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Antifungal Susceptibility Patterns of Environmental Dermatophytes to Conventional Agents

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Abstract

Dermatophytes present in soil serve as important environmental reservoirs capable of causing superficial fungal infections in humans. The increasing resistance of dermatophytes to conventional antifungal agents poses a significant challenge to effective treatment. This study evaluated the antifungal susceptibility patterns of dermatophytes isolated from garden soils to commonly used conventional antifungal agents. Soil samples were collected from various locations and processed for the isolation of dermatophytes using standard culture techniques. Pathogenic isolates were identified through macroscopic, microscopic, biochemical, and molecular characterization. Antifungal susceptibility testing was performed using the microdilution method to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of selected antifungal agents, including ketoconazole, fluconazole, griseofulvin, cotrimazole, terbinafine, and tinidazole. Four pathogenic dermatophyte species were identified: *Trichophyton mentagrophytes*, *Paraphyton cookei*, *Trichophyton rubrum*, and *Trichophyton tonsurans*. Susceptibility testing revealed generally low sensitivity of the isolates to most conventional antifungal agents. Terbinafine demonstrated the highest antifungal activity, showing lower MIC and MFC values compared to other agents. Variations in susceptibility patterns were observed among the isolates, indicating possible differences in resistance mechanisms. Dermatophytes isolated from environmental soil sources exhibit varying levels of resistance to commonly used antifungal agents. Terbinafine remains the most effective antifungal agent among those tested. Continuous surveillance of antifungal susceptibility patterns is essential to guide effective therapeutic strategies.

Keywords: Dermatophytes; Antifungal resistance; Environmental fungi; Minimum inhibitory concentration; Minimum fungicidal concentration; Terbinafine

Introduction

Dermatophytes present in soil can serve as reservoirs of infection in humans. The geographic distribution of dermatophytes varies depending on climatic conditions and soil characteristics. Loamy soil harbors a significant number of dermatophytes due to their saprophytic nature, and humans especially farmers frequently interact with loamy soil during agricultural activities, predisposing them to infection (Basavarajappa *et al.,* 2022). The prevalence of superficial mycotic infections has risen to the extent that skin mycoses now affect approximately 20–25% of the global population, making them one of the most common infectious diseases worldwide (Putta *et al.,* 2016; Baghi *et al.,* 2016; Sabthrarishi *et al.,* 2017; Prabha *et al.,* 2019; Basavarajappa *et al.,* 2022).

Recently, fungal infections have attracted increased attention from physicians and microbiologists, largely due to the growing number of immunocompromised individuals, including patients with HIV/AIDS, those receiving immunosuppressive therapy for malignancies, and organ transplant recipients. Furthermore, the widespread use of broad-spectrum antibiotics has contributed to the increased occurrence of fungal infections. Dermatophytosis often becomes chronic, disfiguring, and socially stigmatizing. Epidemiological data regarding the exact prevalence and incidence of dermatophyte species responsible for infections remain limited because many patients are treated based solely on clinical diagnosis without laboratory confirmation.

The classical presentation of tinea infection is characterized by lesions with central clearing surrounded by an advancing red, scaly, and elevated border. Although typical of ringworm infections, this presentation is frequently confused with other dermatological conditions, making laboratory diagnosis essential. The prevalence of dermatophytosis is influenced by environmental conditions, personal hygiene, and individual susceptibility, which vary across different geographical locations (Putta *et al.,* 2016; Baghi *et al.,* 2016; Sabthrarishi *et al.,* 2017). Notably, several conventional antifungal agents (fluconazole, ketoconazole, miconazole, tinidazole, terbinafine, among others) previously optimized for treating dermatophytosis have become less effective due to increasing microbial resistance. Additionally, the toxicity

associated with antifungal drugs remains a major concern. These issues highlight the urgent need for alternative therapeutic options for dermatophytosis (Putta *et al.,* 2016; Baghi *et al.,* 2016; Sabthrarishi *et al.,* 2017)

Materials and Methods

Isolation of Dermatophytes from Garden Soil

Sample collection: One hundred (100) soil samples were collected from Umuoma community in Ihiala Local Government Area, Anambra State. Samples were randomly collected from different soil types (loamy, clay, and sandy soils) using a soil auger sterilized with 70% ethanol (prepared by mixing 70 mL ethanol with 30 mL distilled water). Soil samples were collected at a depth of 10 cm and transferred into sterile polyethylene bags (Umeaku *et al.,* 2019). Samples were transported to the Department of Microbiology Laboratory, COOU, for immediate analysis.

Culturing and Purification of the Isolates : A stock suspension of the soil samples was prepared by weighing 1 g using a weighing balance (DC-300) and transferring it into 100 mL of sterile distilled water in a Pyrex conical flask. Tenfold serial dilution was performed by dispensing 9 mL of sterile distilled water into test tubes and transferring 1 mL of the stock suspension into the first tube. The mixture was vortexed, and 1 mL was serially transferred into subsequent tubes. The final 1 mL aliquot was discarded. Aliquots (1.0 mL) were aseptically inoculated onto Potato Dextrose Agar supplemented with 0.5 mg/mL cycloheximide using the pour plate method. Plates were incubated in an inverted position at 32 ± 2°C for 7 days. Distinct colonies were subcultured by streaking single colonies onto sterile plates. All plates were prepared in triplicate and incubated inverted at 32 ± 2°C for 7 days (Yew et al., 2014).

Molecular Characterization of the Selected Fungal Isolates

Extraction and Purification of DNA: All strains were cultured on Sabouraud Dextrose Agar (BIOTECH) and incubated at room temperature (30 ± 2°C) for 5 days. Genomic DNA was extracted and purified using the Zymo Research DNA MiniPrep™ kit (Category No. D6005, Irvine, California, USA) according to the manufacturer's instructions, as described by Iheukwumere *et al.* (2018).

Determination of DNA Quality: DNA quality was assessed using a NanoDrop

spectrophotometer. One microlitre (1 µL) of the extracted DNA was placed into the measurement chamber, and absorbance values at 260/280 nm were recorded through a connected computer interface, as described by Iheukwumere *et al.* (2018).

Amplification of DNA and Gel Electrophoresis of PCR Products: PCR amplification was performed using a Mastercycler Nexus Gradient thermal cycler (Eppendorf). The reaction mixture consisted of primers (20 µL), template DNA (20 µL), nuclease-free water (72 µL), and master mix (108 µL) containing Taq polymerase, dimethyl sulfoxide (DMSO), magnesium chloride ($MgCl_2$), and deoxynucleotide triphosphates (dNTPs). The mixture was homogenized using a vortex mixer. PCR cycling conditions included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 21 seconds, and a final extension at 72°C for 10 minutes. Amplified products were separated by electrophoresis on 1.0% agarose gel using a 1 kb DNA ladder as a molecular size marker. After staining with 3 µL nucleic acid stain (GR Green), gels were visualized using a gel documentation system, as described by Iheukwumere et al. (2018).

DNA Sequencing of rRNA Fragment: Amplified PCR products generated using universal primers were sequenced using an ABI DNA sequencer (Applied Biosystems Inc.) at the International Institute of Tropical Agriculture (IITA), Ibadan, following the method described by Iheukwumere et al. (2018).

Computational Analysis: Computational analysis was conducted using the modified method of Mohammed et al. (2011). Chromatograms generated from sequencing were cleaned to obtain high-quality nucleotide regions. Cleaned sequences were aligned using pairwise alignment tools. Consensus sequences generated from forward and reverse strands were subjected to Basic Local Alignment Search Tool (BLAST) analysis using the National Center for Biotechnology Information database. Isolates showing ≥95% sequence similarity were considered identified. Maximum scores, total scores, and accession numbers were recorded.

Susceptibility Patterns to Conventional Antifungal Agents

Preparation of 0.5 McFarland Standard: The 0.5 McFarland standard was prepared using 1.175% barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) and 1% sulfuric acid (H_2SO_4). Specifically, 1.175 g of $BaCl_2 \cdot 2H_2O$ was dissolved in 100 mL distilled water. Separately, 1 mL of H_2SO_4 was added to 99 mL distilled water to prepare 1% H_2SO_4 solution. Then, 0.5 mL of $BaCl_2 \cdot 2H_2O$ solution was added to 99.5 mL of 1% H_2SO_4, forming a turbid suspension due to $BaSO_4$ precipitation. This turbidity corresponds to the 0.5 McFarland standard and is equivalent to 1×10^8 CFU/mL (Balouiri *et al.,* 2016).

Preparation of Test Isolates: Dermatophyte isolates were subcultured on SDA plates to obtain pure cultures. Normal saline (0.85%) was prepared by dissolving 0.85 g NaCl in 100 mL distilled water and sterilizing at 121°C for 15 minutes. Fungal colonies were flooded with sterile normal saline and gently scraped using sterile forceps. The suspension was transferred into a conical flask and teased using a sterile needle. The mixture was filtered using Whatman No. 1 filter paper. The filtrate was diluted with sterile normal saline to match the turbidity of the 0.5 McFarland standard (1×10^8 CFU/mL) (Balouiri *et al.*, 2016).

Microdilution Method: A micro-well dilution technique was employed. Aliquots (100 µL) of each antifungal eluate were dispensed into micro-wells, followed by the addition of 100 µL of standardized fungal suspension. The contents were mixed thoroughly and incubated for 24 hours. After incubation, resazurin indicator was added, and wells were examined for growth or inhibition (EUCAST, 2003).

Determination of Minimum Inhibitory Concentration (MIC): The MIC was determined as the lowest concentration that inhibited visible fungal growth. Concentrations were recorded according to the method described by Balouiri *et al.* (2016).

Determination of Minimum Fungicidal Concentration (MFC): Wells showing no visible fungal growth were subcultured onto freshly prepared peptone water and incubated at 28°C for 48 hours. The lowest concentration showing no growth after subculture was recorded as the MFC (Balouiri *et al.,* 2016).

Results

The nature of the nucleic acid extracted from the isolates is presented in Table 1. The results showed that the absorbance

ratio at 260 nm and 280 nm ranged between 1.80 and 1.90, indicating high purity of the extracted nucleic acid and confirming that the genetic material was DNA. Molecular characterization based on polymerase chain reaction amplification and gene sequencing revealed the presence of *Trichophyton mentagrophytes* strain UKJ 594119 (TMU5), *Paraphyton cookei* strain IFM40904 (PCM4), *Trichophyton rubrum* strain CBS118892 (TR), and *Trichophyton tonsurans* strain DSM112177 (TTD1) (Table 2). The antifungal susceptibility pattern of the fungal isolates showed variations in their response to conventional antifungal agents. Ketoconazole exhibited the highest antifungal activity against isolate N, with a minimum inhibitory concentration (MIC) of 0.008, followed by isolate Q (0.016), while the lowest activity was observed against isolate M (0.500).

Ketoconazole showed no inhibitory effect on isolate P. Similarly, fluconazole demonstrated the highest antifungal activity against isolate N (0.004), followed by isolate Q (0.008), whereas the lowest activity was recorded against isolate M (0.500). Griseofulvin exhibited greater antifungal activity against isolate N (0.063) compared to isolate Q (0.125), while no inhibitory effect was observed against isolates M and P.

Cotrimazole also showed higher antifungal activity against isolate N (0.008) than isolate Q (0.016), with no observable effect on isolates M and P. Terbinafine demonstrated the highest antifungal activity against isolate N, followed by isolate Q, while the least activity was observed against isolate M. Tinidazole exhibited greater antifungal activity against isolate N (0.008) compared to isolate Q (0.016), whereas no inhibitory effect was observed against isolates M and P. Meanwhile, Sabouraud dextrose broth showed no antifungal activity against any of the fungal isolates (Table 3). The antifungal susceptibility pattern of the fungal isolates showed variations in their minimum fungicidal concentration (MFC) values. Ketoconazole exhibited similar antifungal activity against isolates N and Q, with an MFC value of 0.063. However, ketoconazole showed no fungicidal effect against isolate P.

Similarly, fluconazole demonstrated greater antifungal activity against isolate Q (0.031) than isolate N (0.063). Griseofulvin also exhibited higher antifungal activity against isolate Q (0.205) compared to isolate N (0.500).

Cotrimazole showed greater antifungal activity against isolate Q (0.063) than isolate N (0.125), while no fungicidal effect was observed against isolates M and P. Terbinafine demonstrated the highest antifungal activity against isolates N and Q compared to isolate M. Tinidazole exhibited greater antifungal activity against isolate N (0.031) than isolate Q (0.063), while no fungicidal effect was observed against isolates M and P (Table 4).

Table 1: Nature of nucleic acid extracted from the isolates

Isolate Code	Conc (mg/mL)	A_{280}	A_{260}	260/280
M	86.40	0.5294	0.9741	1.84
N	78.80	0.5160	0.9391	1.82
P	87.20	0.5271	0.9646	1.83
Q	72.30	0.4540	0.8399	1.85

Table 2: Molecular characteristics of the isolates

Code	Max Score	Total Score	Query Cover	E-Value	Percent Identity (%)	Accession Number	Description
M	1423	1423	100	0.0	100	MN064822.1	*Trichophyton mentagrophytes* strain UKJ 594119 (TMU5)
N	1339	1339	100	0.0	100	AF150738.1	*Paraphyton cookie* strain IFM40904 (PCM4)
P	1681	2340	100	0.0	100	JX431933.1	*Trichophyton rubrum* strain CBS118892 (TR)
Q	1552	1552	100	0.0	100	OM326334.1	*Trichophyton tonsurans* strain DSM112177 (TTD1)

Table 3: Minimum inhibitory concentration of conventional antifungal agents against the test isolates

Conventional Antifungal Agents	M	N	P	Q
Ketoconazoile	0.500	0.008	0.000	0.016
Fluconazole	0.500	0.004	0.000	0.008
Griseofulvin	0.000	0.063	0.000	0.125
Cotrimazole	0.000	0.008	0.000	0.016
Terbinafine	0.250	0.001	0.000	0.002
Tinidazole	0.000	0.008	0.000	0.016
SD Broth	0.000	0.000	0.000	0.000

SD= Sabouraud dextrose broth

M= *Trichophyton mentagrophytes* strain UKJ 594119 (TMU5) N= *Paraphytoncookie* strain IFM40904 (PCM4); p= *Trichophyton rubrum* strain CBS118892 (TR); Q= *Trichophyton tonsurans* strain DSM112177 (TTD1)

Table 4: Minimum fungicidal concentration of conventional antifungal agents against the test isolates

Conventional Antifungal Agents	M	N	P	Q
Ketoconazoile	0.000	0.063	0.000	0.063
Fluconazole	00.000	0.063	0.000	0.031
Griseofulvin	0.000	0.500	0.000	0.205
Cotrimazole	0.000	0.125	0.000	0.063
Terbinafine	0.500	0.016	0.000	0.016
Tinidazole	0.000	0.031	0.000	0.063

M= *Trichophyton mentagrophytes* strain UKJ 594119 (TMU5) N= *Paraphyton cookie* strain IFM40904 (PCI4); p= *Trichophyton rubrum* strain CBS118892 (TR); Q= *Trichophyton tonsurans* strain DSM112177 (TTD1)

Discussion

Fungal infections were previously neglected to some extent, as they were considered common conditions limited mainly to superficial tissues such as the skin, hair, and nails. However, in recent times, these infections have increased in severity, resulting in pronounced abnormalities and significant damage to affected tissues. The poor efficacy of some conventional antifungal agents (ketoconazole, fluconazole, griseofulvin, cotrimazole, terbinafine, and tinidazole) against the fungal isolates investigated in this study may be attributed to the presence of resistance mechanisms, as indicated by the minimum inhibitory concentration (MIC) values obtained. Similar MIC patterns have been reported by several researchers (Arshah *et al.,* 2015; Shalaby *et al.,* 2016; Baghi *et al.,* 2016; Putta *et al.,* 2016; Dabas *et al.,* 2017; Sabtharishi *et al.,* 2017; Konda *et al.,* 2017; Datt *et al.,* 2018; Prabha *et al.,* 2019; Basavarajappa *et al.,* 2022). However, the overall MIC obtained in this study was lower (0.5 µg/mL) than the MIC value (>1 µg/mL) reported by Basavarajappa *et al.* (2022) for fluconazole, indicating that fluconazole demonstrated greater potency against the fungal isolates evaluated in the present study. This variation may be attributed to differences in fungal strains used in the respective studies. Similar observations have been documented by several researchers (Arshah *et al.,* 2015; Shalaby *et al.,* 2016; Baghi *et al.,* 2016; Putta *et al.,* 2016; Dabas *et al.,* 2017; Sabtharishi *et al.,* 2017; Konda *et al.,* 2017; Datt *et al.,* 2018; Prabha *et al.,* 2019; Basavarajappa *et al.,* 2022). The poor fungicidal activity of the conventional antifungal agents investigated may also be attributed to antifungal resistance, as reflected in the minimum fungicidal concentration (MFC) values obtained. This finding is consistent with reports from several researchers (Arshah *et al.,* 2015; Shalaby *et al.,* 2016; Baghi *et al.,* 2016; Putta *et al.,* 2016; Dabas *et al.,* 2017; Sabtharishi *et al.,* 2017; Konda *et al.,* 2017; Datt *et al.,* 2018; Prabha *et al.,* 2019; Basavarajappa *et al.,* 2022), who documented substantial antifungal resistance among dermatophytes exposed to conventional antifungal agents.

Conclusion

The susceptibility pattern of the pathogenic fungal strains to conventional antifungal agents was generally low; however, terbinafine demonstrated the highest antifungal activity, as evidenced by its superior minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values.

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