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Original Article

Molecular typing and antifungal susceptibility study of *Aspergillus* spp. in intensive care unit (ICU) patients in Indonesia

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Abstract

Introduction: *Aspergillus* exhibits a wide variation of susceptibility against antifungals according to genetic and environmental factors. Identification to the species level is necessary for appropriate treatment. Our objective was to determine the *Aspergillus* species involved in invasive pulmonary aspergillosis (IPA) among ICU patients in Jakarta, Indonesia.

Methodology: The incidence of IPA in ICU patients at six hospitals in Jakarta from October 2012 – January 2015 was investigated. It involved a collection of endotracheal aspirates (ETA), nasal swabs and environmental samples around the hospitals, phenotypic screening, molecular characterization, and antifungal susceptibility testing.

Results: Of the 405 patients investigated, 31 patients (7.7%) were diagnosed with putative IPA, from whom 45 *Aspergillus* isolates were collected. *Aspergillus* isolates were identified from pulmonary secretions in 24 patients, from nasal swabs in 7 patients and from both pulmonary secretions and nasal swabs in 7 patients. The phenotypic method showed 33 isolates of *Aspergillus flavus* (73.4%), nine *Aspergillus fumigatus* (20%), two *Aspergillus niger* (4.4%), and one *Aspergillus nidulans* (2.2%) isolate. Molecular identification showed 27 isolates of *A. flavus* (60.0%), eight isolates of *A. fumigatus* (17.8%), two isolates of *A. niger* (4.4%) and one isolate of *A. nidulans* (2.2%), while seven isolates (15.6%) were cryptic species or mixed isolates.

Conclusions: Susceptibility testing showed all isolates were susceptible to amphotericin B, azoles and micafungin. *Aspergillus flavus* was the main causative organism in IPA cases in Jakarta, followed by *A. fumigatus*.

Key words: *Aspergillus*; molecular typing; susceptibility.

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Introduction

Invasive pulmonary aspergillosis (IPA) has increasingly been documented as a major cause of morbidity and mortality in patients with underlying immunodeficiencies, including critically ill patients in the Intensive Care Unit (ICU). The disease is characterized by acute invasion of the bronchi or lung parenchyma by *Aspergillus* hyphae [1,2]. *Aspergillus* section *Fumigati*, section *Flavi*, section *Nigri* and section *Terrei* are responsible for causing mist cases of IPA. Those terms represent the complexes or sections of cryptic species (closely related species that are difficult to differentiate based on morphology) [3]. *Aspergillus fumigatus* is widely reported as the leading cause of IPA in Europe and America. Meanwhile, *Aspergillus* section *Flavi* complexes are documented as the main cause for IPA in tropical and sub-tropical climate zones [4–6].

Identification of *Aspergillus* to the species level is of utmost importance since each species has a different antifungal susceptibility. However, phenotypic identification is inadequate, because the microscopic

characteristics among different species may be identical. Molecular methods could, therefore, shorten the species identification procedure and contribute to the selection of appropriate antifungal treatment [7]. Species other than *A. fumigatus* and cryptic species have increasingly been isolated from clinical specimens during the past two decades. The epidemiological changes are related to the increasing number of immunocompromised patients, advances in fungal detection and identification, as well as the extensive use of broad-spectrum antifungal drugs in clinical practice and agriculture. Those situations pose a great challenge to IPA management. In fact, the emergence of triazole resistance among *Aspergillus* species has become a global public health menace nowadays [8–10].

Unfortunately, published data on the etiology of IPA in Indonesia and the antifungal susceptibility pattern of *Aspergillus* species are scarce. This study, therefore, aimed to determine the etiology of IPA in ICU patients based on morphological and molecular tests, as well as the antifungal susceptibility patterns of the identified isolates.

Methodology

This study was part of a prospective multi-center cohort study to observe the incidence of IPA in ICU patients at six hospitals in Jakarta, Indonesia, from October 2012 – January 2015. Informed consent forms were signed by all participants. The study was carried out in accordance with the principles of the Helsinki Declaration, and approved by the Ethics Committee of the Faculty of Medicine Universitas Indonesia. There were six participating hospitals: Cipto Mangunkusumo Hospital, Persahabatan Hospital, Sulianti Saroso Hospital, Dharmais Cancer Hospital, Universitas Kristen Indonesia Hospital, and PGI Cikini Hospital.

Patients and specimens

Subjects in this study were patients suspected of IPA who met the inclusion criteria, as previously described [11]. The case group included patients who met the diagnostic criteria of probable/putative IPA (according to European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC) and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) [12] and AspICU [1] criteria), while the control group was randomized amongst patients who did not suffer from IPA. Probable IPA was diagnosed in patients with all of: 1) at least one of these conditions including prolonged use of corticosteroids, treatment with other immunosuppresants, a history of neutropenia, receipt of an allogeneic stem cell transplant, or inherited severe immunodeficiency, 2) at least one of these chest imaging findings including cavity, air-crescent sign, or dense, well-circumscribed lesion(s) with or without halo sign, 3) at least one of these following mycological criteria including direct microscopy/culture/cytology on sputum, broncho-alveolar lavage fluid, nasal swab, pulmonary secretions showed fungal elements or positive culture of *Aspergillus* or detection of galactomannan in serum or pulmonary secretions. Patients/families who declined the informed consent to join the study and patients who received antifungal therapy within the previous month were excluded from the study.

The consecutive sampling method was used to obtain samples for *Aspergillus* identification and susceptibility testing. There were 31 IPA patients (probable), and the control group was four-fold (124 patients). Sixty-two environmental isolates from indoor air of wards and outdoor air around the hospitals were also examined to consider the possible source of infection. Settle plates was performed as a form of passive air sampling [13]. Petri dishes were placed in several places inside ICU and outside ICU around hospitals for 15 minutes, 1 m from the floor, and 1 m away from the walls, as a modification methods of previously described method of air sampling [14].

Collection of clinical and environmental specimens, phenotypic identification and susceptibility testing were carried out in the Mycology Laboratory of Parasitology Department, Faculty of Medicine, Universitas Indonesia (FMUI), Jakarta, Indonesia, while molecular characterization was performed in the Microbiology Laboratory of Canisius-Wilhelmina Ziekenhuis (CWZ), Nijmegen, the Netherlands. The control isolate for *A. flavus* microsatellite typing examination was (NRRL 3357). The control isolates for antifungal susceptibility were *A. flavus* ATCC 204304, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, and *Trichophyton mentagrophytes* ATCC MYA 4439.

Isolation and identification of Aspergillus

The clinical specimens were cultured onto Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, MI, USA) according to Mertz *et al*. [15]. Culture plates were observed daily for growth for up to 10 days. Phenotypic identification of *Aspergillus* isolates were carried out based on macroscopic and microscopic morphology according to the standard operating procedure of the Mycology Laboratory, Parasitology Department, FMUI.

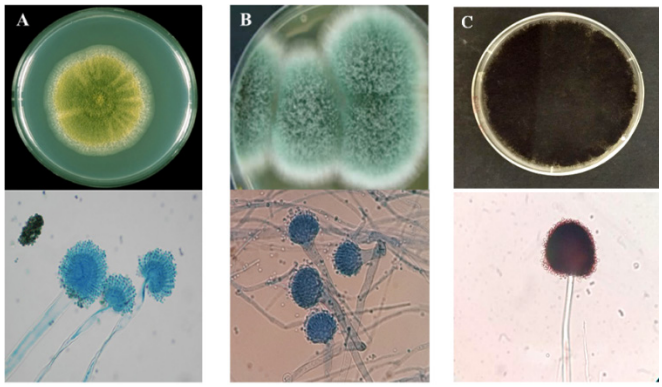
Microsatellite typing

Aspergillus DNA was isolated and extracted as previously described by de Valk *et al*. [16]. For *A. fumigatus*, microsatellite typing was carried out using a panel of nine short tandem repeats of *A. fumigatus* (STR*Af*). The amplification of three di-, tri-, or tetra-nucleotide repeat markers was performed using three multiplex polymerase chain reactions (PCRs), specifically M2, M3, and M4, according to de Valk *et al*. [17]. Repeat numbers in each marker were determined using *A. fumigatus Af*293 as reference. *Aspergillus flavus* microsatellite typing was performed as described previously by Khodavaisy *et al*. [18]. The microsatellite data were analyzed using BioNumerics version 7.5 (Applied Maths, St. Martem-Latum, Belgium). Previously reported *A. flavus* microsatellite data [19] were used to compare with those from the current study. On the genotypic map produced, percentages indicated the number of corresponding markers; for example, two lines with six of the nine corresponding markers showed a 66.7% similarity.

Antifungal susceptibility testing

In vitro antifungal susceptibility testing was carried out based on the method of Clinical and Laboratory Standards Institute, CLSI M38-A2 (2008) for filamentous fungi. The antifungal agents tested were amphotericin B (AMB), itraconazole (ITC), voriconazole (VRC), posaconazole (PSC) (Sigma-Aldrich, St. Louis, MO, USA), isavuconazole (ISA; Basilea Pharmaceutica, Basel, Switzerland), and micafungin (MICA; Astellas Pharma Inc., Osaka, Japan). The concentration range for the azoles and AMB was 0.03-16 μg/mL, while for micafungin it was 0.015-8 μg/mL. The susceptibility plates were incubated at 37 °C for 24-48 hours and read immediately after or on the next few days. The minimum inhibitory concentration (MIC) for AMB or each azole was determined as the lowest concentration of the antifungal drug that prevented any visible growth (100% inhibition), while the minimum effective concentration (MEC) for micafungin was determined as the lowest concentration of the antifungal drug which showed the growth of rounded compact hyphae compared to the unchanged hyphal growth in the control well. We used the screening plates of antifungal susceptibility tests [20] as described in a previous paper before using CLSI method. Three screening plates containing antifungal and one drug-free agar control well were prepared for each isolate. The inoculum of *Aspergillus* isolates with volume of 25 μL was poured into the plates with subsequent incubations at 34 °C – 37 °C. Evaluations were performed after 48 hours [20].

Figure 1. Morphology of macro-colony of: (A) *Aspergillus flavus*; (B) *Aspergillus fumigatus*; and (C) *Aspergillus niger* on Sabouraud Dextrose agar (top images), and its micro-colonies on slide agar (bottom images).



Results

Out of the 405 patients investigated, 31 (7.7%) were diagnosed as probable IPA patients. There were 45 *Aspergillus* isolates obtained from 38 patients, of these seven patients had *Aspergillus* isolates from pulmonary secretions and nasal swabs, 24 patients had *Aspergillus* isolates only from pulmonary secretions, and seven patients had *Aspergillus* isolates solely from nasal swabs. The phenotypic profile of *Aspergillus* identified by the classical method is shown in Figure 1.

The identification of *Aspergillus* species by the classical method was substantiated with molecular identification by ITS sequencing (Table 1). Classical identification showed nine isolates of *A. fumigatus* (20%), 33 isolates of *A. flavus* (73.4%), two isolates of *A. niger* (4.4%), and one isolate of *A. nidulans* (2.2%).

Table 1. Identification of *Aspergillus* from clinical isolates by phenotypic method and microsatellite typing.

Identification results	Clinical isolate (n = 45)				Information
	Phenotypic method		Microsatelite typing		
	n	%	n	%	
A. flavus	33	73.4	27	60.0	Microsatelite typing was carried out
A. fumigatus	9	20.0	8	17.8	
Aspergillus sp.	-	-	7	15.6	
A. niger	2	4.4	2	4.4	
A. nidulans	1	2.2	1	2.2	

Molecular identification of those 45 isolates showed that eight isolates were *A. fumigatus* (17.8%), 27 isolates *A. flavus* (60%), two isolates of *A. niger,* and one isolate of *A. nidulans,* and seven other isolates (15.6%) were *Aspergillus* cryptic species or mixed isolates. In this study, *A. flavus* was the predominant species.

The microsatellite genotypes of Indonesian *A. flavus* isolates were compared with isolates from the Netherlands, India, Kuwait, and the Czech Republic to assess the genotypic diversity (Figure 2). The Indonesian *A. flavus* isolates clustered separately and had heterogeneous, variable microsatellite profiles. Some Indonesian isolates were connected by a dotted thick line with isolates from India and Kuwait, indicating that similarity was observed in six of the nine loci studied. It can be assumed that Indonesian isolates were more closely related with Indian and Kuwaiti isolates, but the genetic correlations needs to be further explored.

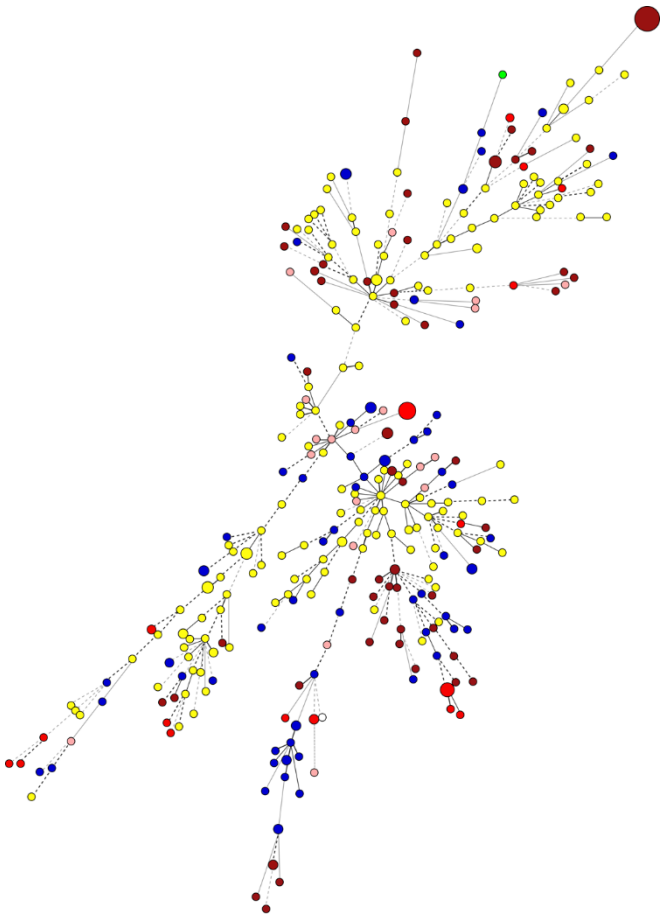
Molecular profiles of *A. fumigatus* showed that Indonesian isolates did not have genotypic similarities with isolates from India, Iran, and Australia. Some clinical isolates of *A. flavus* and *A. fumigatus* showed intersection patterns with the environmental isolates, but the genotypic similarities remain to be investigated.

The antifungal susceptibility results of the 31 clinical isolates showed that almost all *Aspergillus* isolates were susceptible to antifungal drugs. Only one *Aspergillus* spp. isolate was initially considered resistant. However, molecular characterization of the 'resistant' isolate showed that it was a mixed colony of *Penicillium citrinum* and *Aspergillus tamarii*. Thus, the susceptibility testing did not yield any resistant *A. fumigatus* or *A. flavus* isolate in this study. The antifungal minimum inhibitory concentrations (MIC) of seven clinical isolates are shown in Table 2.

Discussion

In this study, *A. flavus* was the main species in causing IPA in Jakarta. This is the first report of such observation from Jakarta, and it represents a different etiology of IPA to what is known for some other countries [21–24]. It might be related with the geographical and climatic conditions in Indonesia, causing *A. flavus* to grow more easily and become the most common species isolated from the environment.

Figure 2. Molecular profile of *Aspergillus flavus* microsatellite typing.



Red nodes, Indonesian clinical isolates; green nodes, Indonesian environmental isolates; blue nodes: Dutch patient isolate (cystic fibrosis); yellow nodes, Indian isolates; brown nodes: Kuwaiti isolates; peach nodes: Czech Rep. isolates. Solid thick line: 1 of 9 markers show the difference; thin solid line: 2 of 9 markers show the difference; dotted thick line: 3 of 9 markers show the difference; thin dashed line: 4 of 9 markers show the difference; thin dotted line >4 of 9 marker shows the difference.

Table 2. Antifungal susceptibility values of clinical *Aspergillus* isolates.

***Aspergillus* species**	**Specimen origin**	**AFT screening**	**Antifungal MIC (µg/mL) of:**					
			AMB	**ITC**	**VRC**	**PSC**	**ISAVU**	**MICA**
Aspergillus flavus	ETT Aspirate	VRC	1	0.125	0.5	0.125	0.5	0.016
Aspergillus fumigatus	ETT Aspirate	ITC	1	0.125	0.5	0.063	0.063	0.016
Aspergillus flavus	Nasal swab	VRC	1	0.125	0.5	0.125	0.5	0.016
Aspergillus flavus	ETT Aspirate	ITC	0.5	0.063	0.125	0.063	0.063	0.016
Aspergillus fumigatus	Nasal swab	ITC+VRC	0.125	0.125	0.125	0.063	0.5	0.016
Aspergillus fumigatus	Nasal swab	ITC+VRC	0.25	0.25	0.25	0.063	0.5	0.016
Aspergillus nidulans	Nasal swab	ITC+VRC+PSC	0.25	0.25	0.25	0.063	0.5	0.016

The result is similar to results from other studies in Asia, Africa and the Middle East [25]. *Aspergillus flavus* was the most common cause of invasive aspergillosis in India, Pakistan, Qatar, and Iran. The most common presentations of invasive aspergillosis due to *A. flavus* is invasive rhinosinusitis and pulmonary forms [6].

The pathogenicity and clinical syndromes caused by *A. flavus* and *A. fumigatus* are correlated to the size of conidia, the structure of conidia's outer surface, and fungal pigments. The size of *A. flavus* conidia (3-6 µm) is greater than that of *A. fumigatus* (2-3.5 µm), making *A. flavus* more easily deposited in the upper airway to become a major cause of aspergillosis in the sino-orbital or rhino-cerebral region. The speed of *Aspergillus* conidia sedimentation of different sizes might play an important role as well [26].

The isolation of *Aspergillus* from clinical material must be identified to species level, considering that each species has different antifungal susceptibility patterns [27]. Molecular identification has advantages over the phenotypic classical method, including better sensitivity and specificity, higher accuracy in determining the species level, and ability to detect fungi directly from clinical materials. However, one key obstacle to using molecular techniques is lack of standardization, causing limited use in daily practice [28]. Additionally, the limitations of molecular typing are the high cost of the tests and the limited access of these facilities in most of the mycology laboratory in Indonesia.

Molecular typing showed that *A. flavus* and *A. fumigatus* isolates from Jakarta appeared to be scattered and not clustered in one particular place and mostly connected by thin dashed lines. In addition, the *Aspergillus* distribution paths from various places did not show the same linkages or origin. It seems that *A. flavus* and *A. fumigatus* strains from Jakarta have no genotypic similarities with those from other countries, and genotypic diversity between isolates was also discovered. The different genotypic profiles can be related to differences in genetic traits and fungal characteristics, including fungal pathogenicity and sensitivity to drugs [29].

There were seven *Aspergillus* isolates that failed microsatellite typing with the *A. fumigatus* and *A. flavus* specific markers. Those isolates were thought to be the cryptic species or mixed isolates that were difficult to separate. Cryptic species in *Aspergillus* section *Fumigati* are reported to be more resistant to antifungal agents. Furthermore, cryptic species in *Aspergillus* section *Flavi* were also more resistant to antifungal drugs [24].

Antifungal susceptibility testing is very important in the management of IPA, as it is able to predict therapeutic failure. In this study, one clinical *Aspergillus flavus* isolate was found to be resistant to itraconazole and voriconazole. However, sequencing analysis showed that the isolate was a mixed colony of *A. tamarii* and *Penicillium citrinum. Aspergillus tamarii* is a cryptic species of *A. flavus*, and was identified as a cause of superficial aspergillosis, including keratitis [30]. Furthermore, *P. citrinum* is a contaminant fungus and known as an azole resistant species, but rarely reported as a cause of infection [23]. Fatal infections of this fungi was found in an acute myeloid leukemia patient who had pneumonia and post-induction chemotherapy pericardial tamponade [31]. We concluded that all isolates of *A. flavus* and *A. fumigatus* in this study were still susceptible to the antifungal drugs tested.

Aspergillus infection might occur in community environment or hospital environment (nosocomial), or even both [32]. At the time of this research, building renovations and reconstruction were being carried out in Cipto Mangunkusumo Hospital, Persahabatan Hospital, and Cikini Hospital. This situation might become a potential source of nosocomial *Aspergillus* transmission because the possibility of the dust during hospital reconstruction might carry transient *Aspergillus* spores [33]. Conducting antifungal susceptibility profiling of *Aspergillus* from the environment is also indispensable if we would be able to anticipate the emergence of resistant species that could complicate IPA management [34].

In ICU patients who are at risk of IPA, the isolation of *Aspergillus* from the airways, including nasal cavity, should be carefully considered as an early indication of infection, and not just colonization. Aisner *et al*. [35] reported that isolation of *Aspergillus* from the nasal cavity of acute leukemia patients has a 90% correlation with IPA. Isolation of *Aspergillus* from nasal culture of leukemia patients when they are entering the hospital for chemotherapy could be a poor prognosis due to the high likelihood of IPA [36]. Chronic obstructive pulmonary disease (COPD) is the most common underlying condition of IPA in ICU patients. In certain circumstances, *Aspergillus* colonization in the respiratory airway of COPD patients may lead to invasive infection [37].

Conclusions

The identification of *Aspergillus* species based on phenotypic method is relatively insensitive since microscopic characteristics of several different species may be identical. Molecular methods might accelerate species identification procedures, more sensitive and accurate, so that the selection of appropriate antifungal drugs can be determined more promptly. This study underlines *A. flavus* as the main cause of IPA in Jakarta, followed by *A. fumigatus*. All isolates were still sensitive to antifungal agents. The identification of *Aspergillus* from clinical and environmental isolates is important, as well as the antifungal susceptibility testing, to achieve better IPA management.

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