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Article

Case Report of Indoor Air Antimicrobial Resistant Fungi Circulating in a Ghanaian Referral Autopsy Unit

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Abstract: Background: The public health risk associated with the autopsy unit is high and information on indoor microbial air contamination is limited. Airborne pathogenic fungi thrive in moist indoor environments similar to autopsy room conditions. They spread through spores, cause infections in immunocompromised patients, and exhibit resistance to antimicrobials, making them a public health threat. This study profiled fungi circulating in the indoor air of a referral autopsy unit in Accra, Ghana.

Methods: The different sections of the unit were sampled using the standard open plate technique. Fungal isolates obtained were phenotypically and molecularly characterized. The resistance to fluconazole and itraconazole, as well as a germicide used in routine cleaning of the unit, was tested using broth microdilution assays. **Results:** Fungi isolated include species of clinically relevant strains of *Candida* and *Aspergillus*; also, *Penicillium* spp. and *Mucor* spp. They exhibited high levels of resistance to fluconazole and itraconazole, as well as Denzal® germicide used in routine cleaning of the autopsy unit. **Significance:** This study is the first to profile the fungal diversity of an autopsy unit in Ghana, and the findings suggest that workers might be exposed to fungi with high pathogenic potential that could cause infections.

Keywords: autopsy; postmortem; azoles; antifungal; fungi; referral-autopsy rooms

Introduction

The autopsy room is a sensitive and high-risk sections of the hospital unit. Globally, microbial profiles of autopsy rooms are not well documented. However, diverse microbial populations have been associated with autopsy procedures [1]. This has increasingly contributed to the occupational risks of the autopsy section of the hospital. Pollution of indoor air, including autopsy room can be attributed to bacteria and airborne fungi, especially filamentous fungi that grow in moist indoor environments [2]. Fungi are a threat to public health, especially those implicated in diseases including species of *Aspergillus*, *Candida*, and *Cryptococcus* [3,4]. These fungi were recently described as WHO Critical Fungal Pathogens with high levels of resistance to antimicrobial agents [5].

Fungal infections are on the rise, with an estimated 150 million severe cases resulting in 1.7 million annual death [6]. Humans are exposed to pathogenic fungi via dispersed *Aspergillus* spores in air or *Penicillium* moulds in food [7]. Pathogenic fungi pose a higher risk to immunocompromised patients with cancer, tuberculosis, diabetes, HIV/AIDS and secondary infections [8,9]. In Ghana, about 4% of the

population suffer from severe fungal infections including chronic pulmonary and invasive aspergillosis, asthma and pneumocystis pneumonia, candidiasis and vulvovaginal annually [10,11]. Also, Cryptococcal meningitis, *Pneumocystis jirovecii* pneumonia and histoplasmosis affect AIDS patients [11]. This has contributed to fungi-associated public health-risks in Ghana, especially with fungal increasing resistance to antimicrobials.

In West African countries including Ghana, there is little or no information on the fungal profiles of autopsy units. In a referral hospital autopsy unit in Accra-Ghana, there was a case report of possible exposure of workers to microbial agents, especially airborne pathogenic fungi which could cause infections. This study, therefore, profiled the airborne fungi within a Ghanaian referral hospital autopsy unit and provide insights into their antimicrobial resistance.

Materials and Methods

Study Design and Sample collection

A referral hospital autopsy unit in Accra, Ghana was profiled for fungi. The autopsy (10 x 17 meters) has different compartments including the autopsy suites, staff offices, reception, secretariat and washrooms. There is an average of twenty working staff with equal and unrestricted access to the autopsy suites to perform autopsy. The autopsy rooms are disinfected with Denzal® germicide before and after autopsy. Ten air samples were collected (in duplicate with two per section) under aseptic condition using the standard open plate technique previously described [12]. Briefly, sterile plates containing Saboraud Dextrose agar (SDA) were placed in different sections of the autopsy unit (offices, reception, washroom, secretariat, autopsy/postmortem room, dissection room). The plates were opened for 1-3 h during postmortem session, with sealed or unopened plate as controls. These plates were aseptically transported to the laboratory on ice for analysis at the Molecular Biology Unit, Department of Biochemistry, Cell and Molecular Biology, University of Ghana. The agar plates were incubated at 25 °C for 5-7 days.

Characterization of Fungal Isolates

Phenotypic and Morphological Characterization

Characteristic fungal colonies were isolated, successively sub-cultured and morphologically identified based on shape, form, elevation, margin and colour as displayed by the isolates on the SDA plates (S1). This was followed by lactophenol cotton blue (0.1 mg cotton blue, 40 mg phenol crystals, 80 ml Glycerol, 40 ml distilled water, 40 ml lactic acid) staining. Briefly, a small portion of the mycelium of the fungal culture was placed on a sterile slide with drops of lactophenol blue and carefully covered with a clean sterile coverslip. The stain was allowed to stand at room temperature for 3-5 min and examined under a light microscope at 40X magnification (S1).

DNA Extraction and Internal Transcribed Spacer PCR Amplification

Guanidine Hydrochloride (GHCl) DNA extraction method was used. Briefly, fungal cultures were suspended in sterile distilled water, centrifuged (5,600 rpm, 5 min) and transferred to a tube containing 450 μ l of lysis solution (1 M Tris of pH 8, 0.5 M EDTA, 0.5 M NaCl, 10% SDS, distilled water, RNase A). The sample was bead beat with 50 mg of silica beads solution in a Disruptor Genie™ (Scientific Industries, US. Pat. 5707861) for 15 min, incubated (65 °C, 20 min) and centrifuged (2 min, 5,600 g). 400 μ l of the supernatant was added to 150 μ l of 5 M potassium acetate in a 2 ml microcentrifuge tube and cooled (1 h, -20 °C). This was further centrifuged (30 min, 5,600 g) and 400 μ l of the supernatant added to 600 μ l of 1 M GHCl. 700 μ l of the mixture was transferred to a spin filter collection tube and centrifuged (2 min, 5,600 g). The flow-through discarded and 500 μ l of wash buffer (1 M Tris of pH 8.0, 0.5 M EDTA, 5 M NaCl, absolute ethanol,

distilled water) was added and centrifuged (2 min, 5,600 g). 500 μ l of ice-cold ethanol was added and centrifuged (5,600 g, 2 min). This was incubated at 25 °C for 15 min and DNA was eluted with 200 μ l of sterile distilled water. ITS regions within fungal isolates was PCR profiled using ITS-1 (5' TCCGTAGGTGAAACCTGCGG 3'), ITS -2 (5' GCTGCGTTCTTCATCGATGC 3'), ITS-4 (5' TCCTCCGCTTATTGATATGC 3') and ITS-5 primers (5' GGAATAAAAGTCGTAACAAAGG 3'). A total reaction of 15 μ l included 8.4 μ l of OneTaq 2X, 0.4 μ l of each primer, 3.8 μ l of nuclease-free water and 2 μ l of template DNA. The reaction was run in a thermocycler (Biometra-T professional TRIO Thermocycler, Sheffield, UK) at an initial denaturation (94 °C, 5 min), followed by 35 cycles of denaturation (94 °C, 45 s), annealing (50 °C, 1 min), final and a final extension (72 °C, 10 min). The PCR products were resolved on ethidium bromide stained 1.5% agarose gel electrophoresis (100 V for 35 min) and visualized by UV on an Amersham™ Imager 600 (S1). The amplicons purified with a QIAquick PCR kit before Sanger sequencing (Eurofins Genomics, India) and isolates identified using NCBI BLAST algorithm.

Antifungal and Disinfectant Resistance Profiling

Broth micro dilution was performed in increasing two-fold concentrations. 10 μ g/ml, 20 μ g/ml and 40 μ g/ml concentrations of fluconazole and itraconazole were tested against RPMI standardized fungi culture. The fungal strains were also tested against the recommended working concentration (0.1:6.4 v/v); also, double (0.2:6.4 v/v), triple (0.3:6.4 v/v) and quadruple (0.4:6.4 v/v) concentrations of Denzal® germicide routinely used for autopsy cleaning. To the test wells of a 96-well plate, 100 μ l of the RPMI standardized inoculum was added to 100 μ l of the antifungal or germicide, and the test was conducted in triplicates in two independent experiments. A control without the antifungal or germicide, containing 100 μ l of the test strain and 100 μ l of RPMI was included and incubated (25 °C, 24-48 h, 150 rpm). After incubation, resistance profile was determined by measuring absorbance at 600 nm using a Varioskan™ LUX multimode microplate reader. Data were interpreted according to CLSI standards for antifungal agents and resistance expressed as a percentage of the test to the controls [13].

Statistical Analysis

Data were processed with MS Excel (**version**) and presented in graphs/tables using descriptive statistics (with SPSS 16.0 and GraphPad 6.0). The data were subjected to simple t-test and one-way ANOVA with significance level at $p < 0.05$.

Results and Discussion

Autopsy Air Harbors Diverse Fungi Isolates

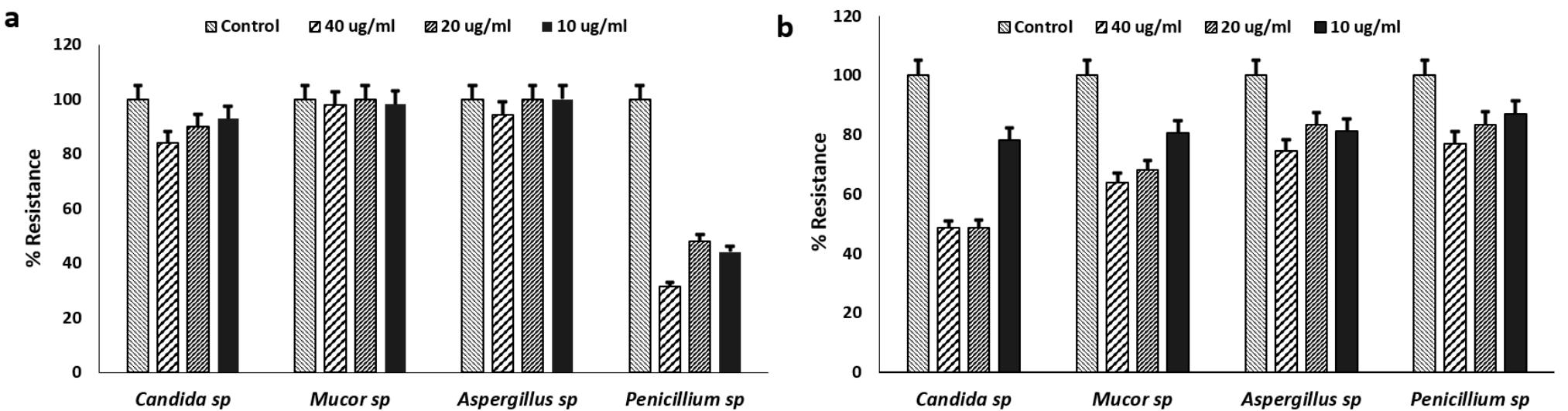
Fifteen fungal isolates of four genera (Yeast, *Mucor*, *Aspergillus* and *Penicillium*) were obtained and identified from the ten air samples collected (**Figure 1**). Yeast and *Mucor* spp. were obtained from the autopsy suite, the offices and the reception; *Mucor* spp., *Aspergillus* spp. and *Penicillium* spp. from the secretariat and *Aspergillus* spp. from the washroom (**Table 1**). These fungal isolates have also been previously isolated in hospital indoor air [14–16]. The Yeasts were majorly *Candida albicans* which has been implicated in human infections, its presence in the autopsy room is alarming; however, it might be attributed to its opportunistic nature to survive outside the host in moist environments. *Aspergillus niger* and *A. fumigatus* found in the washroom and secretariat are opportunistic pathogens implicated in pneumonia and aspergillosis, especially in immunodeficient individuals [17]. *Aspergillus* spp. have genetic architecture to survive in different environments, including harsh conditions such as in the presence of antimicrobials and extremely high temperature [18]. *Mucor*, a fungus commonly distributed in the autopsy unit have been implicated in rare but severe fungal infection, mucormycosis [19]. *Mucor* spp. also increases

the severity of secondary infections with immunocompromised patients [20]. *Penicillium* spp. are associated with keratitis, pneumonia and UTIs [21,22].

Table 1. Identification and Characterization of Fungal Isolates.

Section/No of Samples	Isolates	Form	Elevation	Margin	Colour	Probable Isolates	ITS PCR	ITS Sequencing
Autopsy	A1	Circular	Raised	Undulate	White	Yeast	+	<i>Candida albicans</i>
Suite/2*	A3	Circular	Raised	Undulate	White	Yeast	+	<i>Candida albicans</i>
	A5	Circular	Flat	Entire	White	<i>Mucor</i> spp	+	<i>Mucor</i> spp.
	A7	Circular	Raised	Undulate	White	Yeast	+	<i>C. albicans</i>
	A8	Circular	Raised	Undulate	White	Yeast	+	<i>Candida</i> spp.
Offices/2	C4	Circular	Flat	Undulate	White	Yeast	+	<i>Candida</i> spp.
	C6	Circular	Flat	Entire	White	<i>Mucor</i> spp	+	<i>Mucor</i> spp.
	C8	Circular	Flat	Entire	White	<i>Mucor</i> spp	+	<i>Mucor</i> spp.
Washroom/2	D2	Circular	Raised	Entire	Dark	<i>Aspergillus</i> spp	+	<i>Aspergillus</i> spp
	D3	Circular	Flat	Entire	Dark	<i>Aspergillus</i> spp	+	<i>A. niger</i>
	D4	Circular	Raised	Entire	Dark	<i>Aspergillus</i> spp	+	<i>A. fumigatus</i>
Secretariat/2	E5	Irregular	Raised	Filiform	Dark-green	<i>Penicillium</i> spp	+	<i>Penicillium</i> spp
	E7	Circular	Flat	Entire	Dark	<i>Aspergillus</i> spp	+	<i>A. niger</i>
	E8	Circular	Raised	Entire	Dark	<i>Aspergillus</i> spp	+	<i>A. niger</i>
	E9	Circular	Flat	Entire	White	<i>Mucor</i> spp	+	<i>Mucor</i> spp
Reception/2	F1	Circular	Flat	Entire	White	<i>Mucor</i> spp	+	<i>Mucor</i> spp
	F3	Irregular	Flat	Entire	Creamy-white	Yeast	+	<i>C. albicans</i>

2* - two samples per site (in duplicate), + - Positive for ITS1/ITS2 or ITS4/ITS5.



Autopsy Rooms Fungal Strains are Highly Resistant to Antimicrobials

Relative to the control (strains without antifungal agents), fungal isolates survived 10 μ g/ml, 20 μ g/ml and 40 μ g/ml fluconazole and itraconazole (Figure 1a). Yeast and species of *Mucor* and *Aspergillus* showed 80-100% levels of resistance to fluconazole, and 50-100% to Itraconazole. This is similar to previous studies that indicated highly resistant *Candida* spp. to these antifungals, with clinical *C. auris* showing approximately 90% resistance to fluconazole [23,24]. *Penicillium* spp. showed relative low levels of resistance (30-50%) to fluconazole as compared to Itraconazole at all concentrations (Figure 1b). *Aspergillus* spp. and *Penicillium* spp. have been reported to have intrinsic resistance to azole (itraconazole and fluconazole) antifungals, especially in European and Asian countries similar to the observation in this study [25,26]. In addition, resistance of *Mucor* spp. to itraconazole as observed in this study, has also been associated with septicemia [27]. This also suggests that there is possibility for microbial spread within the hospital environment where the autopsy unit is situated, raising concerns of risks to public health.

The isolated fungal strains were also tested against the germicide used for routine cleaning of the autopsy unit at 0.1/6.4 v/v, 0.2/6.4 v/v, 0.3/6.4 v/v and 0.4/6.4 v/v (Figure 2). Relative to the controls, the recommended standard working Denzal® germicide concentration (0.4/6.4 v/v) showed a relative effectiveness against *Mucor* spp. and *Aspergillus* spp. with reduction in microbial growth; however, *Candida* spp. and *Penicillium* spp. were resistant. All the fungal strains showed 40-100% levels of resistance to the Denzal® germicide at all concentrations. This is of high occupational risks as germicide is a cresylic creosote with 27% w/v phenols with high fungicidal activities. The germicide was ineffective against the fungal strains at recommended working concentration and increased concentrations. This suggests increased risk of exposure of the autopsy staff to these pathogenic fungi, as the cleaning and disinfection regimen of the unit is likely unable to inhibit spores generated during autopsy, those resident on fomites and dispersed into the air.

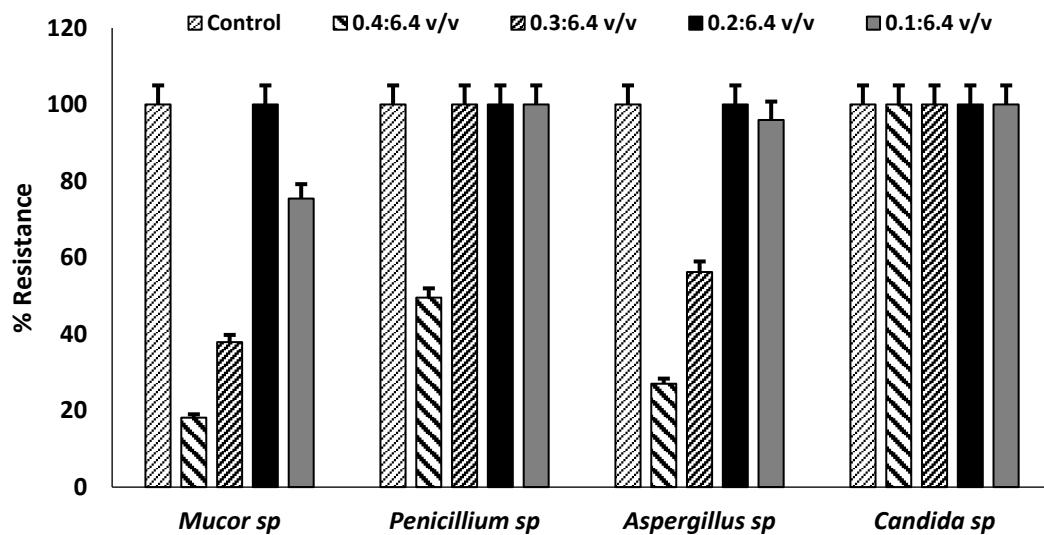


Figure 2. Resistance Profiles of Fungal Isolates to Denzal® germicide. High levels of percentage resistance at different recommended and two-fold concentrations with significance at $p<0.05$ by one-way ANOVA. Error bars represent percentage error of the mean of three replicates of each value.

Conclusions

This is the first study to provide baseline data on fungal profiles of a Ghanaian hospital referral autopsy unit. The fungal isolates dispersed into autopsy indoor air are of clinical relevance with increasing occupational risks. Species of *Yeast*, *Penicillium*, *Mucor* and *Aspergillus* have been implicated in diverse human infections, and could pose a threat to autopsy workers and visitors. The high levels of antimicrobial resistance displayed by the isolated fungi is a threat to public health; necessitating a need for consistent surveillance of autopsy unit with proper cleaning and disinfection practices.

Author Contributions: MO, KA, LM and AI conceptualized. MO and AI designed the study. AI, MO, and FG obtained, processed, analyzed and interpreted the data. AI and MO prepared the first draft of the manuscript. AI and LM revised the draft for important intellectual content. All the authors approved the final draft of the manuscript for submission.

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Data Availability: https://figshare.com/articles/journal_contribution/S1_Supplementary_Data-_Fung_Characterization_pdf/23943102.

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Conflicts of Interest: The authors declare no conflict of interests.

Ethics Statement: No human or animal samples were obtained or analyzed. This is a part of a bigger study profiling different sections of Ghanaian hospital environments as approved by Ghana Health Service (GHS-ERC01/02/19).

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