and Dorosevich A. Pathology of HIV/AIDS: lessons from autopsy series. In HIV and AIDS - updates on biology, immunology, epidemiology and treatment strategies. Edited by Dumais N. Rijeka, Croatia: InTech; 2011. pp. 373-392.

[4] Dignani MC. Epidemiology of invasive fungal diseases on the basis of autopsy reports. F1000Prime Rep 2014; 6: 81.

[5] Lewis RE, Cahyame-Zuniga L, Leventakos K, Chamilos G, Ben-Ami R, Tamboli P, Tarrand J, Bodey GP, Luna M and Kontoyiannis DP. Epidemiology and sites of involvement of invasive fungal infections in patients with haematological malignancies: a 20-year autopsy study. Mycoses 2013; 56: 638-645.

[6] De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Muñoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE; European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 2008; 46: 1813-1821.

[7] Halliday CL, Kidd SE, Sorrell TC and Chen SC. Molecular diagnostic methods for invasive fungal disease: the horizon draws nearer? Pathology 2015; 47: 257-269.

[8] Knoke M, Bernhardt H and Schwesinger G. Is there a need for autopsies in the management of fungal disease? Mycoses 2008; 51: 291-300.

[9] Pfaller MA, Pappas PG and R. WJ. Invasive fungal pathogens: Current epidemiological trends. Clin Infect Dis 2006; 43: S3-S14.

[10] Kume H, Yamazaki T, Togano T, Abe M, Tanuma H, Kawana S and Okudaira M. Epidemiology of visceral mycoses in autopsy cases in Japan: comparison of the data from 1989, 1993, 1997, 2001, 2005 and 2007 in Annual of Pathological Autopsy Cases in Japan. Med Mycol J 2011; 52: 117-127.

[11] Shimodaira K, Okubo Y, Nakayama H, Wakayama M, Shinozaki M, Ishiwatari T, Sasai D, Nemoto T, Takahashi K, Ishii T, Saji T and Shibuya K. Trends in the prevalence of invasive fungal infections from an analysis of annual records of autopsy cases of Toho University. Mycoses 2012; 55: 435-443.

[12] Suzuki Y, Kume H, Togano T, Kanoh Y and Ohto H. Epidemiology of visceral mycoses in autopsy cases in Japan: the data from 1989 to 2009 in the Annual of Pathological Autopsy Cases in Japan. Med Mycol 2013; 51: 522-526.

[13] Larbcharoensub N, Srisuma S, Ngernprasertsri T, Aroonroch R, Chongtrakool P, Santanirand P, Chirachariyavej T and Sirikulchayanonta V. Invasive fungal infection in Ramathibodi Hospital: a ten-year autopsy review. J Med Assoc Thai 2007; 90: 2630-2637.

[14] Uppin MS, Anuradha SV, Uppin SG, Paul TR, Prayaga AK and Sundaram C. Fungal infections as a contributing cause of death: an autopsy study. Indian J Pathol Microbiol 2011; 54: 344-349.

[15] Faksri K, Kaewkes W, Chaicumpar K, Chaimanee P and Wongwajana S. Epidemiology and identification of potential fungal pathogens causing invasive fungal infections in a tertiary care hospital in northeast Thailand. Med Mycol 2014; 52: 810-818.

[16] Guarner J and Brandt ME. Histopathologic diagnosis of fungal infections in the 21st century. Clin Microbiol Rev 2011; 24: 247-280.

[17] White TJ, Bruns TD, Lee SB and Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. New York: Academic Press, 1990.

[18] Burton JL and Underwood J. Clinical, educational, and epidemiological value of autopsy. Lancet 2007; 369: 1471-1480.

[19] McPhee SJ. Maximizing the benefits of autopsy for clinicians and families. What needs to be done. Arch Pathol Lab Med 1996; 120: 743-748.

[20] Perkins GD, McAuley DF, Davies S and Gao F. Discrepancies between clinical and postmortem diagnoses in critically ill patients: an observational study. Crit Care 2003; 7: R129-132.

[21] Alsharif M, Cameron SE, Young JA, Savik K, Henriksen JC, Gulbahce HE and Pambuccian SE. Time trends in fungal infections as a cause of death in hematopoietic stem cell transplant recipients: an autopsy study. Am J Clin Pathol 2009; 132: 746-755.

[22] Donhuijsen K, Petersen P and Schmid WK. Trend reversal in the frequency of mycoses in hematological neoplasias: autopsy results from 1976 to 2005. Dtsch Arztebl Int 2008; 105: 501-506.
[23] Koch S, Hohne FM and Tietz HJ. Incidence of systemic mycoses in autopsy material. Mycoses 2004; 47: 40-46.
[24] Chamilos G, Luna M, Lewis RE, Bodey GP, Chemaly R, Tarrand JJ, Safdar A, Raad II and Kontoyiannis DP. Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989-2003). Haematologica 2006; 91: 986-989.
[25] Colombo TE, Soares MM, D'Avilla SC, Nogueira MC and de Almeida MT. Identification of fungal diseases at necropsy. Pathol Res Pract 2012; 208: 549-552.
[26] Lehrnbecher T, Frank C, Engels K, Kriener S, Groll AH and Schwabe D. Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. J Infect 2010; 61: 259-265.
[27] Hostetter MK. Handicaps to host defense. Effects of hyperglycemia on C3 and Candida albicans. Diabetes 1990; 39: 271-275.
[28] Chinn RY and Diamond RD. Generation of chemotactic factors by Rhizopus oryzae in the presence and absence of serum: relationship to hyphal damage mediated by human neutrophils and effects of hyperglycemia and ketoacidosis. Infect Immun 1982; 38: 1123-1129.
[29] Eucker J, Sezer O, Graf B and Possinger K. Mucormycoses. Mycoses 2001; 44: 253-260.
[30] Balloy V, Huerre M, Latge JP and Chignard M. Differences in patterns of infection and inflammation for corticosteroid treatment and chemotherapy in experimental invasive pulmonary aspergillosis. Infect Immun 2005; 73: 494-503.
[31] Kim HJ, Park YJ, Kim WU, Park SH and Cho CS. Invasive fungal infections in patients with systemic lupus erythematosus: experience from affiliated hospitals of Catholic University of Korea. Lupus 2009; 18: 661-666.
[32] van Burik JH, Leisenring W, Myerson D, Hackman RC, Shulman HM, Sale GE, Bowden RA and McDonald GB. The effect of prophylactic fluconazole on the clinical spectrum of fungal diseases in bone marrow transplant recipients with special attention to hepatic candidiasis. An autopsy study of 355 patients. Medicine (Baltimore) 1998; 77: 246-254.
[33] Dekio F, Bhatti TR, Zhang SX and Sullivan KV. Positive impact of fungal histopathology on immunocompromised pediatric patients with histology-proven invasive fungal infection. Am J Clin Pathol 2015; 144: 61-67.
[34] Schuetz AN and Walsh TJ. Importance of fungal histopathology in immunocompromised pediatric patients: It's not just "Aspergillus" anymore. Am J Clin Pathol 2015; 144: 185-187.
[35] Stergiopoulou T and Walsh TJ. Clinical pharmacology of antifungal agents to overcome drug resistance in pediatric patients. Expert Opin Pharmacother 2015; 16: 213-226.
[36] Hammond SP, Bialek R, Milner DA, Petschnigg EM, Baden LR and Marty FM. Molecular methods to improve diagnosis and identification of mucormycosis. J Clin Microbiol 2011; 49: 2151-2153.
[37] Rickerts V. Identification of fungal pathogens in formalin-fixed, paraffin-embedded tissue samples by molecular methods. Fungal Biology 2015. doi:10.1016/j.funbio.2015.07.002.
[38] Babouee Flury B, Weisser M, Prince SS, Bubendorf L, Battegay M, Frei R and Goldenberger D. Performances of two different panfungal PCRs to detect mould DNA in formalin-fixed paraffin-embedded tissue: what are the limiting factors? BMC Infect Dis 2014; 14: 692.
[39] Lau A, Chen S, Sorrell T, Carter D, Malik R, Martin P and Halliday C. Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. J Clin Microbiol 2007; 45: 380-385.
[40] Tarrand JJ, Lichterfeld M, Warraich I, Luna M, Han XY, May GS and Kontoyiannis DP. Diagnosis of invasive septate mold infections. A correlation of microbiological culture and histologic or cytologic examination. Am J Clin Pathol 2003; 119: 854-858.