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Diversity of Fungal Colonization in Respiratory Tract of Naïve Lung Cancer and The Emergence of Voriconazole Resistant *Aspergillus*

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ABSTRACT

Fungal spores in the air can be inhaled and enter the human respiratory tract. The entry of fungi into the respiratory tract can cause colonization or infection depending on the host immune response. Fungal colonization is the first step into debilitating fungal disease in humans, especially in immunocompromised groups. The increased rate of drug-resistant fungi has been reported in human disease and the environment. This study aims to examine the diversity of fungal colonization in humans and the rate of fungal resistance to voriconazole. This cross-sectional study was done in patients with naïve lung cancer who had not been previously treated with any cancer therapy nor given antifungal agent. Induced sputum from 70 subjects was collected and inoculated in the Sabouraud Dextrose Agar medium. Macroscopic and microscopic examinations were performed to identify fungal species. Voriconazole susceptibility tests were done using the disc diffusion method. This study found *Candida albicans*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Penicillium* sp. among the most common lower respiratory tract colonies. This study also found the colonization of up to 5 species in a single subject. A high rate of voriconazole-resistant *Aspergillus* sp. was found (42.4%) among 59 isolates tested. Given that these subjects had never taken antifungal agents previously, the high rate of voriconazole resistance might be attributed to the environment, such as community and agriculture. Mitigation of antifungal use in the agricultural sector, fungal diversity in the environment, and clinical study of fungal colonization/ infection in other high-risk groups are needed.

1. Introduction

Fungi are ubiquitous microorganisms commonly found in the human environment. Fungi can produce small inhalable spores or conidia that are able to survive in certain environments over time (Rozaliyani *et al.* 2019; van Rhijn and Bromley 2021). Climate change, natural disaster and agricultural use of antifungals might drive environmental pressures affecting the evolution of traits, including virulence and antifungal resistance (Hall *et al.* 2018; van Rhijn and Bromley 2021). These conditions could enhance the possibility of spore aerosolization and lead to inhalation of the conidia into the respiratory tracts of humans (van Rhijn and Bromley 2021). Colonization occurs when fungi are identified on the body surface without causing disease and the early sign of fungal infection and fungal diseases. However, the fungal disease is relatively uncommon in healthy human beings (Rozaliyani *et al.* 2019; van Rhijn and Bromley 2021). Airway colonization is common in immunocompromised hosts such as individuals with HIV/AIDS, cancer, and mechanically ventilated patients, and this often leads to fungal infection/ disease. Therefore mitigation of colonization among immunocompromised hosts is important (Jose and Brown 2016; Hall *et al.* 2018).

We have conducted a cross-sectional study to identify fungal colonization among newly diagnosed

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lung cancer/naïve treatment that might play an important role in lung mycosis. Induced sputum from naïve lung cancer was cultured and fungal isolates were identified for each subject. Further antifungal voriconazole drug susceptibility test was done.

2. Materials and Methods

2.1. Patients Selection

Subjects were recruited at Persahabatan National Respiratory Referral Hospital from June 2019 to June 2020. The study's inclusion criteria included non-small cell lung cancer (confirmed either histologically or cytologically), patients aged more than 18 years, and had not been previously treated with any systemic cancer therapy (newly diagnosed naïve lung cancer). Lung cancer staging was based on the 8th Edition of the TNM classification for non-small cell lung cancer. Data were obtained from patient history, questionnaires, and medical records from the hospital.

2.2. Evaluation of Fungal Colonization in the Respiratory Tract

2.2.1. Induced Sputum

Specimens for fungal culture were obtained from sputum induction. Sputum induction is a simple and safer technique to sample the lower respiratory tract and bronchus compared to direct sampling with bronchoscopy.

The subjects were instructed to rinse their mouths with chlorhexidine gluconate 0.2% to clean and reduce the upper airway and mouth contamination. Then hypertonic saline (2-3 ml NaCl 3%) solution was nebulized with a nebulizer, and the subject was asked to perform tidal breathing for 10 to 15 minutes to inhale the particles. After nebulization, the subjects were asked to perform complete inspiration for 5 minutes intervals, followed by coughing and expectoration (Weiszhar and Horvath 2013). The fresh sputum was collected and sent to the mycology laboratory of the Department of Parasitology, Faculty of Medicine Universitas Indonesia.

2.2.2. Fungal Culture

The specimen was cultured in a Sabouraud Dextrose Agar (SDA) mixed with chloramphenicol as an antibiotic. Using the high-volume culture method, fungi samples were isolated in two Petri dishes poured with the entire volume of sputum collected. The Petri dishes were incubated at 32-37°C (room temperature) and at 48°C until fungal growth was noted. The incubation at 48°C was also done to facilitate the growth of *A. fumigatus* specifically. The fungal growth was observed for 14 days and then evaluated for species identification and antifungal sensitivity test (Jorgensen and Pfaller 2015).

2.2.3. Fungal Identification

Species identification was performed by observing fungal colonies macroscopically and microscopically. Macroscopic examination assessed the colony form, color, bottom surface, and edge form. Fungal colonies were taken with a sterile inoculating loop to make slides. Microscopic examination assessed the fungal element and sporulation (spores, hyphae, or yeast) with a microscope. The fungi were identified microscopically with a Lactophenol Cotton Blue (LPCB) stain. The fungal structure was identified under 40× object-glass magnification (Jorgensen and Pfaller 2015).

2.2.4. Antifungal (Voriconazole) Susceptibility Test

Voriconazole is newer class of azole and reserved as first-line therapy for invasive aspergillosis. Voriconazole is available in tablet, suspension, and IV forms and is categorized as a broad-spectrum antifungal (Jenks and Hoenigl 2018).

The antifungal voriconazole drug susceptibility test was done in *Aspergillus* sp. isolates. *Aspergillus* sp. suspension was smeared onto the Mueller-Hinton Agar twice; thus, the fungi were equally distributed. Voriconazole disc (1 µg) was added to the agar surface followed by incubation at room temperature for 2 days. The susceptibility test result was categorized based on the modified Clinical and Laboratory Standards Institute (CLSI) M44-A2 criteria by measuring the inhibition zone diameter. Calipers were used to measure the inhibition zone diameter. The inhibition zone diameter is categorized into sensitive/susceptible if zone diameter is ≥17 mm, intermediate if zone diameter is between 14-16 mm, and resistant if zone diameter is ≤13 mm (Espinel-Ingroff *et al.* 2007; Arikan 2007) .

2.3. Statistical Analysis

Data were analyzed using SPSS version 25th. The diversity of fungal colonization and antifungal sensitivity test were presented as descriptive statistics. A comparative test using Chi-square was performed to identify risk factors or clinical

presentations that affect the culture results of *Aspergillus* sp.

3. Results

3.1. Demographics

Among 70 subjects of newly diagnosed, treatment naïve lung cancer patients, 61.4% (43 subjects) were male. In most cases, the age was 18-65 years old (72.9%), with a mean age of 59.83±9.18 years old. Active smokers were identified in 42 subjects (60%). The histopathology characteristics of lung cancer were mostly adenocarcinoma with advanced lung cancer stage IIIB-IV (78.6%). Clinical symptoms in the last three months experienced by the subjects included dyspnea (81.4%), chest pain (71.4%), and weight loss (71.4%). Comorbidities commonly found were Diabetes Mellitus (18.6%), previous tuberculosis (12.9%), and asthma/COPD (1.4%),

3.2. Fungal Species

In this study, there were 140 fungal isolates grown and identified in the induced sputum from 70 newly diagnosed naïve lung cancer.

The macroscopic examination was used to determine the fungal species. The isolate was differentiated by colony form, bottom surface, edge form, and color as shown in Table 1. The representative of macroscopic fungal colonies grown in SDA are shown in Figure 1.

Microscopic examination was used to observe fungal structure and sporulation (yeast, spores, yor hyphae) and help identify the species. Figure 2 shows the microscopic images of the fungal isolate under the LPCB mount.

Sixty-seven *Aspergillus* sp. isolates were found (61.4%), consisting of *Aspergillus niger* in 31 isolates (22.1%), *Aspergillus fumigatus* in 25 isolates (17.9%), and *Aspergillus flavus* in 11 isolates (7.9%). *Candida* sp. were found in 65 isolates, of which *Candida albicans* was dominant (54 isolates). Details of identified fungal species are presented in Table 2.

Interestingly, the induced-sputum sample from one subject yielded more than one fungal species. One individual subject harbored five types of fungal colonies. Details of fungal diversity from induced-sputum naïve lung cancer are shown in Table 3.

3.3. Antifungal Susceptibility Test to Voriconazole

Isolates with positive *Aspergillus* sp. were tested with antifungal voriconazole susceptibility.

Among 67 *Aspergillus* isolates, 59 could be tested for antifungal susceptibility, ie 21 isolates of *A. fumigatus*, 10 isolates of *A. flavus*, and 28 isolates of *A. niger*. Thrity-four of the 59 isolates of *Aspergillus* (57.6%) were still susceptible/sensitive to voriconazole. Surprisingly, a high proportion of isolates showed voriconazole resistance from naïve/newly diagnosed lung cancer samples: *A. fumigatus* 28.6%, *A. niger* 14.3%, and *A. flavus* 10%. The details of the voriconazole susceptibility test are shown in Table 4.

4. Discussion

Fungus is everywhere. There are around 1.5 million fungal species in the world, but only several hundred of them are known to be pathogenic to humans (Köhler *et al.* 2014). Environmental changes due to climate change, human activities, or natural disasters could change the natural ecology of fungi around the human environment that benefits its growth and survival (van Rhijn and Bromley 2021). Fungi produce spores/conidia with diameters small enough to be potentially inhaled by humans. Particulate matters, relative humidity, rainfall, and temperature might affect the concentration and survivability of fungal spores (Yan *et al.* 2016; Songnuan *et al.* 2018).

Humans inhale air from the environment continuously. The mucosa of the human lower respiratory tract is usually sterile, but it could become colonized with microorganisms, including fungi (Jose and Brown 2016). Asymptomatic

Table 1. Macroscopic spesies identification characteristics

Species	Colony form	Bottom surface	Edge form	Color
Candida spp.	Smooth and creamy colonies	none	none	white
A. fumigatus	Velvety-powdery growth	White or pale-yellow	Often with white margin	Blue-green
A. flavus	Floccose to granular	Cream	White or yellow mycelia	Bright yellow-green
A. niger	Granular with radial folds	Cream	Less intense sporulation	Black or puple-black
Penicillium spp.	Glabrous	Cream to pale brown	White edges	Light green

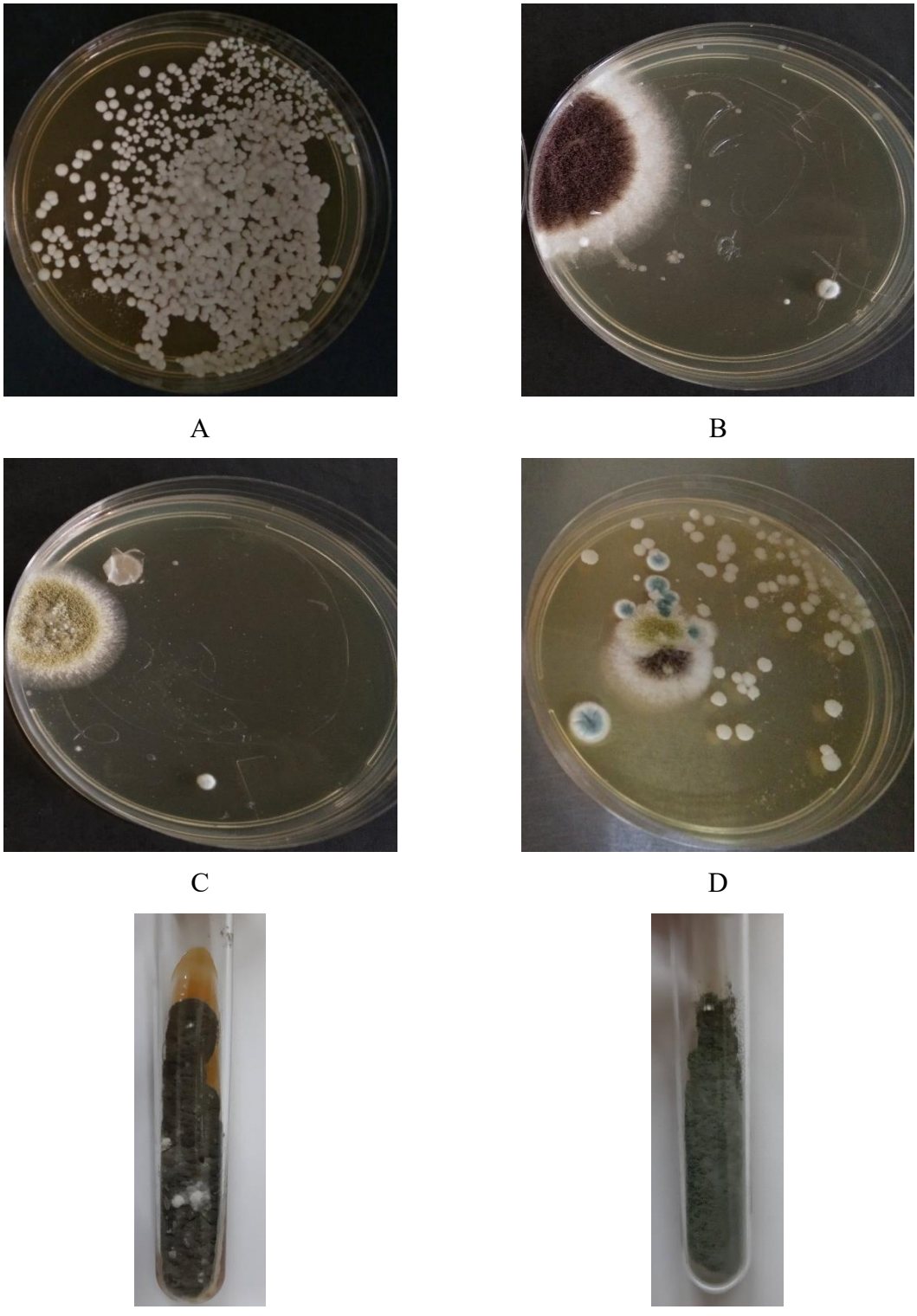
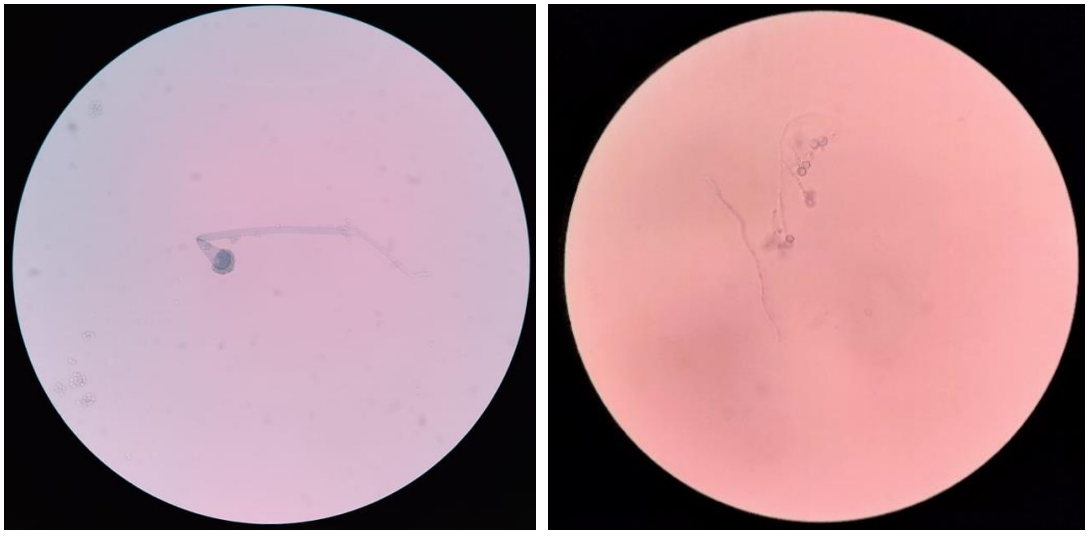


Figure 1. Macroscopic fungal identification (A) *Candida* spp., (B) *Aspergillus niger*, (C) *Aspergillus flavus*, (D) *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, and *Penicillium* spp., (E) *Aspergillus fumigatus*, (F) *Penicillium* spp.



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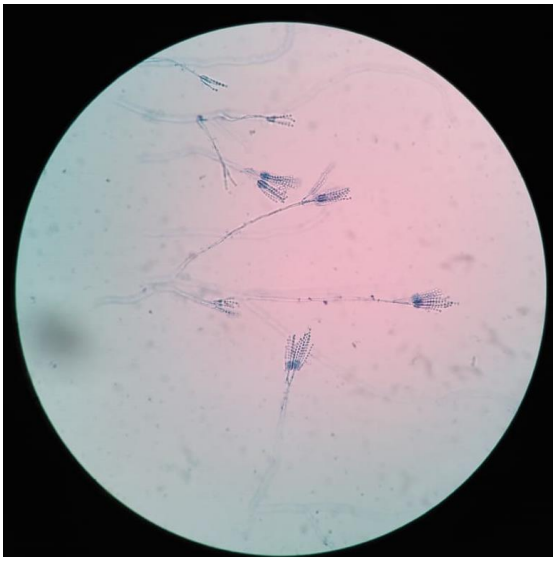

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Figure 2. Microscopic fungal identification (A) Conidia of *Aspergillus fumigatus* with a columnar conidial head, (B) ovoid blastoconidia of *Candida albicans*, (C) the phialides of *Penicillium* spp. with acerose to flask-shaped form and globose conidia

Table 2. The fungal species recovered from sputum of 70 naïve lung cancer patients

Fungal species	Number of fungal isolates	Percentage (%)
Candida albicans	54	38.6
Aspergillus niger	31	22.1
Aspergillus fumigatus	25	17.9
Aspergillus flavus	11	7.9
Penicillium spp.	8	5.6
Candida glabrata	7	5.0
Candida parapsilosis	3	2.1
Candida krusei	1	0.7
Total	140	100.0

Table 3. Diversity of fungal colonization from 70 sputum specimens of naïve lung cancer patients

Colonization fungal species	Number of specimens	
	N	%
Colonization of five types of fungal species		
A. fumigatus + *A. niger* + *C. albicans* + *C. glabrata* + *Penicillium* spp.	1	1.4
Colonization of four types of fungal species		
A. flavus + *A. niger* + *C. albicans* +*Penicillium* spp.	1	1.4
A. fumigatus + *A. niger* + *C. albicans* + *C. parapsilosis*	1	1.4
A. fumigatus + *A. flavus* + *A. niger* + *Penicillium* spp.	2	2.9
Colonization of three types of fungal species		
A. fumigatus + *C. albicans* + *Penicillium* spp.	1	1.4
A. niger + *C. albicans* + *Penicillium* spp.	3	4.3
A. fumigatus + *A. flavus* + *C. albicans*	1	1.4
A. fumigatus + *A. niger* + *C. albicans*	5	7.1
A. fumigatus + *A. niger* + *C. glabrata*	1	1.4
A. flavus + *A. niger* + *C. albicans*	1	1.4
A. fumigatus + *A. flavus* + *A. niger*	2	2.9
Colonization of two types of fungal species		
A. fumigatus + *C. albicans*	7	10.0
A. fumigatus + *C. parapsilosis*	1	1.4
A. flavus + *C. albicans*	1	1.4
A. niger + *C. albicans*	8	11.4
A. niger + *Penicillium* spp.	1	1.4
A. fumigatus + *A. flavus*	1	1.4
A. fumigatus + *A. niger*	2	2.9
A. flavus + *A. niger*	2	2.9
C. albicans + *C. glabrata*	3	4.3
C. albicans + *C. parapsilosis*	1	1.4
Colonization of one species		
A. niger	1	1.4
C. albicans	19	27.1
C. glabrata	2	2.9
C. krusei	1	1.4
No fungal colonization	1	1.4
Total	70	100.0

Table 4. The susceptibility tests profile of *Aspergillus* sp. isolates against voriconazole

Species	Sensitive	Intermediate	Resistance	Number of isolates
Aspergillus fumigatus	9 (42.9%)	6 (28.6%)	6 (28.6%)	21
Aspergillus flavus	8 (80%)	1 (10%)	1 (10%)	10
Aspergillus niger	17 (60.7%)	7 (25%)	4 (14.3%)	28
Total	34 (57.6%)	14 (23.7%)	11 (18.6%)	59

colonization is defined as the appearance of fungi on the body surface without causing disease. Human fungal disease is uncommon in healthy individuals due to their competent innate and adaptive immunity (Denning and Chakrabarti 2017). However, colonization might be the first step before developing the fungal disease in individuals at risk (Jose and Brown 2016; Hall *et al.* 2018). Fungal colonization can be found in the gastrointestinal tract and sometimes spill over to upper respiratory tracts in the human body. The most common fungal species that colonize the gastrointestinal tract, including oral cavity, are *Candida, Saccharomyces*, and *Cladosporium* sp. (Hall *et al.* 2018).

Lower respiratory tract colonization might reflect the spread from the oral mucosa or upper respiratory tract and are common among hospitalized patients. Identification of fungal colonization in the respiratory tract is important to detect fungi with the potential to progress into fungal disease, especially in high-risk individuals (Biswas *et al.* 2010). Induced sputum, tracheal aspirates, or samples through bronchoscopy are the common techniques to identify lower respiratory tract colonization. Regular sputum is not

recommended since it has numerous oral and upper airway contamination. If done properly, the sample from induced sputum is similar to samples obtained through bronchoscopy and can reflect the condition in the lower respiratory tract. Induced sputum is also more favorable since it is simple, less invasive, low risk, and affordable (Escribano *et al.* 2015). This is the first study in Indonesia to evaluate fungal colonization in lower airways among naïve, newly diagnosed lung cancer patients that have not been given any prior systemic cancer therapy.

We found *Candida* sp., *Aspergillus* sp., and *Penicillium* sp. as the common fungal colonization species. This study found that only one subject had no fungal colonization. As expected, *Candida* sp. was found in a high percentage of subjects. *Candida* sp. is one of the most common fungal species in the human body, and its appearance might not reflect fungal disease (Pendleton *et al.* 2017; Rozaliyani *et al.* 2019). *Candida* in this study is not included as pathogen because it is normal flora in oral cavity. A previous study from China with 389 *Candida* isolates from invasive candidiasis patients revealed most of them (>90%) sensitive to voriconazole with exception of 3.6% of *C. parapsilosis* showed resistance to voriconazole (Liu *et al.* 2014). Interestingly, 67 *Aspergillus* sp. isolates were found (61.4%), consisting of *Aspergillus niger* in 31 isolates (22.1%), *Aspergillus fumigatus* in 25 isolates (17.9%), and *Aspergillus flavus* in 11 isolates (7.9%). The prevalence of *Aspergillus* sp. colonization in this study is higher than in Italy (Carpagnano *et al.* 2014), in which 27.9% of lung cancer patients show colonization of *Aspergillus niger* (11.6%), *Aspergillus ochraceus* (6%), and *Penicillium* sp. (9.3%). In China, pulmonary aspergillosis was found in 59.1% of immunosuppressed patients. However, the sample population included patients who had undergone prior chemotherapy/radiotherapy (Zhang 2017).

Aspergillus sp. is a saprophytic fungus, mainly living on decaying vegetation in soil, and produces inhalable small spore/conidia (Kosmidis and Denning 2015). *Aspergillus* is one of the most important fungal species since it has the potential to induce a various spectrum of *Aspergillus* disease in humans, especially in immunodeficient/immunocompromised individuals. *Aspergillus* infection in humans depends on the interaction between host immune response, structural lung damage, and the *Aspergillus* sp. virulence. It could appear as allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis or invasive aspergillosis, with some cases showing grave prognoses (Kosmidis and Denning 2015).

The subjects in this study were naïve/untreated lung cancer subjects. The possibility that the colonization was hospital-acquired and treatment-related was less likely since they had spent short time in hospital. Additionally, the patients never been received any systemic cancer treatment such as chemotherapy or radiotherapy nor received any systemic antifungal agent. Nonetheless, the fungal diversity in the hospital environment requires further control as it might colonize hospitalized patients who spend more time in the hospital. Colonization might also reflect fungal variability in their surrounding environment (van Rhijn and Bromley 2021). We could not rule out these possibilities since we had not sampled the fungi from the environment of these subjects.

Airborne fungal data is limited in Indonesia. A study in the university libraries in Yogyakarta, Indonesia, found *Aspergillus, Cladosporium*, and *Penicillium* sp. were commonly found (Rahmawati *et al.* 2018). In Bangkok, airborne fungal spore concentration was varied between months. The average spore concentration in one year was 11,211.89 spores/m^3. The lowest spore concentration was found in the summer. The fungal spores that were mostly found were *Cladosporium* sp. (49.37%). *Aspergillus* and *Penicillium* sp. counted 885.31 spores/m^3 (7.90%) (Songnuan *et al.* 2018). Air quality levels, particulate matter, humidity, and temperature were also correlated with fungal spore concentration and fungal diversity in the air, as shown in Beijing (Yan *et al.* 2016). A study in the underground stations of St Petersburg, Rusia, found that although fungal density in the air was within acceptable levels, numerous fungal species had still grown. The most common fungal species were *Acremonium* sp., *Aspergillus* sp., *Cladosporium* sp., and *Penicillium* sp. (Bogomolova and Kirtsideli 2009).

The emerging of voriconazole resistant *Aspergillus* sp. The alarming finding from this study is the high prevalence of voriconazole-resistant *Aspergillus* since it has a potential impact on human and animal health (Rivero-Menendez *et al.* 2016; Revie *et al.* 2018). There are limited antifungal drugs available for fungal disease in humans. Antifungals can be divided into three classes, namely azoles, echinocandins,

and polyenes. Voriconazole is a newer class of azole groups with a broad spectrum of anti-fungal activity, including *Aspergillus* sp., *Candida* sp., *Coccidioides* sp., *Histoplasma* sp., *Penicillium* sp., *Scedosporium* sp., and *Fusarium* sp. The mechanism of action of voriconazole is inhibition of ergosterol synthesis in fungal cells through inhibition of cytochrome P450 (CYP 450)–dependent 14α-lanosterol demethylation (Saravolatz *et al.* 2003; Ullmann *et al.* 2018). It is reserved for severe fungal infection in humans and fungal infection in immunocompromised patients such as AIDS, organ transplant recipients, hematologic cancers, and bone marrow transplants (Saravolatz *et al.* 2003; Ullmann *et al.* 2018).

This study showed that among 67 *Aspergillus* isolates, 59 could be tested for antifungal susceptibility, ie 21 isolates of *A. fumigatus*, 10 isolates of *A. flavus*, and 28 isolates of *A. niger*. A total 34 of 59 isolates of *Aspergillus* sp. (42.4%) showed resistance to voriconazole, including 28.6% *A. fumigatus*, 14.3% *A. niger*, and 10% *A. flavus* isolates. *Aspergilllus* resistance in the world might varied depending on local environmental and geographical condition. *Aspergillus* resistance to voriconazole has been reported in Canada, China, India, the United States, and Europe, but mostly in a hospital setting (Howard and Arendrup 2011). In the hospital setting, the incidence of *Aspergillus* resistance to voriconazole increased up to 16.2%, especially in *Aspergillus fumigatus* (Fuhren *et al.* 2015).

The voriconazole resistance is mostly related to the alteration of cyp51A gene that encodes sterol 14alpha demethylase (Howard and Arendrup 2011). Multiple factors could induce the emergence of drug resistance fungal strain. For example, long-term exposure to the drug creates selective pressure for resistance, population size, a pool of pre-existing resistance strains, and the fitness cost associated with resistance (Brockhurst *et al.* 2019). In the clinical setting, azole-resistant strains could arise from long-term use of azoles as an antifungal such as in aspergilllosis. But recent data has highlighted the emerging azole-resistant strain in naïve patients with no history of long-term antifungal exposure (Yan *et al.* 2009; Zhang 2017).

The possibility that the emerging drug-resistant *Aspergillus* was derived from the environment is of concern to many experts (Snelders *et al.* 2009; Berger *et al.* 2017; van Rhijn and Bromley 2021). Review articles strongly suggest the role of extensive agricultural use of antifungals such as azoles in the emergence of the azole-resistant strain in the field (Snelders *et al.* 2009; Berger *et al.* 2017; Brauer *et al.* 2019). This resistant strain could be easily transmitted to human hosts. Studies in Italy (Prigitano *et al.* 2019), the Netherlands (Schoustra *et al.* 2019), and China (Cao *et al.* 2020) analyzed *Aspergillus* sp. in the environment and clinical settings. Using the current genetic association study, the studies have found some samples show the cross-resistance with agro-chemical and medical azoles.

Surveillance of the emerging drug-resistant fungi in the environment, community, and high-risk groups is needed, especially in tropical countries, such as Indonesia, which is home to various fungal species. Mitigation of antifungal use for common diseases in the community and antifungal use in agriculture is also imperative. This mitigation is important to identify potential emerging fungal diseases that have consequences to both human and animal health.

In conclusion, fungal colonization can be found in the airways of naïve lung cancer patients in Indonesia. Species mostly identified in this study were *Candida* sp., *Aspergillus* sp., and *Penicillium* sp. *Aspergillus* sp. is of concern since it could induce a varying spectrum of *Aspergillus* infections in humans. A high prevalence of voriconazole-resistant *Aspergillus* was observed, which might be a potential challenge to human health since limited antifungal drugs are available, and the azole class is the drug of choice in most fungal diseases. There are possibilities that the resistant strain was acquired from the environment and agriculture. Further studies are needed to mitigate this potential emerging disease that could impact human and animal health.

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PPM.00.02/2019 and the Ethics Committee of Persahabatan National Respiratory Referral Hospital, Ref number 70/KEPK-RSUPP/09/2019. Informed consent was obtained from all subjects included in the study. All authors declare no conflict of interest.

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