ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26nov494

Antifungal Susceptibility Pattern of Moulds Isolated from Wounds of Students in Some Tertiary Institutions in Imo State, Nigeria

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Publication Date: 2026/11/21

Abstract: The Antifungal susceptibility pattern of moulds isolated from wounds of students studying at Imo State University, Owerri and University of Agriculture and Environmental Sciences both in Imo State were studied. Fifty wound samples were aseptically collected, isolated, characterized and identified using culturable and non culturable standard microbiological methods. Culturable methods of morphological and biochemical characteristics and non culturable molecular characteristics using 18S rRNA sequencing method was employed. Six antifungal agents namely amphotericin B, fluconazole, itraconazole, ketoconazole, voriconazole and nystatin were employed in performing antifungal susceptibility pattern using the disc diffusion method. The results of isolation and characterization of both culturable and non culturable methods showed the following moulds namely; *Aspergillus flavus, Aspergillus tamarii, Aspergillus niger, Penicillium chrysogenum, Fusarium solani, and Rhizopus stolonifers*. The most prevalent mould was *Aspergillus flavus* (28%) while the least prevalent moulds was *Fusarium solani* (6%). The different moulds were susceptible to amphotericin B (28.00±1.154), Itraconazole (35.00 ±1.732), ketoconazole (38.00 ±2.081) and voriconazole (33.00 ±2.886), while the different moulds were resistant to fluconazole (11.00 ±0.577) and Nystatin (12.00 ±1.527). The antifungal agent of choice was ketoconazole (38.00 ±2.081) because it gave the greatest inhibitory potential when compared with other antifungal agents. The different antifungal agents apart from fluconazole (11.00 ±0.577) and Nystatin (12.00 ±1.527) gave good inhibitory potentials and can therefore be used to treat wounds. There is need to adhere strictly to prescription when using the antifungal agents in order to ensure proper care and treatment of wound infections.

Keywords: *Antifungal agents, Aspergillus Species, Moulds, Students and Wounds.*

How to Cite: Ohabughiro, N. B. (2026) Antifungal Susceptibility Pattern of Moulds Isolated from Wounds of Students in Some Tertiary Institutions in Imo State, Nigeria. *International Journal of Innovative Science and Research Technology,* 11(11), 939-942. https://doi.org/10.38124/ijisrt/26nov494

I. INTRODUCTION

Fungal infections and diseases of man are commonly observed in tropical regions (Bazira, *et al*., 2023). Wound infection is one of such fungal infections seen among students in tertiary universities. Wounds are injuries (cut, scratches, scrapes, punctured skins, surgery, sutures, and stitches) that results in the breakage of the skin or other tissues in the body. Accidents are the primary cause of wounds (National Library of medicine, 2023). Fungal and mould colonization of wounds occurs as a result of moist conditions as well as other favourable factors being present. Fungal and mould colonization of wounds can result to mild, serious and even fatal infections and diseases (Sharma and Nonzom, 2021).

This results to diverse human infections ranging from superficial, subcutaneous and systemic fungal infections. These infections usually various because of age, gender and occupational types and other factors (Morais *et al*., 2010). Clinically, wounds can be said to be infected when there are the presence of purulent materials, change in colour, presence of foul odor even in the absence of positive culture confirmation. Wounds are colonized by microorganisms whether they are infected or not. Microorganisms may not be easily detected in wounds when the patient is on antimicrobial therapy or when the organism is a fastidious microbe (Nwachukwu *et al*., 2009*) [11]*. The isolation, characterization and antifungal pattern plays a key role in the management of wound infections. This study aims to determine the antifungal susceptibility pattern of moulds isolated from wounds of students in some tertiary institutions in Imo State, Nigeria.

II. MATERIALS AND METHODS

- *Sample Collection*

A total of fifty (50) wound swab sticks samples were aseptically collected from both male and female students of Imo State University, Owerri and University of Agriculture and Environmental Sciences both in Imo State. These

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26nov494

wound samples were immediately taken to the Imo State University, Microbiology laboratory for analysis.

➢ *Isolation of Moulds*

The agar used was sabouraud dextrose agar (SDA). The wound swab samples were serially diluted to different dilution factors of 10^{-3} 10^{-4} and 10^{-5}. A concentration of 50mg/l of chloramphenicol an antimicrobial agent was added to prepared plates containing 15ml of sabouraud dextrose agar so as to inhibit the growth of bacteria. 0.1ml of lactic acid was also added to prevent the growth of yeasts (Avasthi and Bhadauria, 2011). An aliquot of the diluent 0.1ml was inoculated onto the prepared plate and incubated at 28 ± 2 ℃ for 7 days in an incubator and was examined daily to observe for fungal growth. The fungal growths observed were subcultured and the results were recorded in colony unit per gram. Antifungal sensitivity test was done on Mueller Hinton agar (Beuchat, 1992).

➢ *Morphological and Microscopic Identification of Moulds*

The colonial morphology and microscopic examinations were used to identify isolated moulds. Lactophenol cotton blue was used to stain the grown fungi mounted on a clean grease free slide then covered with a cover slip. This was then subjected to microscopic examination using x100 magnification in order to identify fungal structure, morphology of the fungal colony and characteristics of fungi spores (Tortorano, *et al*., 2014).

➢ *Molecular Characterization of Fungi Spores*

The Zymo research fungal DNA miniprep extraction kit was employed for the extraction of the deoxyribonucleic acid (DNA). Agarose gel electrophoresis method was used to qualitatively extimate the genomic DNA extracted. Polymerase chain reaction machine was further employed to amplify the extracted DNA. The amplified DNA fragments were subjected to sequencing protocol. The sequencing protocol involved the PCR products which were cleaned using the protocol of EXoSAP. Nimagen brilliant dye terminator cycle sequencing kit was then used to sequenced the DNA fragments in accordance to the manufacturer's instructions (Platt, *et al*., 2007).

➢ *Determination of Antifungal Susceptibility Test*

Clinical and Laboratory Standard Institute method of CLSI M38-A standard for moulds was employed to determine the antifungal susceptibility tests. This was done using the standardized reference method of disk diffusion proposed by the clinical and laboratory standard institute. Six antifungal agents were employed for this tests and they as follows; Amphotericin B, Fluconazole, Itraconazole, Ketoconazole, Nystatin and Voriconazole. Oxoid disc of the antifungal agents were asceptically placed with sterile pair of forceps on the fungal plates. The plates of fungal were then incubated at 28 ℃ for 4 to 7 days and observed daily for determination of their sensitivity to antifungal agents. (Espinel-Ingroff *et al*., 2010).

➢ *Statistical Analysis*

Statistical analysis was done using the analysis of variance (ANOVA).

III. RESULTS

The frequency (percentage) number of students, males and females, from Imo State University, Owerri (IMSU) and University of Agriculture and Environmental Sciences (UAES) both in Imo State from which wound samples were collected were shown in table 1. The moulds isolated from different wound samples were first identified both morphologically and microscopically is seen in table 2. They were further identified genetically by characterizing molecularly using sequencing of 18SrRNA gene using ITS1 and ITS4 primers. The resultant mould species identified were *Aspergillus flavus, Aspergillus tamarii, Aspergillus niger, Penicillium chrysogenum, Fusarium solani, and Rhizopus stolonifers* as shown in table 3. Table 4 shows the frequency and occurrence of the isolated moulds, where the most prevalent mould was *Aspergillus flavus* (28%) while the least prevalent moulds was *Fusarium solani* (6%).

The susceptibility of the different moulds to various antifungal agents were shown in table 5. The antifungal agents being, Amphotericin B, Fluconazole, Itraconazole, Ketoconazole, Nystatin and Voriconazole. The different moulds showed highest sensitivity to Ketoconazole, Itraconazole, Voriconazole and Amphotericin B, while the different moulds showed resistance to Nystatin and fluconazole. The antifungal agent of choice was Ketoconazole (38.00 ±2.081) because it gave the greatest inhibitory potential when compared with other antifungal agents. Ketoconazole, Amphotericin B, Itraconazole, and Voriconazole antifungal agents gave good inhibitory potentials to moulds growth. Nystatin and fluconazole did not give a good inhibitory potential against the growth of the different moulds.

The analysis of variance done showed that there was no significant difference ($P< 0.05$) between Ketoconazole, Amphotericin B, Itraconazole, and Voriconazole. There was a significant difference ($P> 0.05$) between Ketoconazole, Amphotericin B, Itraconazole, Voriconazole and Nystatin and Fluconazole.

Table 1 Frequency (Percentage) of Male and Female Students (Wound Samples) in the Two Institutions.

Students	IMSU (%)	UAES (%)	Total (%)
Male	17 (61)	13 (59)	30 (60)
Female	11 (39)	9 (41)	20 (40)
Total of each Institution	28 (100)	22 (100)	50 (100)
Grand Total of both Institutions	28 (56)	22 (44)	50 (100)

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26nov494

Table 2 Morphological and Microscopic Characteristics of Identified Moulds.

Morphology of Colonies	Microscopic	Probable fungi
Greenish colonies which may turn brown	Septate hyphae	*Aspergillus flavus*
Dark brown or rusty colonies	Conidia heads are in chains	*Aspergillus tamarii*
Black or Dark colonies	Septate hyphae	*Aspergillus niger*
Blue or greenish colonies	Branched septate hyphae	*Penicillium chrysogenum*
White to grayish white	unbranched Septate hyphae	*Fusarium solani*
White cotton to blackish brown colonies	Non septate hyphae	*Rhizopus stolonifers*

Table 3 Molecular Characterization and Identification of Isolated Moulds.

Sequence identity	Percentage (%)	NCBI match	Moulds
NR111041.1	99	*Aspergillus flavus* NR135325	*Aspergillus flavus*
AF004929.1	100	*Aspergillus tamarii* MN339986	*Aspergillus tamarii*
AY373852.1	91	*Aspergillus niger* AY373852	*Aspergillus niger*
NR138306.1 Penicillium	99	*Penicillium chrysogenum* MH793845	*Penicillium chrysogenum*
SC 660122	97	*Fusarium solani* SC 169388	*Fusarium solani*
RS 4846	98	*Rhizopus stolonifers* RS 4846	*Rhizopus stolonifers*

Table 4 The Frequency and Occurrence of the Isolated Moulds.

MOULDS	FREQUENCY	PERCENTAGE (%)
Aspergillus flavus	43	28
Aspergillus tamarii	30	20
Aspergillus niger	24	16
Penicillium chrysogenum	18	12
Fusarium solani	9	6
Rhizopus stolonifers	27	18
Total	151	100

Table 5 The Susceptibility of the Different Moulds to Various Antifungal Agents.

Moulds	Amphotericin B (20 µg/ml)	Flucanozole (25 µg/ml)	Itraconazole (10 µg/ml)	Ketoconazole (10 µg/ml)	Nystatin (22 µg/ml)	Vorioconazole (1 µg/ml)
Aspergillus flavus	24.00 ±1.154(S)	9.00 ±0.277(R)	34.00 ±2.886(S)	36.00 ±1.732(S)	10.00 ±0.577(R)	30.00 ±1.154(S)
Aspergillus tamarii	28.00 ±1.154(S)	11.00 ±0.277(R)	33.00 ±2.886(S)	38.00 ±2.081(S)	12.00 ±1.527(R)	32.00 ±1.154(S)
Aspergillus niger	21.00 ±0.577(S)	8.00 ±0.155(R)	32.00 ±1.154(S)	34.00 ±2.886(S)	11.00 ±0.577(R)	28.00 ±1.154(S)
Penicillium chrysogenum	15.00 ±1.527(S)	6.00 ±0.155(R)	30.00 ±1.154(S)	32.00 ±1.154(S)	11.00 ±0.577(R)	25.00 ±1.527(S)
Fusarium solani	14.00 ±0.577(I)	4.00 ±0.155(R)	28.00 ±1.154(S)	31.00 ±1.154(S)	10.00 ±0.577(R)	21.00 ±0.577(S)
Rhizopus stolonifers	25.00 ±1.527(S)	11.00 ±0.577(R)	35.00 ±1.732(S)	38.00 ±2.081(S)	11.00 ± 0.577(R)	33.00 ±2.886(S)

Key: Susceptible - (S), Resistant (R) and intermediate (I) measured in mm.
End point reading of the Minimum Inhibition Concentration (MIC): • Susceptible ≥ 17 mm (azole) and ≥15 mm (amphotericin B and Nystatin) • Intermediate, 14 to 16 mm (azole) and 13 to 14 mm (amphotericin B and Nystatin) • Resistant, ≤ 13 mm (azole) and ≤ 12 mm (amphotericin B and Nystatin).

IV. DISCUSSIONS

This work tested the antifungal susceptibility of different moulds using the standard disk diffusion method which concurred with the work of Lopez, *et al*., 2006 that disk diffusion method of susceptibility testing for different antifungal agents was a good method for testing moulds susceptibility. This study found out that ketoconazole was the most sensitive antifungal agent among different antifungal agents tested and this result agreed with the research of Agbulu, *et al*., 2015 that ketoconazole antifungal agent had the highest level of sensitivity against different moulds tested when compared with other antifungal agents .The work of (Kahan *et al*., 2018), showed that amphotericin B was the main antifungal agent against invasive fungal infections , this differ from this work that found out that ketoconazole was the antifungal agent of choice against fungi wound infections. The study of (Kamal, *et al*., 2018) also observed that all isolated moulds were sensitive to ketoconazole and amphotericin B and observed a high resistance to fluconazole. These findings agreed with this research work that showed high sensitivity of the different moulds to antifungal agents of ketoconazole and amphotericin B. The findings of (Nisha, *et al*., 2023) and

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26nov494

(Kindu, *et al*., 2019) observed that *Aspergillus* species were susceptible to azole antifungal agents like ketoconazole, voriconazole and itraconazole but the *Aspergillus* species were resistant to fluconazole. These findings agreed with this work that showed high sensitivity of *Aspergillus* species to ketoconazole, voriconazole and itraconazole and high resistance of *Aspergillus* species to fluconazole antifungal agent.

The work of (Elizabeth, *et al*., 2020) found out that itraconazole had high level of sensitivity against moulds and high level of resistance of fluconazole against different moulds tested. This finding agreed with this research that showed high sensitivity of itraconazole and high resistance to fluconazole against different types of moulds.

V. CONCLUSION

Different types of moulds like *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Penicillium chrysogenum*, *Fusarium solani*, *and Rhizopus stolonifers* were isolated from wounds of students studying at higher institutions in owerri. *Aspergillus flavus* being the most prevalent moulds. Among the antifungal agents, moulds were most sensitive to Ketoconazole. Amphotericin B, Itraconazole, and Voriconazole antifungal agents also gave good inhibitory potentials to moulds growth while Nystatin and fluconazole did not give a good inhibitory potential against the growth of the different moulds. Antifungal agents can be effectively used to treat moulds infections in wounds. Therefore, there is need to adhere strictly to prescription when using the antifungal agents in order to ensure proper care and treatment of wound infections.

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