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Susceptibility of Medical Fungal Isolates to Commonly used Antifungal Agents

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The susceptibility of fungal isolates associated with indoor air of medical institutions to commonly used antifungal agents were investigated using the open plate technique on Sabouraud Dextrose Agar plates. The well in agar diffusion technique was used in determining the antifungal susceptibility. The antifungal agents used were fluconazole and ketoconazole and these drugs were prepared into four concentrations. Result showed reduced antifungal activity as the concentration decreased. Results of fluconazole against the fungal isolates showed that *Aspergillus flavus, Aspergillus niger*, *Candida* sp, and *Mucor* sp were all sensitive at 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml while *Rhizopus* sp was only sensitive at the 5mg/ml concentration. Antifungal activity of ketoconazole showed that *Aspergillus flavus, Candida* sp, *Mucor* sp and *Rhizopus* sp were all sensitive at the 5, 2.5, 1.25 and 0.63 mg/ml concentrations while *A. niger* was susceptible to ketoconazole at 5 and 2.5 mg/ml concentrations. The MIC of Ketoconazole against *Candida* sp, *Aspergillus flavus*, *Mucor* sp and *Rhizopus* sp was 0.63 mg/ml. while the MIC of *Aspergillus niger* was at 2.5 mg/ml. There was highly significant difference (P≤0.05) in the response of the fungal isolates to fluconazole and ketoconazole in all concentrations of the drugs. Result also showed that both drugs were more effective against *Candida* sp and because of the effectiveness of ketoconazole on other isolates, it is recommended for use in treating mycoses caused by these fungi from this site.

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1. INTRODUCTION

Bioaerosols impact heavily on human health via the promotion of infectious diseases, allergy, and asthma including neurological diseases in susceptible individuals. These could alter normal hospital activities and since they were contacted within the hospital, they are commonly referred to as Hospital acquired infections or Nosocomial infections [1]. Nosocomial infections are problematic and most of the infections are connected with person to person contact with an estimated 1 in 10 patients acquiring an infection when hospitalized [2]. Although most of these infections are transmitted through the air by inhalation of pathogens present in the air, thus, the quality of the air within the hospital environment is something the hospital board need not overlook.

In the hospital, mixtures of chemical and biological contaminants (bioaerosols) are types of harmful materials which are of major concern on the health of humans who comes in contact with them [3]. For the comfort of patients, including other persons within the hospital and to control the emission of hazardous substances, the hospital and other health care institutions need to be properly ventilated [4]. The quality of air within the hospital building or the health care environment in respect to its biological constituent is a major concern because patients could be a source of pathogenic microbes thereby disseminating infectious particles to personnel or persons within the building [5]. Thus, inhalation of these microbial constituents presents in the air may cause diseases especially in susceptible and immune compromised patients. According to Stetzenbach [6], inhalation of pathogenic microorganisms exposes the upper and lower respiratory tracts of humans to a variety of airborne particles and vapors. Susceptible individuals within this environment could also be infected. Fungi which range from yeast to molds could thrive in harsh environments including areas with low pH, and high humidity and their spores which are forms through which they reproduce are constantly disseminated or dispersed by the air (Prescott *et al.,* 2011). Thus, they could also be found in indoor environments including the hospitals or health care institutions. Tormo *et al.* [7] reported that the epidemiology of indoor fungi is related to many factors including the temperature, moisture content, ventilation and the organic matter that make up the building materials, and that their correlation between the amount of spore and the risk of infection by these spores is generally accepted. These factors including the number of persons both healthy and unhealthy and seasonal variations could contribute to the availability of fungal spores within the hospital building. Aspergillosis which is an invasive pulmonary disease that is caused by *Aspergillus* species has been reported [4]. Other systemic infections caused by fungi are airborne and could be transmitted by inhaling aerosols containing these spores. For instance, histoplasmosis caused by the fungus *Histoplasma capsulatum* is a systemic infection that results from the inhalation of spores of the fungi. Previous study on indoor air has focused more on the antibiotic resistant pattern of bacterial isolates within the indoor air with less attention given to the susceptibility of fungal isolates within the indoor air to antifungal drugs. Thus, this study was aimed at evaluating the susceptibility of fungal isolates associated with the indoor air in health institutions.

2. MATERIALS AND METHODS

2.1 Description of the Study Area

The study area is Port Harcourt metropolis. The sampling locations were the Model Primary Health Centre, Rumuigbo, the Mini Mile 3 Model Primary Health Centre and the Kelsey Harrison Hospital. The model primary health care centre, Rumuigbo is located in Rumuigbo along the Rumuokoro high way immediately after the Obi Wali round about in Obio/Akpor local government area. Its coordinates are 4.850^{o}N and 6.991^{o}E. The Mini Mile 3 model primary health care centre is situated along the building materials axis of the Mile 3 market in Port Harcourt Local Government area. Its coordinates are 4.806^{o}N and 6.992^{o}E. The Kelsey Harrison Hospital which was formerly the Niger hospital is located along Emenike Street of the Diobu axis in Port Harcourt Local Government area. It is a two storeyed building consisting of more than eleven hospital wards. It is one of the major specialist hospitals in the state that is owned by the Rivers State government. Four wards which included the Outpatient ward, Children ward, Postnatal ward and the Injection room were the sites selected for the investigation in the Primary Health Centres. This is because these sites were frequently used and readily accessed. The Outpatient ward,

Female ward, Injection room, Accident and Emergency ward and the Maternity ward were the sites selected for study in the Kelsey Harrison Specialist Hospital.

2.2 Isolation of Indoor Air Fungi

Freshly prepared sterile Sabouraud Dextrose Agar (SDA) plates in duplicate were exposed 1m above ground level to air in the different wards for 15minutes to allow microbial particles within the wards to settle on the surface of the medium by gravity. These plates were transported to the microbiology laboratory and incubated for 3-5 days at 25℃ [8]. Counts were made for plates that showed significant growth at the end of incubation.

Discrete fungal colonies on SDA plates were picked with sterile inoculating needle and cultivated on freshly prepared pre-dried SDA plates. Pure cultures of the isolates were obtained by subculturing fungal spores and colonies continuously on freshly prepared medium until it was ascertained that there were no contaminants. Pure fungal cultures were stored on SDA slants and stored in a refrigerator. These served as stock cultures for further use. Pure fungal isolates obtained were characterized culturally and microscopically. The cultural characteristics examined included; colour, shape, size, spore type and texture while the microscopic characterization was done by transferring fungal spores or piece of mycelium on clean microscope slide containing drop of lactophenol blue stain, slides were later covered with cover slip and were viewed under the microscope at X10 and X40 magnification [9]. Fungal identity was confirmed by referencing fungal characteristics with those recorded in the fungal identification manual [10].

2.3 Preparation of Antifungal Concentrations

The two antifungal drugs used in this study were fluconazole (Flucox, phamatex, 150mg) and Ketoconazole (Axoral, 200mg). About 150mg of Fluconazole was dissolved in 150ml conical flask containing 30ml sterile distilled water, while 200mg of Ketoconazole was dissolved in 150ml conical flask containing 40ml sterile distilled water. This gave rise to a 5mg/ml concentration of the various drugs. Subsequent two-fold serial dilutions gave rise to 2.5mg/ml, 1.25 5mg/ml and 0.63 5mg/ml for both drugs respectively.

2.4 Determination of Antifungal Susceptibility

The agar well diffusion was carried out by employing the method adopted by [11]. About 48 hours old fungal broth culture of each isolate were swabbed evenly on the surface of the freshly prepared pre-dried SDA plates in duplicates and allowed for 5 minutes to dry. A 6mm well cutter was sterilized by dipping in 70% ethanol and heating on a Bunsen burner flame and used to bore holes on the seeded plates after which varied concentrations of the drugs were introduced into the well and incubated at 25℃ [8] for 48 hours.

2.5 Statistical Analysis

The zone diameters were recorded and presented in excel work sheet. The mean and standard deviations were calculated using SPSS (version 22). ANOVA was used in checking for significance while the Duncan test was used to separate the means in areas were there was a significant difference.

3. RESULTS AND DISCUSSION

Results showing the zone of inhibition of the fluconazole and ketoconazole against the fungal isolates are presented in Tables 1 and 2, respectively. Result showed varied response to the different antifungal agents. Results of fluconazole against the fungal isolates showed that *Aspergillus flavus, Aspergillus niger*, *Candida* sp, and *Mucor* sp were all sensitive at 5mg/m1, 2.5mg/m1, and 1.25mg/m1 while *Rhizopus* sp was only sensitive at the 5mg/ml concentration. This was also observed as the MIC of the drug against *Rhizopus* sp. Although fluconazole at 0.63mg/ml was not sensitive to *Aspergillus flavus, Aspergillus niger* and *Mucor* sp, it was very sensitive to *Candida* sp. Thus, *Candida* sp was the most susceptible with the least MIC. Minimal inhibitory concentration of *Aspergillus niger, Aspergillus flavus and Mucor* sp were observed to be 1.25mg/ml while MIC against *Rhizopus* sp was observed to be 5mg/ml. There was highly significant difference ($P \leq 0.05$) in the response of the fungal isolates to fluconazole in all concentrations.

Result of the antifungal activity of ketoconazole on the fungal isolates showed that *Aspergillus flavus, Candida* sp, *Mucor* sp and *Rhizopus* sp were all sensitive at the 5, 2.5, 1.25 and

0.63mg/ml concentrations. *A. niger* was susceptible to ketoconazole at 5 and 2.5mg/ml concentrations. The MIC of Ketoconazole against *Candida* sp, *Aspergillus flavus*, *Mucor* sp and *Rhizopus* sp was 0.63mg/ml. while the MIC of *Aspergillus niger* was at 2.5mg/ml (Table 2). The antifungal activity of ketoconazole on fungal isolates showed significant differences (P≤0.05) at all concentrations in the respective fungal isolates. The antifungal activity on *Candida* sp was significantly higher than those recorded for other fungal isolates at all concentrations.

A major problem faced in the medical sector is the resistance posed by microorganisms against antimicrobial agents (Smolinski *et al.,*2003). In other to understand new and emerging trends of resistance shown by microorganisms, knowing the susceptibility patterns of various isolates to commonlyused antimicrobial agent could be vital.

Table 1. Response of fungal Isolates to Fluconazole

Fungal Isolates	Zone Diameter (mm)			
	5mg/m1	**2.5mg/m1**	**1.25mg/m1**	**0.63mg/m1**
Aspergillus flavus	29.50±6.36^{bc}	27.50±0.71^{d}	21.50±0.71^{d}	0.00±0.00^{a}
Aspergillus niger	18.50±0.71^{b}	5.50±0.71^{b}	1.50±0.71^{b}	0.00±0.00^{a}
Candida sp	40.00±0.00^{d}	32.00±0.00^{e}	29.00±0.00^{e}	18.00±0.00^{b}
Mucor sp	24.50±0.71^{c}	19.50±0.71^{c}	10.50±0.71^{c}	0.00±0.00^{a}
Rhizopus sp	1.00±0.00^{a}	0.00±0.00^{a}	0.00±.00^{a}	0.00±0.00^{a}

**Means with same superscript show no significant difference (P≤0.05)*

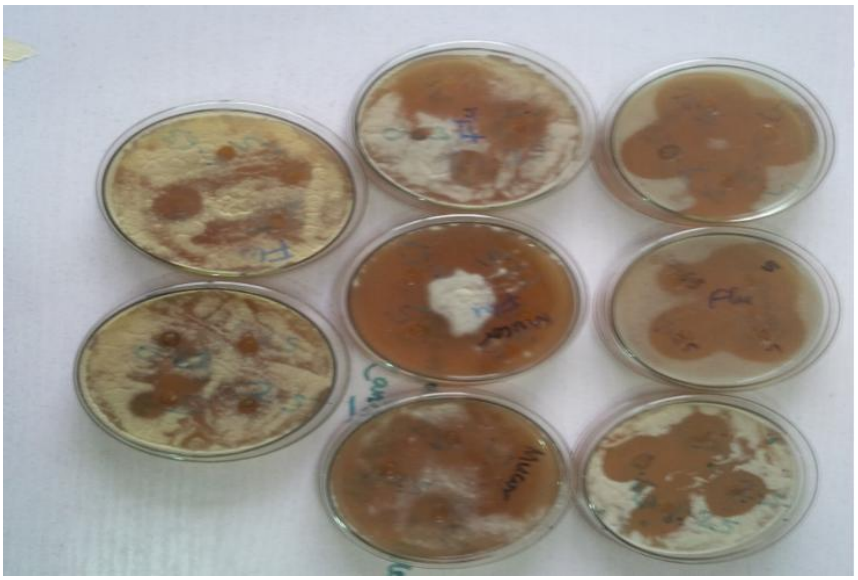

Plate 1. overlaid plates showing the antifungal activity of fluconazole and ketoconazole on some fungal isolates

Table 2. Response of fungal Isolates to Ketoconazole

Fungal Isolates	Zone Diameter (mm)			
	5mg/m1	**2.5mg/m1**	**1.25mg/m1**	**0.63mg/m1**
Aspergillus flavus	28.50±0.71^{c}	24.50±0.71^{d}	20.50±0.71^{d}	18.50±0.71^{d}
Aspergillus niger	23.50±0.71^{b}	3.50±0.71^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Candida sp	40.50±0.71^{e}	37.50±0.71^{e}	29.50±0.71^{e}	27.50±0.71^{e}
Mucor sp	31.50±0.71^{d}	20.50±0.71^{c}	15.50±0.71^{c}	10.50±0.71^{c}
Rhizopus sp	15.50±0.71^{a}	10.50±0.71^{b}	8.50±0.71^{b}	3.50±0.71^{b}

**Means with same superscript show no significant difference (P≤0.05)*

The susceptibility pattern of some fungi isolated from the various study locations were tested against two known antifungal agents (fluconazole and Ketoconazole). According to Talaro [12], a defined zone of inhibition for fungal agents has not been developed because antifungal tests are hard and sometimes not necessary. *Aspergillus niger, Mucor, A. flavus and Candida* were very susceptible to fluconazole at the different drug concentration. *Aspergillus niger, Mucor,* and *A. flavus* had the same MIC values of 1.25mg/ml, while MIC values for *Candida* was 0.63 mg/ml. It was observed that *Candida* species unlike other fungal isolates were highly sensitive to fluconazole. The use of fluconazole in treatment of infections caused by the yeast *Candida* sp is well documented [1]. Thus, this could explain the high zone of inhibition recorded against *Candida* sp by the antifungal agent. Fluconazole acts by inhibiting the enzyme CYP P450 14 α-Demethylase which converts lanosterol to ergosterol [1,13]. Also, for ketoconazole antifungal agent, the MIC values for *Candida, A. flavus, Mucor,* and *Rhizopus* were 0.63 mg/ml, while the MIC values for *A. niger* was observed as 2.5 mg/ml. At 5mg/ml, *Candida* isolates were more susceptible followed by *Mucor* species. Ketoconazole antifungal agents are broad spectrum that is used in treating cutaneous mycoses as well as candidiasis. They function by interrupting the permeability of the fungal cell membrane thereby stopping the synthesis of fungal sterol [1]. They are also classified as azoles which has been known to impact on the integrity of fungal membranes, changing their shape and limiting their growth [13,14]. Despite the high susceptibility of some isolates like the yeast and *A. niger* including *A. flavus* to the respective drugs, other isolates like *Rhizopus* sp showed resistance at low concentrations and were not highly sensitive especially to fluconazole even at high concentration. This could be signs of emerging resistance either as a result of modification of drug binding site, genetic modification or the use of efflux pumps to flush drug concentrations. Resistance to existing antifungal agent has progressed due to the extensive use of antifungal agents and the limited arsenal linked with the growing frequency of opportunistic infections (Scorzoni *et al.,* 2017). Antifungal resistance can be triggered by a variety of factors, including a drop in effective therapeutic concentration, alterations or overexpression of drug targets, and metabolic bypasses [15]. Thus, this could also be the reason why some of the fungal isolates in the current study are resistant to the antifungal drugs especially at lower concentrations.

4. CONCLUSION

The antifungal activity of fluconazole and Ketoconazole showed that both drugs were more effective against *Candida* sp. Also, the findings showed that ketoconazole was more effective in-vitro for treatment of the fungal infections as it displayed higher antifungal activity to the fungal isolates compared to fluconazole. Because of this, ketoconazole could be the drug of choice in treating fungal infections caused by these isolates.

CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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