

Antifungal Susceptibility and Virulence of *Aspergillus fumigatus* Environmental Strains from a Public Tertiary Hospital in Metro Manila, Philippines

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RESEARCH ARTICLE

Abstract

Background and Objectives: The increase in the number of invasive *Aspergillus* infections has been observed among immunocompromised and hospitalized patients. In the Philippines to date, no published data focused on the prevalence of *Aspergillus* species or any other thermotolerant fungal species in a hospital environment. This research served as a primary study to characterize the antifungal susceptibility of environmental strains of *Aspergillus fumigatus* from a hospital facility against three antifungal agents and to determine the virulence of these isolates on BALB/c mice using an animal survival assay.

Methodology: Ten environmental strains of *A. fumigatus* were isolated from three air-conditioned wards in a medical facility using Andersen Air Sampler. The antifungal susceptibility profile of the isolates was determined against Voriconazole, Amphotericin B and Caspofungin. The virulence of these isolates was also tested on BALB/c mice using an animal survival assay. Moreover, the lung tissues of infected BALB/c mice were subjected to histopathological analyses using Gomori Methenamine Silver stain (GMS) and Hematoxylin & Eosin (H&E) stains.

Results: Etest result for antifungal susceptibility testing showed that two of the ten isolates were resistant to Amphotericin B (AF2-A and AF-3A); one isolate resistant to Voriconazole (AF2-A) and an isolate that manifested non- susceptibility to Caspofungin m(AF2-A). Epidemiological cut-off values were determined for each antifungal following the M38-A2 CLSI guidelines. BALB/c mice median survival analysis revealed that the isolate with the highest Minimum Inhibitory Concentration (MIC= 4.89 µg/ml) for Voriconazole resulted in the most number of mortality with the least number of observation days. GMS AND H&E histopathology slides showed fungal elements embedded on left lung lobe of mice.

Conclusion: This study showed that there were strains of *Aspergillus fumigatus* from a hospital indoor air which were considered as resistant strains to Voriconazole, Amphotericin B, and Caspofungin (AF2-A and AF3-A). Lung tissues of infected mice showed characteristics of bronchopneumonia.

Keywords: Antifungal susceptibility, survival analysis, environmental isolates, Etest, Gomori Methenamine Silver Stain

Introduction

There is a growing burden of fungal infections and there are more than three hundred million people who suffer from invasive fungal infection globally, resulting in almost 1,350,000 deaths. The increase in the number of invasive *Aspergillus* infections has been observed among immunocompromised individuals and hospitalized patients [2]. These *Aspergillus* species are saprophytic in nature but considered as opportunists to susceptible host. However, the control of fungal diseases is further complicated with the emergence of antifungal drug resistance to Voriconazole,

Amphotericin B and Caspofungin, the three main drug of choice for aspergillosis. These resistance mechanisms were seen in both clinical and environmental isolates. In-house patients of the medical facility who are exposed to the fungal elements of this *Aspergillus fumigatus* may suffer from simple allergies to systemic and invasive infections particularly the immunocompromised patients. The fungus can grow and proliferate in the lung tissues and other organ systems of high-risk patients. The previously mentioned scenario can seriously aggravate the existing condition of

patients with the development of invasive pulmonary aspergillosis. Medication regimens available will not be able to sufficiently address the treatment of patients infected with these drug-resistant fungal strains, thus resulting in higher mortality cases in affected patients. In the Philippines to date, no published data focused on the prevalence of *Aspergillus* species or any other fungal species in a hospital environment. This research characterized the antifungal susceptibility of *Aspergillus fumigatus* strains from a hospital facility against Voriconazole, Amphotericin B, and Caspofungin using Etest Method. This served as the first fungal virulence study in the country on BALB/c mice with description on the histopathology of *A. fumigatus* infected lung tissues. This study may offer a source of information for the hospital Infection Control Committee on the characteristics of environmental isolates found in the medical institution.

Methodology

Ethical Considerations

The study protocol was initially submitted to the University of the Philippines Manila Review of Ethics Board (UPM-REB) for archival purpose. This was also submitted and approved by the University of the Philippines Manila Institutional Animal Care and Use Committees (IACUC) and Bureau of Animal Industry (BAI) for the review of animal care and procedures included in the research. In addition, the research protocol was also reviewed and approved by the Institutional Biosafety and Biosecurity Committee (IBBC). The study did not commence without prior approval of the mentioned committees.

Isolation of *Aspergillus fumigatus*

The indoor air sampling was conducted in three randomly selected air-conditioned wards and another three in 16 non-air-conditioned wards at a public tertiary hospital in Metro Manila. The three wards under the Department of Surgery (Ward 2-Male Surgery, Ward 4- Female Surgery, and Ward 6-Pediatrics Surgery) were the sampling sites for the non-air-conditioned wards (NACWs). These wards house patients that have undergone invasive medical procedures that predisposed these individuals to opportunistic infections. On the other hand, ACW 1 (Room 434), ACW 2 (Room 435) and ACW 3 (Room 436), also located at the central block building of the hospital were the sampling sites for air-conditioned wards. The main lobby and the nurse station of the hospital served as the comparison/control areas of the study for the NACWs and ACWs respectively.

The researcher coordinated with the Philippine Astronomical, Geophysical and Astronomical Services Association (PAG-ASA) through e-mail and direct phone call to seek reference information about the meteorologic condition and physical parameters including temperature and relative humidity in the sampling sites for the particular time on the sampling day. The researcher also measured the temperature and relative humidity inside the NACWs and ACWs with calibrated digital thermometer and hygrometer respectively. NACWs with apparent higher environmental temperature (AVE: 35.9 °C) have lower average number of *A. fumigatus* isolates (72.66 CFU/ m³) when compared to ACWs (AVE: 26.4 °C) having a mean total of 150.66 CFU/ m³. Another established physical factor affecting fungal growth was relative humidity. Evidently, NACWs with lower relative humidity (AVE: 44%) yielded a lower number of *A. fumigatus* isolates when compared to ACWs (AVE: 58%) with higher relative humidity.

Indoor air sampling was conducted using six-stage Andersen air sampler loaded with Malt Extract Agar (MEA) with chloramphenicol plates (20g Malt Extract, 15g Agar, 0.200g chloramphenicol, 1 L deionized water). In one sampling process, six MEA agar plates were used yielding 12 plates including the duplicate. After the sampling procedure, the seeded Malt Extract Agar (MEA) plates with chloramphenicol were incubated at 37°C at the Department of Medical Microbiology Laboratory incubator (Walk-in Lab line S.N 147). On the third and fifth day of incubation, the colonies considered morphologically distinct or showing typical characteristics of suspected fungi were inoculated and compared with each other, then inoculated or sub-cultured to ensure that only one isolated strain representative of each morphological group found was selected. Lactophenol blue stained Scotch-preparations were done for the microscopic analyses. The taxonomic identification of fungi performed, considered the morphological characteristics of the vegetative mycelium and the reproductive structures.

The environmental sampling conducted among the eight sampling sites using Andersen Air Sampler has collectively isolated seven thermotolerant fungal organisms. The following fungi were identified through morphological-microscopic analysis: *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Curvularia sp.*, *Penicillium sp.*, *Alternaria sp.* and *Rhizopus sp.* The most abundant isolate was *Aspergillus fumigatus* for both NACWs and ACWs. Overall, ACWs showed higher number of fungal colony-forming units as compared with NACWs. The abundance of *Aspergillus fumigatus* in ACWs and as the most common aetiologic agent of nosocomial mycosis, this organism was selected for antifungal

profiling and virulence testing. These *A. fumigatus* strains were stored inside a secured cabinet at the Department of Medical Microbiology Laboratory at room temperature in MEA plates. The fungal organisms were revived by transferring the isolates into fresh malt extract agar medium.

Molecular identification of Aspergillus fumigatus

The isolates were molecularly identified as *Aspergillus fumigatus* by the Philippine Genome Center at the University of the Philippines Diliman, Quezon City. The fungal genomic DNA extraction of ten strains submitted in Malt Extract Agar slants was conducted following the Triton X-100-Ampure DNA extraction protocol. The isolated DNA from the strains were then subjected to gene amplification using Polymerase Chain Reaction (PCR). The primers used were ITS1 and ITS4 18S primers. Lastly, the capillary electrophoresis was conducted using ABI 3730xl DNA analyzer using 50 cm- 96 cm capillary array, POP7TM polymer and 3730xl data collection software version 3.1.

Etest Antifungal Susceptibility Testing of Aspergillus fumigatus

The Etest antifungal susceptibility testing was performed in accordance with the manufacturer's (bioMerieux) recommendations and following the Clinical and Laboratory Standards Institute (CLSI) M38-A2 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi guidelines in 2008. Strains were grown on Malt Extract Agar slants at 37°C for seven days to ensure adequate sporulation. Spore suspensions were prepared in sterile saline and adjusted to a concentration of 10^8 spores/ml, corresponding to 89% transmittance and 0.0506 absorbance value at 530 nm using the spectrophotometer (Spectronic 20D+). The agar formulation utilized for Etest was RPMI 1640 (American Biorganics, Buffalo, N.Y.) supplemented with 1.5% agar and 2% glucose and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma, St. Louis, Mo.). The 90-mm-diameter petri plates contained RPMI 1640 agar at a depth of four milliliters. The plates were inoculated by dipping a sterile swab into the spore suspension and streaking it across the surface of the agar in three directions. The plates were air dried at ambient temperature for 15 minutes before applying the Amphotericin B, Voriconazole and Caspofungin Etest strips. The plates were incubated at 37°C and were run in duplicate. Moreover, *Aspergillus fumigatus* ATCC 204305 strain was the control strain used in the study.

The results of the Etest were read after 48 hours for Voriconazole and Amphotericin B and after 24 hours for

Caspofungin. The Etest MIC for Amphotericin B and Voriconazole was read as the drug concentration at the point where dense colonial growth intersects the strip. On the other hand, Minimum Effective Concentration (MEC) was read for Caspofungin as the intersection between the lawn of confluent colonies and the presence of distinct microcolonies. Etest analyses for these ten *A. fumigatus* strains and one ATCC strain, (ATCC 204305) were done in duplicate for each of the antifungal drugs (Voriconazole, Amphotericin B, and Caspofungin). The three antifungal drugs used in this study were recommended by Etest manufacturer as a panel of antifungals for *Aspergillus fumigatus* susceptibility testing. Aside from the primary investigator, the Etest results were also validated by Ms. Mary Ann C. Sison, DMM, MPH, University Researcher II at the Department of Medical Microbiology-College of Public Health, University of the Philippines Manila. The researcher computed for the average MIC and MEC values for all of the isolates and were expressed as geometric mean.

Virulence Testing on BALB/c Mice of Three Aspergillus fumigatus Strains from air-conditioned Wards with the highest MICs for Voriconazole

Among the ten *A. fumigatus* strains subjected to antifungal susceptibility testing, only three with the highest MIC for Voriconazole (AF2-A: 4.89 µg/ml, AF3-A: 0.268 µg/ml and AF4-A: 0.217 µg/ml) were utilized for the animal survival analysis phase of the study. Among the three highest MIC results for Voriconazole, AF2-A was the only resistant strain according to the cut-off value of CLSI. The animal study protocol was submitted and approved by the University of the Philippines Manila Institutional Animal Care and Use Committees (IACUC) and Bureau of Animal Industry (BAI) for the review of animal care and procedures included in the research.

The animal laboratory room was located at the fourth level of the College of Public Health, UP Manila. During the one-week acclimatization period, a commercially available rabbit pellet consisting of (20% fibre, 21% protein, 0.7% Calcium and Phosphorus, 2.4-5% Fat) was the mice diet. Distilled water was supplemented with 200 µg/ ml of doxycycline for prophylaxis after the first dose of cyclophosphamide was administered. Drinking water was provided *ad libitum*. During the acclimatization period, health monitoring of the laboratory animals was conducted to check for the presence of ectoparasites and endoparasites. The fur pluck technique (tape test) was administered for the detection of ectoparasites on the animal's coating such as fur mites belonging to genus *Radfordia*, *Myobia*, and *Myocoptes*.

Endoparasite detection was done using perianal tape test, in which three representative animals were randomly picked for checking of intestinal microorganisms such as *Syphacia* eggs. Moreover, the weight, presence of alopecia, and mucosal condition were also monitored during this period.

Physical randomization was conducted among the forty BALB/c mice. Test mice were assigned into four groups resulting in ten BALB/c mice each for the three experimental groups and one control group. A day after the acclimatization period, immunosuppression of BALB/c mice commenced. All mice received a total dose of 250 mg/kg cyclophosphamide by two 0.02 ml intraperitoneal injections scheduled at day one (150mg/kg) and on day four (100 mg/kg) via intraperitoneal route [3].

Intranasal Inoculation of Fungal Inocula

On the day of infection, fungal conidia were harvested by washing the slant culture with five milliliters of normal saline solution. The conidia were gently scraped from the mycelium with a plastic pipette. Five milliliters of saline + conidia was decanted into a 25ml plastic conical tube and was shaken for ten seconds using a gyratory vortex mixer. Spore suspensions were adjusted using Spectronic 20D+ (220 V, 340-950 nm) to a concentration of 3×10^8 spores/ml [4]. The mice were anesthetized with 0.02ml of (0.0025 mg/kg) of Zoletil 50 via intramuscular injection using insulin syringe. For the experimental group and control group, a single droplet of 30 μ l (0.03 ml) of inoculum and 0.03 ml of 0.9% saline solution respectively were administered via the intranasal route. The mice inhaled the droplet involuntarily. After inhalation, the nares were disinfected with 70% alcohol and were returned to their respective cages.

Survival Analysis and Histopathology

Animal survival was observed twice a day (morning and late afternoon) for 15 days. The mice were poked lightly with forceps to see any reaction to physical stimuli. A dead mouse is immediately removed from its cage. Animal necropsy was done inside the Animal Laboratory Room within an hour after death. There were two five millimeter excisions done on the left lung lobe tissue of the mouse and these tissues were fixed in 10% buffered formalin. The first four mice that died from each of the three experimental groups were subjected to necropsy. One five-millimeter lung tissue was fixed in 10% buffered formalin for Gomori Methenamine Silver (GMS) staining and another five-millimeter lung tissue from the same mouse was prepared for Hematoxylin & Eosin (H&E) staining. The same procedure was performed in the control group and four mice were

euthanized to prepare samples for both GMS and H&E staining. A total of 16 slides for GMS and 16 for H&E for all the four groups were made. Sectioned lung tissues for GMS and H&E staining were submitted and processed at the Histopathology Division, Lung Center of the Philippines. The presence of fungal elements and histopathological changes in the lung tissues were recorded. The reading and the conduct of the five-point infarct scoring was done by Dr. Alice Alma C. Bungay, Assistant Professor of the Department of Medical Microbiology, UP Manila. Scores ranged from 0-5 with the score roughly representing the percentage of tissue involvement (0= 0%, 1= 10%, 2=20%, 3= 30%, 4=40% and 5=50%). The animals were overdosed with sevoflurane-soaked cotton, enclosed in an anesthetizing jar, and were disposed of properly. To describe the survival time of BALB/c mice, Kaplan-Meier survival curve was produced. Point 95% confidence interval estimate of the median survival time was also computed. All statistics were generated using Stata MP version 14.

Results

Molecular Identification of Aspergillus fumigatus

The species identity of the ten environmental strains of morphologically identified *Aspergillus fumigatus* was confirmed using capillary sequencing method. All of the resulting sequences of the strains (ranged from 473-616 base pairs) were confirmed molecularly as *Aspergillus fumigatus* when processed using Basic Local Alignment Search Tool (BLAST). The isolates showed percent identities of 97% to 99% to sequenced databases.

Antifungal Susceptibility Testing

All environmental strains grew well on the RPMI 1640 culture media plates used giving definitive endpoints. The MIC geometric mean values obtained from the ten isolates for the panel antifungals (Voriconazole, Amphotericin B and Caspofungin) were summarized in Table 1. As seen in Table 1, AF2-A (isolated from Room 435) has the highest MIC geometric mean value of 4.89 μ g/ ml among the strains from air-conditioned wards followed by AF3-A (0.268 μ g/ ml) from Room 435, AF4-A (0.217 μ g/ ml) from Room 436, AF1-A (0.19 μ g/ ml) from Room 434, and AF5-A (0.108 μ g/ ml) which is isolated from Room 436. On the other hand, the *A. fumigatus* isolates from non-air-conditioned wards, AF6-N (isolated from Ward 2) and AF8-N (isolated from Ward 4), showed the highest MIC geometric mean value at 0.342 μ g/ ml. This was followed by AF10-N (0.217 μ g/ ml), AF7-N, and AF9-N with the same MIC value at 0.125 μ g/ ml. In accordance with the epidemiological

Table 1. Minimum inhibitory concentration (MIC) geometric mean values of ten *Aspergillus fumigatus* strains and ATCC 204305 strain in µg/ml

Isolate	Voriconazole	Amphotericin B	Caspofungin
ATCC (204305)	0.775	1.73	0.003
AF1-A	0.19	<	0.006
AF2-A	4.89	4.89	>
AF3-A	0.268	9.79	<
AF4-A	0.217	<	0.006
AF5-A	0.125	0.75	<
AF6-N	0.342	0.75	<
AF7-N	0.125	1.0	0.002
AF8-N	0.342	1.22	0.006
AF9-N	0.125	1.73	0.005
AF10-N	0.217	0.612	0.006

***Epidemiological cut-off values following CLSI M38-A2: *Aspergillus fumigatus* isolates were considered as Wild Type (WT)/ susceptible when MIC/MEC is (≤ 1 µg/ml) for Voriconazole, (≤ 2 µg/ml) for Amphotericin B and (≤ 0.06 µg/ml) for Caspofungin (>) highest value on MIC scale, no inhibition ellipse, (<) lowest value on MIC scale, does not intersect strip. Text in red signifies resistant strains to respective antifungals.

cut-off value set by CLSI M38-A2 for *A. fumigatus* susceptibility testing in Voriconazole, an MIC reading of less than or equal to one (≤ 1) µg/ml is considered non-drug resistant strain. All of the ten *A. fumigatus* specimens were considered non-drug resistant except AF2-A with 4.89 µg/ml MIC value. Moreover, the *A. fumigatus* ATCC 204305 showed susceptibility to Voriconazole with 0.775 µg/ml MIC mean value.

The strain AF3-A showed the highest MIC geometric mean value for all the specimens at 9.79 µg/ml. Among the ten environmental strains, two isolates (AF2-A, 4.89 µg/ml and AF3-A, 9.79 µg/ml) were considered resistant to Amphotericin B based on the CLSI M38-A2 epidemiological cut-off value for susceptible strains (≤ 2 µg/ml). These two resistant strains were both isolated from the indoor air environment of Room 435. On the other hand, the *A. fumigatus* ATCC 204305 showed susceptibility to Amphotericin B with 1.73 µg/ml MIC mean value.

Lastly, the minimum effective concentration (MEC) of the *A. fumigatus* isolates for Caspofungin was read after 24 hours as the intersection between the lawn of confluent colonies and the presence of distinct microcolonies in the culture media. In accordance with the epidemiological cut-off value set by CLSI M38-A2 for *A. fumigatus* susceptibility testing in Caspofungin, an MEC reading of equal to or less than (≤ 0.06 µg/ml) is considered non-drug resistant. As seen in Table 1, AF2-A (>, highest value on MIC scale, no inhibition ellipse) from Room 435, was the same isolate that showed resistance to the two other antifungals which also displayed non-susceptibility to Caspofungin. On the other hand, *A. fumigatus* ATCC 204305 showed no resistance to Caspofungin with 0.0034 µg/ml MIC mean value.

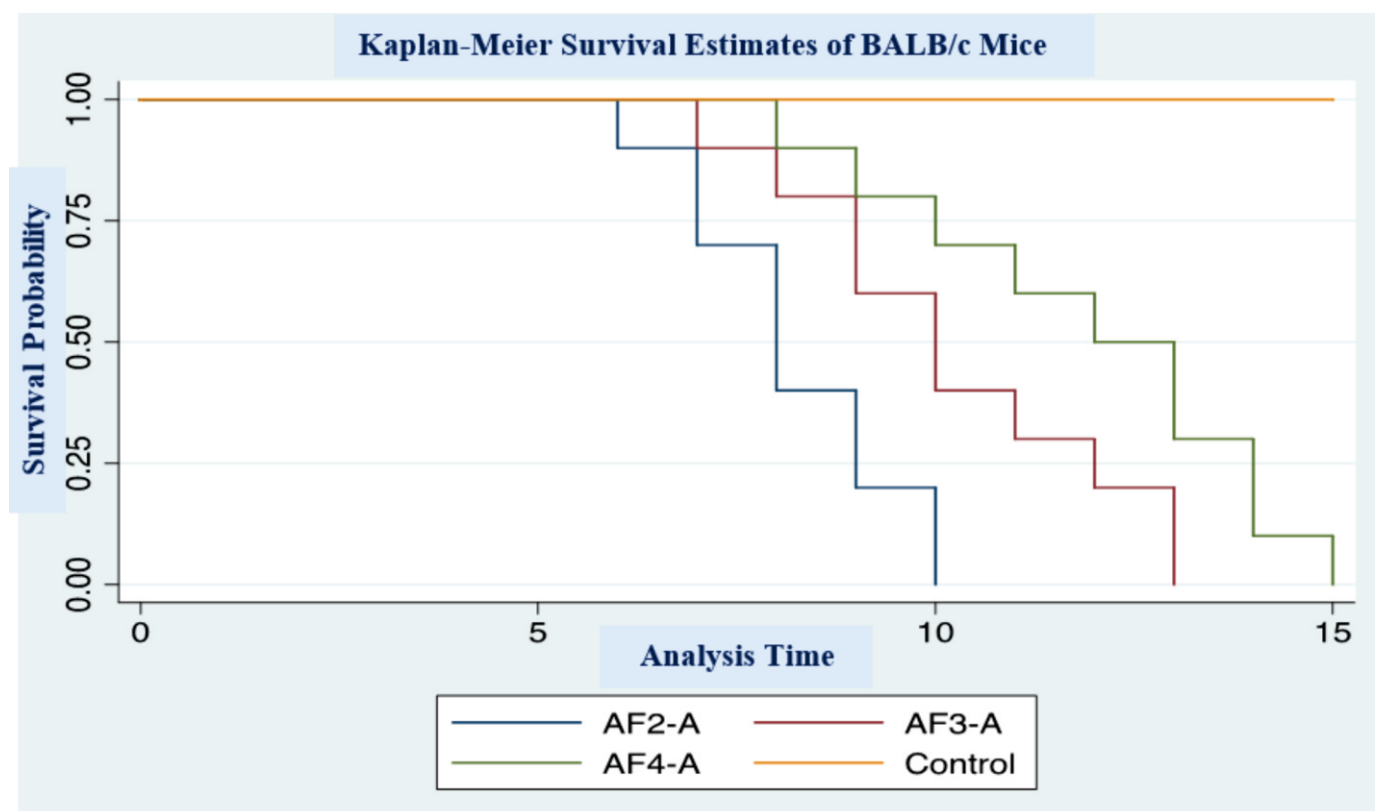


Figure 1. Kaplan- Meier survival curve of BALB/c mice

Table 2. Statistics of the generated Kaplan-Meier curve for the survival analysis of BALB/c mice

Isolate	Subjects	Median Survival (50%)	Standard Error	95% Confidence Interval
AF2-A	10	8	.5163977	6-9
AF3-A	10	10	.7745967	7-12
AF4-A	10	12	1.054093	8-14
Control	10	--	--	--

Virulence Testing (Animal Survival Analysis)

The median survival time of the four groups of BALB/c mice was recorded for 15 days. In the Kaplan-Meier survival curve (Figure 1), the vertical axis indicated the survival probability which represents the percent population of mice that are alive at a specific time point. On the other hand, the horizontal axis showed the time of analysis. Moreover, the varying colors of the curves represent the population of mice infected with isolates AF2-A, AF3-A, AF4-A, and the control group.

As seen in the survival curve (Figure 1), the first mortality case was recorded from the group of BALB/c mice infected with AF2-A strain that resulted to a 90% survival probability of the AF2-A group on the sixth day. On the other hand, the last mice from this group survived until the tenth day only. This gave the AF2-A group a median survival time of eight days as shown in Table 2. The 95% confidence interval that indicates how close the estimated value was to the true mean is also included in the said table.

Consequently, the group of mice inoculated with *A. fumigatus*, AF3-A strain showed a 90% survival probability only after seven days of observation period, meaning that the first case of mortality was seen on the seventh day. The last mice belonging to this group was alive until the 13th day. Moreover, the median survival time of this group was ten days. On the other hand, the third group, AF4-A, had a median survival time of 12 days. In addition, the first mortality of mice in this group was recorded on the eighth-day and the last case on the 15th day. Evidently, as illustrated in the survival curve, the control group (administered with saline solution) showed a 100% survival probability up until the last day of the observation period (15th day).

Histopathology

This study utilized two histologic stains to visualize the effect of the *Aspergillus fumigatus* environmental strains in

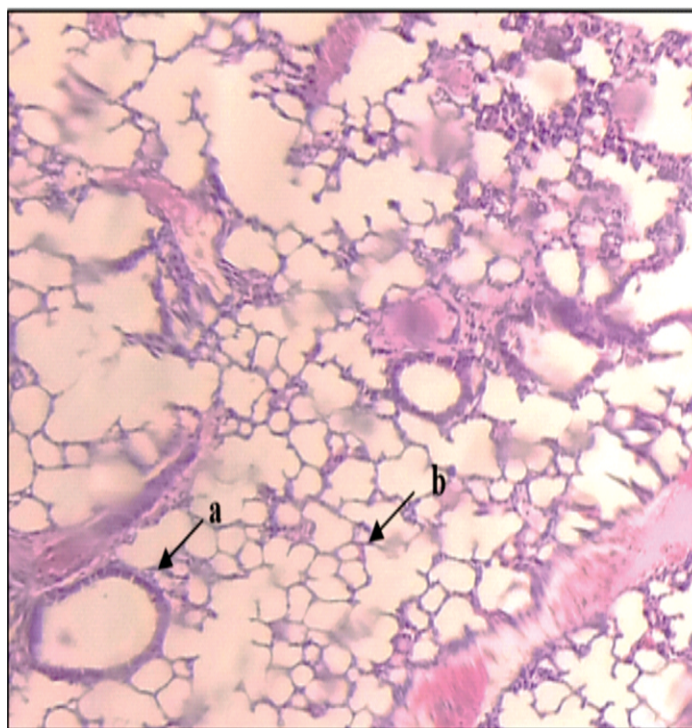


Figure 2. Hematoxylin and Eosin stained histopathology slide of BALB/c mice from the control group showing (a) bronchiolar wall (b) thin lining of pneumocytes around the alveolar walls in LPO [100x]

the lung tissues of infected BALB/c mice. As seen in Figure 2, the cross-section of lung tissue stained with Hematoxylin and Eosin from the control group showed elastic alveolar walls with intact (a) bronchiolar and alveolar epithelium. There were no accumulations of inflammatory cells around the alveoli and bronchioles as seen on the (b) thin lining of pneumocytes around the walls.

In contrast, the results of the histopathological analyses among the *Aspergillus fumigatus* infected mice (Figure 3), showed dilated airspaces and evident rupture of the alveolar walls showing cases of emphysema. The epithelial lining of the bronchiolar and alveolar walls appeared to be desquamated (a) with numerous red blood cells inside the lumen. There was also migration of inflammatory cells from the pleural cavity and accumulation of neutrophils (b) around ruptured walls. The homogenous neutrophilic pink exudate inside the lumen, characteristic pulmonary edema (c) resembled bronchopneumonia. Moreover, conidial spores were also present in the lung tissues of inoculated mice. According to the five-point infarct scoring of Hematoxylin and Eosin stained histopathology slide (cross-section) of the AF2-A inoculated BALB/c mice which died on the sixth day, hemorrhage constituted to 40% of the examined tissue,

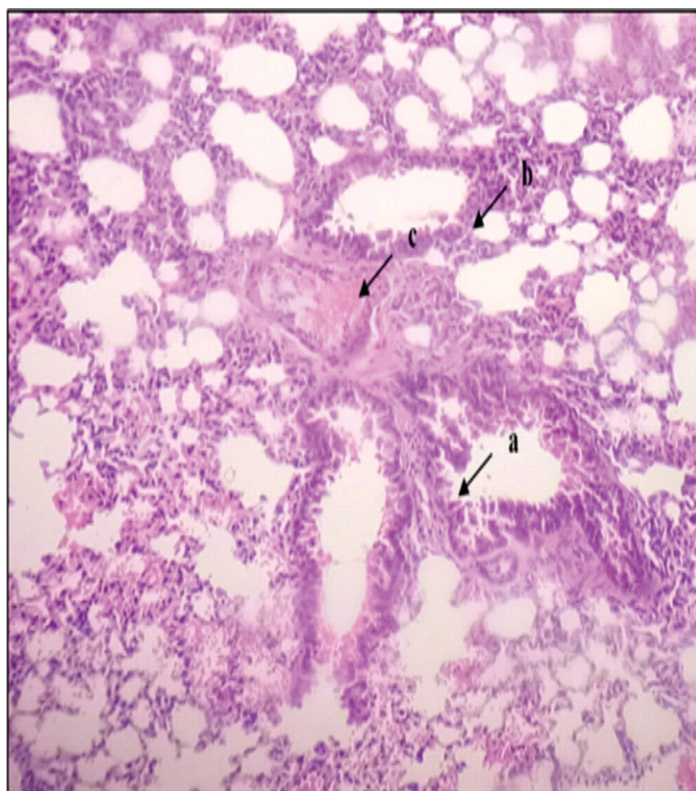


Figure 3. Hematoxylin and Eosin stained histopathology slide (cross-section) of the AF2-A inoculated BALB/c mice which died on the sixth day in LPO [100x] (a) desquamation of epithelial lining of the bronchiolar and alveolar walls (b) accumulation of neutrophils (c) homogenous pink exudate, characteristic of pulmonary edema

necrosis = 10%, edema= 40% and presence of hyphae on 20% of the tissue. These observations were seen in all of the prepared slides (12/12), however, the presence of hyphae is higher in AF2-A inoculated BALB/c mice.

In higher magnification, the homogenous pink exudate surrounded by an accumulation of neutrophils revealed the presence of dichotomously branched septate hyphae of *Aspergillus fumigatus* as illustrated in Figure 4 below. These exudates where most of the fungal propagules were seen, were more prevalent among the lung tissues of BALB/c mice infected with AF2-A followed by AF3-A and AF4-A.

The Gomori Methenamine Silver (GMS) was the second stain used to visualize fungal elements in the lung tissues of mice. Figure 5A showed the lung tissue of mice not infected with *Aspergillus fumigatus* (control). The tissue displayed normal and undamaged bronchiolar and alveolar epithelial walls. The linings of the epithelial walls remained thin and delicate without an evident accumulation of neutrophils or inflammatory cells.

Meanwhile, as seen in Figure 5B, the GMS stained cross-sectional lung tissue of inoculated mice (AF2-A) which died on the sixth day of the observation period showed no visible linings of bronchiolar and alveolar walls that can be attributed to the hyphal invasion of the fungus. Moreover, fungal elements can already be seen even in low power

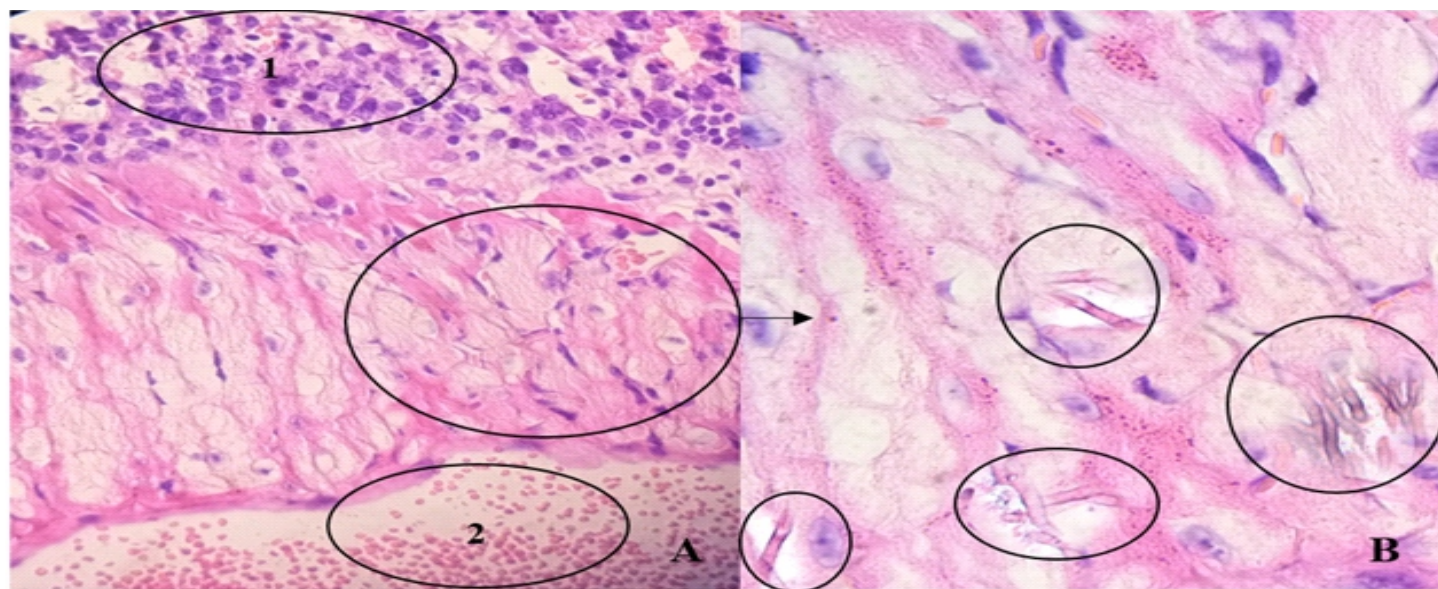


Figure 4. Hematoxylin and Eosin stained histopathology slide of the *Aspergillus fumigatus* infected BALB/c mice which died on the sixth day (A) in high power objective (HPO) [400x] showing hyphal elements and [1] inflammatory cells composed of monocytes, lymphocytes and neutrophils [2] bottom part displayed numerous red blood cells, hemorrhage (B) higher magnification of the same slide in oil immersion objective [1000x] showing typical characteristics of *A. fumigatus*, with dichotomously branched septated hyphae in 45° acute angle

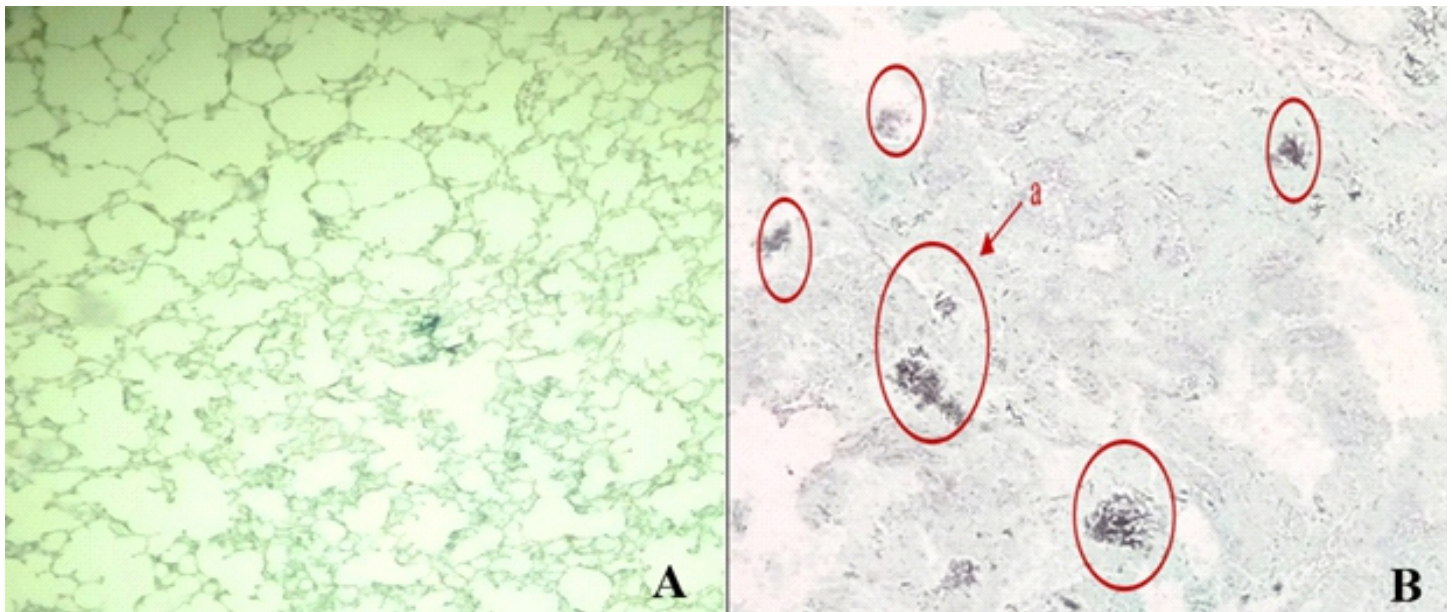


Figure 5. A. Gomori Methenamine Silver stained cross-sectional lung tissue of BALB/c mice from the control group showing intact bronchiolar and epithelial walls in LPO (100x) B. Gomori Methenamine Silver stain of AF2-A group showing (a) masses of fungal hyphae in LPO (100x)

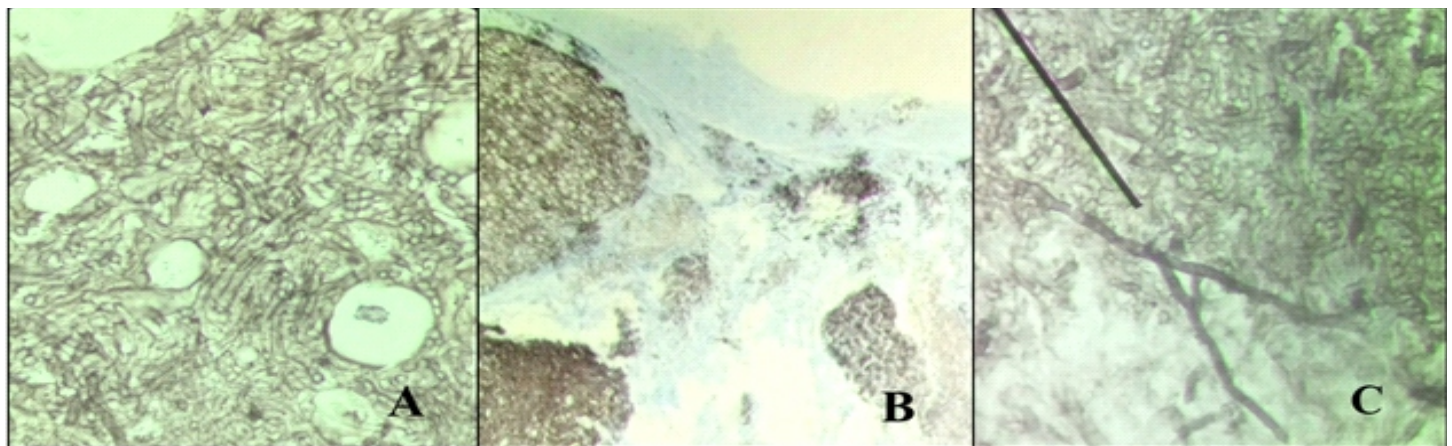


Figure 6. Gomori Methenamine Silver stain of mice from AF2-A group which died after six days of observation period (A) LPO of aggregation of fungal elements [100x] (B) HPO of fungal masses showing overlapping fungal hyphae [400x] (C) OIO of infected lung tissue displaying *A. fumigatus*, dichotomously septated hyphae that branched at an acute angle [1000x]

objective (LPO) magnification showing aggregation or masses of fungal propagules formed in alveolar and bronchiolar tissue. The presence of accumulated fungal hyphae and mycelia appeared brown to black in color against a light green background. The degree of damage, measured in the number of fungal masses in 2.5 mm field of view diameter (10x [LPO] /eyepiece magnification at 25 mm.) among the tissues of infected BALB/c mice showed the highest value in AF2-A, followed by AF3-A and AF4-A. In GMS-stained lung tissue of inoculated mice (AF2-A) which died on the tenth day of the observation period showed a higher number of hyphal elements when compared with the mice which died on the sixth day. These observations

were seen in all of the prepared slides (12/12), however, the presence of hyphae was higher in AF2-A-inoculated BALB/c mice.

Figure 6 shows a more detailed view of the masses of fungal hyphae in the lung tissue of mice. The characteristic morphology of *Aspergillus fumigatus* was evidently displayed showing dichotomously septated hyphae that branched at acute angles. The fungal hyphae were sharply outlined in brown to black color against a light green background. In heavily infected lung tissue of mice, as of AF2-A, the hyphal elements overlapped resulting in the loss of epithelial lining within airways.

Discussion

The air sampling conducted among the three NACWs (Ward 2-Male Surgery, Ward 4-Female Surgery, Ward 6-Pediatrics), three ACWs (Room 434, Room 435, Room 436) and two comparison sites (Main Lobby and Nurse Station) collectively yielded seven fungal isolates which were identified through morphological-microscopic analysis. The following organisms were *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Curvularia sp.*, *Penicillium sp.*, *Alternaria sp.* and *Rhizopus sp.* These organisms have the ability to infect and proliferate inside a host with a compromised immune system.

In using Andersen Air Sampler, particle size discrimination was possible as the air velocity increases through the smaller holes, thus, the species of fungi that can be impacted to each stage of the sampler can be assumed. The first stage allows impaction of 6.8 microns and above of fungal spores was dominated with *Aspergillus niger* having a conidial size of 3-6.5 μm followed by *Aspergillus fumigatus* (2-5.5 μm), and *Alternaria sp.* (7-17 μm). On the other hand, in Stage 2 of the sampler, the three *Aspergillus* species (*Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*) have the most number of fungal colonies, because of the size discrimination capacity of the equipment that allows the lodgement of 2.5-5.5 μm conidial size to 4.7-7 μm size range of holes in this stage. Moreover, the third stage of the sampler was occupied mostly by the three *Aspergillus* species (*Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*). Moving on to the sixth stage of the equipment, it was evident that fungal species with larger conidial size (*Curvularia sp.*, *Alternaria sp.* and *Rhizopus sp.*) were not apparent on this stage, however, the *Aspergillus* species were still observed in this stage.

This study showed that the *A. fumigatus* environmental strain, AF2-A, displayed resistance to the series of antifungals (Voriconazole, Amphotericin B and Caspofungin) used and was considered a drug-resistant strain based on CLSI M38-A2 epidemiological cut-off values. On the other hand, AF3-A also showed non-susceptibility to Amphotericin B.

The observed resistance to Voriconazole of AF2-A isolate could possibly be attributed to the following mechanisms: (a) reduced azole affinity for cyp51A gene, (b) overexpression of cyp51A gene, and (c) reduction in the cellular concentration of target enzyme due to elevated expression of specific drug efflux pumps [5]. The cyp51A also known as Erg11 is a lanosterol 14 α -demethylase that belongs to P450 enzyme

family member. This is a vital enzyme that regulates ergosterol biosynthesis, a component that maintains cell permeability. This study did not characterize which of the three mechanisms particularly resulted in the observed resistance. However, TR34/L98H was the most common mutation in both environmental and clinical samples of *Aspergillus fumigatus* [5,6]. The presence of 34 base pair tandem repeat (TR34) which is always in sync together with a lysine to histidine substitution at codon 98 leads to the overexpression of cyp51A gene. This alteration can induce up to eightfold increase in the expression of the gene. In return, the increase in mRNA levels that correlates with the heightened cellular cyp51A levels results in reduced azole sensitivity [7].

Recently, azole resistance among environmental isolates has also been found in Norway, Iran, Brazil, the Czech Republic, Japan, Kuwait, and Taiwan [8]. This main mechanism of azole resistance among *A. fumigatus* isolates from environmental sources (presence of TR34/L98H alteration in the cyp51A gene) can be attributed to the exposure azole compounds in agriculture that may create mutations in conidia inducing a resistant phenotype [7].

On the other hand, two of the ten strains of *Aspergillus fumigatus* (AF2-A and AF3-A) were resistant to Amphotericin B according to CLSI M38-A2 epidemiological cut-off value. To date, the exact mechanism of *Aspergillus fumigatus* resistance to Amphotericin B is still not certain and this is even true with clinically isolated organisms. Moreover, there are limited publications available on the epidemiology and prevalence of resistant strains of *A. fumigatus* isolated from the environment. It was shown that 3 out of 596 (1.5%) *Aspergillus fumigatus* isolates from hospital air showed an MIC of greater than 2 $\mu\text{g}/\text{ml}$ of Amphotericin B based on CLSI guidelines [9]. These strains were said to be resistant to Amphotericin B and were associated with a high probability of treatment failure. In a more recent study on the potential mechanism of Amphotericin resistance in *A. terreus*, the role of catalase in the development of resistance was explained. They emphasized that catalases are enzymes present among aerobic organisms that play an essential role in the protection of cells from the oxidative damage by converting the harmful hydrogen peroxide (H_2O_2) into H_2O and oxygen (O_2) [10]. The last antifungal drug used in the study was Caspofungin. This particular drug is under the antifungal class of echinocandins that primarily inhibits the synthesis of β -(1,3)-glucan, a major structural component of the fungal cell wall. Caspofungin has a fungistatic effect on

Aspergillus species, that leads to the lysis of hyphal tips. In this study, Etest results of AF2-A on Caspofungin showed no visible inhibition ellipse that corresponded to the resistance of this isolate to the drug. One of the point mutation events that can transform these wild type isolates of *A. fumigatus* to resistant strains is the insertion of a point mutation wherein there is substitution of serine to proline at position 678 (Ser678Pro) in Fks1p. The genetic region of Fks1p corresponds to the most essential subunit of glucan synthase, and this genetic alteration is sufficient to cause resistance in echinocandins which also includes micafungin and anidulafungin [11].

The alterations observed among the health parameters from the acclimatization period to immunosuppression intranasal inoculation of BALB/c mice were due to the immunosuppressive effect of cyclophosphamide that is capable of inhibiting both humoral and cell-mediated immune responses.

The strain AF2-A was the first mouse to die within six days of the observation period and the survival of this group lasted up to ten days only. On the other hand, the first mortality of mice was observed on the seventh and eighth day of the observation period for the AF3-A group and AF4-A group respectively. In the same cyclophosphamide immunosuppression strategy employed in this study, the group of Steinbach *et al.* (2004) also achieved 100% mortality of mice, with animals dying between six to nine days [12]. They utilized the same route of administration and dosage of fungal inocula. Another study that supports the immediate mortality case in this research shows that deaths of intranasally inoculated mice with cyclophosphamide-induced immunosuppression occurred within four to seven days [13]. This group also observed the same way of fungal administration and concentration. Evidently, the control group (administered with 0.03 ml. of 0.9% saline solution) showed no mortality case. The ten mice in this group were subjected to euthanasia.

The two histologic stains used in the study, H&E and GMS, emphasized the damaging effect of the fungus to the lung tissue and the numerous embedded fungal hyphae of *A. fumigatus* respectively. In the H&E slides, hemorrhage, pulmonary edema and migration of neutrophils and inflammatory cells were seen. The pathologic evidences observed were due to the cell-mediated immunity imposed by the immune system of the mice against *A. fumigatus* infection. First, the accumulation of neutrophils was present in the foci of infection. The presence of pulmonary edema,

displayed by characteristic homogenous pink exudate, was due to pulmonary congestion with fungal hyphae. The air sacs were dilated showing evidences of bronchopneumonia. Moreover, the presence of hemorrhage was caused by the destruction of lung tissues by fungal invasion.

Meanwhile, GMS-stained histologic slides elaborately showed the presence of *A. fumigatus* hyphae. Moreover, in these slides masses of fungal hyphae covered almost 50% of the examined tissue. Also, damage in alveolar and bronchiolar epithelia was prominent, thus, alveolar walls were no longer visible. The histologic slide of the first recorded mortality in AF2-A group on the sixth day already showed an invasion of fungal hyphae. The conidia of *A. fumigatus*, when placed in an appropriate milieu for growth, like the lung tissues, starts the process of germination. The thermotolerance ability of this fungus allowed it to thrive in the host at 37°C to 42°C, with an even higher germination rate [14].

The variation in the mortality rates among the three *A. fumigatus* infected BALB/c mice may be due to the inherent virulent factors that were unique to each of these isolates. As previously mentioned, the recorded first death of mice, belonged to the AF2-A group, the same group where the median survival time was least (eight days). Meanwhile, this particular isolate also showed resistance to the panel of antifungals used in this study. The resistance conferred by the AF2-A inoculated BALB/c mice could have possibly contributed to the short survival time observed in this group.

Conclusion

Overall, this study showed that there were strains of *Aspergillus fumigatus* from a hospital indoor air that were considered resistant strains to Voriconazole, Amphotericin B, and Caspofungin (AF2-A and AF3-A). The presence of these fungal species in health institutions can pose a serious concern, particularly to severely immune-compromised patients. Voriconazole, Amphotericin B, and Caspofungin were considered the first-line therapies in the management and prophylaxis of aspergillosis. And the emerging resistance of opportunistic fungi, *Aspergillus fumigatus*, to these antifungals is of public health concern. Moreover, the survival analysis phase of this study reflected the virulence of these environmental isolates in an immune-compromised host. There could have been a linkage between the observed antifungal resistance and virulence exhibited by AF2-A. Evidently, histologic analyses of *A. fumigatus*-infected BALB/c mice showed the damaging effect of fungal invasion and the

concomitant immune response of the host. Edema, haemorrhage, desquamation of epithelial linings, and characteristic bronchopneumonia were the prominent pulmonary effects. According to substantial studies, the immunological process exhibited by BALB/c mice and other murine models to this fungal infection is comparable to the mechanism of immunity observed in man. However, the direct interpretation of murine experimental data to human pathologic events was often difficult due to minimal differences in the immune system and certain biochemical pathways. The generated information will hopefully be helpful for the hospital Infection Control Committee in conducting a more profound epidemiological characterization of environmental isolates found in the medical institution.

For related research work based on the results of this study, the following are the recommendations of the researcher:

1. Concerning the Hospital Infection Control Group:

1.1 Conduct of a regular microbial air sampling to assess the indoor air quality within the hospital facility.

1.2 Monitoring of affected clinical areas (Room 435) for ingress of dust in spite of preventive measures; and for the highest risk groups, use of air sampling to monitor fungal counts.

1.3 Assessment of patients who are at high risk of developing aspergillosis and classification of at-risk patients according to the National Guidelines for the Prevention of Nosocomial Aspergillosis.

1.4 Isolation measures should be taken to protect the growing populations of patients at-risk of acquiring *Aspergillus* infection as a consequence of hospital renovation, construction, or demolition work in or near clinical areas as observed in many areas of the medical facility as of this time of writing.

1.5 Major internal or external works may require transfer of at-risk patients to another part of the hospital if the environment cannot be protected from ingress of airborne fungal spores.

2. Concerning future researchers:

2.1 Increase the duration of microbial air sampling to weeks or months to determine the most prevalent strain for a particular period;

2.2 Increase the number of fungal isolates to be tested to have a more inclusive data on antifungal susceptibility, and determine susceptibility profiles of other *Aspergillus* species with reported cases of nosocomial aspergillosis such as *A. flavus*, *A. niger*, and *A. terreus*.

2.3 Conduct laboratory tests to determine the actual number of leukocytes in the immune-suppressed mice. This is to assess neutropenia among the animals prior to fungal inoculation aside from the physical health determinants.

2.4 The use of molecular assays that can measure fungal burden on examined tissues. This may include performing quantitative nested real-time PCR or galactomannan enzyme immunoassay (EIA).

2.5 The molecular identification of mutations and other genetic alterations on resistant isolates. Information on these factors will be essential in the production of novel antifungal drugs.

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