IDENTIFICATION, PHYLOGENETIC ANALYSES AND ANTIFUNGAL RESISTANCE OF FILAMENTOUS FUNGI ASSOCIATED WITH CHRONIC WOUNDS

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ABSTRACT

Fungal infections can exacerbate chronic wounds, prolong their healing time and increase the risk of complications. This study aimed at identifying filamentous fungi and investigating the antifungal resistance associated with chronic wounds. Sixty-two (62) chronic wound swab samples collected from UNIOSUN Teaching Hospital, Osogbo, Osun State, Southwest, Nigeria, were cultured following standard protocol. Isolated filamentous fungi were identified using phenotypic and molecular approaches. The susceptibility of the isolates to four commercially available antifungal drugs and their multiple antifungal resistance phenotypes were investigated. A total of 17 moulds belonging to 4 genera, i.e. *Aspergillus*, *Cladosporium*, *Fusarium*, and *Sarocladium*, and 8 species were identified. *Aspergillus niger* was the most prevalent mould (7, 41.18%), while *Aspergillus clavatophorous*, *Cladosporium tenuissinum, Fusarium equiseti, Fusarium luffae* and *Sarocladium kiliense* were the least (1, 5.88%). The resistance of the isolates to the test antifungals ranged between 37.5% - 87.5% and was in this order: flucytosine and fluconazole (87.5%), ketoconazole (62.5%), and amphotericin B (37.5%). Ten (10) out of the 16 isolates were multidrug resistant, ranging from 3 to 4 drugs. The highest multiple antifungal resistance pattern (MARP, 4) obtained was KCA-AFY-FLU-AMB. The PCR product of the moulds was between 600 bp and 700 bp. The results from this study reveals high incidence of drug-resistant filamentous fungi in chronic wounds. Thus, there is an urgent need for alternative approaches and innovative treatments to achieve success in the treatment of fungal infections of chronic wounds.

1. INTRODUCTION

Chronic wounds (CWs) are among the clinical cases associated with frequent hospital visits, morbidity, and mortality (Sen, 2021). Majorly, CWs are associated with underlying medical conditions such as diabetes (diabetic ulcers), peripheral arterial disease, and venous insufficiency (arterial/venous ulcers). It also includes wounds arising from pressure ulcers, burns, and non-healing surgical wounds (Kalan et al., 2016). Chronic wounds fail to heal within the expected 4 - 6-week timeframe of normal tissue repair, increasing the risk of infection (Pongrácz et al., 2017). In developing countries, an estimated population of 1 - 4% experience chronic wounds in their lifetime (Rahim et al., 2017).

Diagnosis and the management of chronic wounds are challenging, as they are often polymicrobial, involving multiple species of bacteria and fungi found around the cellulitis of chronic non-healing

(Edmiston et al., 2017). In the hospital setting, bacteria are regarded as important in the clinical treatment and management of chronic wound infections, while less focus is on fungi (Ge & Wang, 2023). Although the signs and symptoms of fungal infections are non-specific and may overlap with those of bacterial infections (Goyal & Zimniak, 2018). A quick and appropriate medical intervention targeting polymicrobial infection can hasten wound healing, forestalling enlargement of the wound area or further aggravation such as systemic infection (Liu et al., 2022).

Fungal infections are becoming increasingly recognized as an important complication of chronic wounds, particularly in immunocompromised individuals and those with multiple morbidities (Short et al., 2023). Fungi are ubiquitous; thus, they colonize and infect the skin and soft tissues, leading to chronic inflammation, delayed wound healing, and systemic infections (Messina et al., 2019). In addition, fungi, through biofilm formation and modulation of host immune cells, assist commensal bacteria in becoming resistant to antibiotics and the host immune response (Ge & Wang, 2023). Thus, forcing wounds to become reservoirs for multi-resistant species, which are considered a potential key factor in microbial bioburden of wounds and ulcers (Ge and Wang, 2023).

Generally, *Candida* species are the widely reported fungal species in non-healing wounds (Bansal et al., 2008; Dowd et al., 2011; Musyoki et al., 2022). Though scarcely reported, filamentous fungi are also wounds where agro-invasion of their hyphae leads to tissue impairment and overwhelming complications such as mycotic aneurysm, nerve compression, mycotic carbuncle and brain ischemia (Kurokawa et al., 2022).

Treatment of fungal infections depends on the four classes of antifungal drugs, i.e. azoles, polyenes, the pyrimidine analogue 5-flucytosine and echinocandins (Robbins et al., 2017). However, many fungi, including the filamentous species, have developed the ability to resist the drugs, leading to treatment failure (Rhodes & Fisher, 2019). In Nigeria, there is a paucity of information on the antifungal resistance of filamentous fungi isolated from chronic wounds. As at the time of this investigation, only Excel et al. (2023) isolated and presumptively identified fungi from chronic wounds in Owerri metropolis, Nigeria. It is in this light that this study aimed at identifying filamentous fungi and determining the antifungal resistance pattern of moulds isolated from chronic wounds.

Materials and Methods

Sample Size and Collection of Samples

A total of 62 wound swabs were collected from patients with chronic wound infections visiting UNIOSUN Teaching Hospital (UTH), Osogbo, Osun State, Southwest Nigeria. The minimum sample size (N) required was estimated using the assumed population formula: $N = \frac{z^2pq}{d^2}$, where z = 1.96 (value of the reference of normal distribution), p = estimated prevalence of 1% filamentous fungi from chronic wounds, q = (1-p), and d = 0.05 (degree of freedom), with attrition value of 10% (Excel et al., 2023).

Wound swabs were collected using sterile Q-tips in a rotatory manner after initial cleaning of the wound edge with sterile saline water. The swab stick was cut into transport media and transported into the laboratory in a cold chain within 6 h of collection. The sample was incubated overnight at 28 °C before further analysis.

Isolation and Presumptive Identification of Filamentous Fungi

The samples were streaked on freshly prepared potato dextrose agar (PDA) and incubated at 28 °C for 5 days. Presumptive identification followed morphology characterization. Macroscopically features including surface colour, reverse colour, growth rate, and colony texture of each isolate were observed. A microscopic examination was performed on a small portion of lactophenol blue-stained mycelium on a clean slide, with a gently placed cover slip, under a light microscope (×40 magnification). The observed images were compared with the published fungal atlas (Barnett and Hunter, 1999).

Molecular Identification of the Isolates

Molecular identification was carried out on representative mould isolates presumptively identified. DNA extraction of a 5-day-old fungal culture was done using the ZR Fungal/Bacterial DNA kit™ (Zymo Research, USA) according to the product manual. Quantification and purity of DNA were done using NanoDrop™ One spectrophotometer (Thermo Scientific, UK). Polymerase chain reaction employed universal primers ITS4 (5′-TCC TCC GCT TAT TGA TATGC-3), ITS5 (5′-GGA AGT AAA AGT CGT AAC AAGG -3′) (White et al., 1990) to amplify the internal transcribed spacer region of the rDNA of each isolate. . This cycling condition is, as stated by Titilawo et al. (2023). PCR products were examined on 1.5% (w/v) agarose gel electrophoresis. The gel representation was observed under a Vilber Lourmat E-Box gel documentation imaging system to validate the size of the amplicons. The estimated PCR product size of the primers is between 600 and 700 bp. Amplicons were cleaned up using an enzymatic method (ExoSAP-IT™ PCR Product Cleanup Reagent (Thermofisher, UK)) according to the manufacturer's guide. Sanger sequencing was carried out using the Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, following the user's manual. Sequence similarities of the generated ITS region were calculated using the Basic Local Alignment Search Tool (BLASTn) for homology to identify the fungal isolates (Altshul et al., 1997). Sequences were deposited in the GenBank® for unique identifier assignment.

Phylogenetic Analysis

Phylogeny was deduced from 34 sequence data using the Neighbour-Joining method (Saitou and Nei, 1987). The genetic distances were generated using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Antifungal Sensitivity Testing

The disk diffusion technique was employed (CLSI, 2024). Each 3-to-5-day-old filamentous fungi was standardized to 0.5 McFarland (1.5 x 10^8 spores/mL) in sterile physiological saline, and 100 µL of the suspension was swabbed on the surface of the PDA plate. Commercially purchased antifungal discs: ketoconazole (15 µg), fluconazole (25 µg), flucytosine (10 µg), and amphotericin B (20 µg) were placed on the inoculated plate and incubated for 48 hours. Zones of inhibition around each antifungal disc were measured in millimetres (mm) and interpreted as susceptible (S), intermediate (I) and resistant (R) according to CLSI recommendations et al. (CLSI, 2009). The frequency of antifungal-resistant isolates was estimated using the formula: (E/F) × 100, where 'E' is the total number of isolates resistant to a drug and 'F' is the total number of isolates involved in the study.

Multiple Antifungal-Resistant Phenotypes and Indexing of the Isolates

Multiple antifungal-resistant phenotypes (MARP) were derived for the isolates exhibiting resistance to three or more antibiotics according to Wose et al. (2022). Multiple antifungal resistance indexes (MARI) for each isolate were also estimated by the expression:

MARI =m/n

Where 'm' represents the number of antifungal agents to which each isolate was resistant and 'n' is the number of antifungal agents tested against each isolate (Wose et al., 2022).

Results

Phenotypic Identification and Occurrence of the Fungal Isolates

Out of the 62 chronic wound samples collected, 17 moulds belonging to 4 genera (*Aspergillus*, *Cladosporium*, *Fusarium*, and *Sarocladium*) and 8 species namely: *Aspergillus aculeatus* (1, 5.88%), *Aspergillus clavatophorous* (1, 5.88%), *Aspergillus flavus* (4, 23.53%), *Aspergillus niger* (7, 41.18%), *Cladosporium tenuissinum* (1, 5.88%), *Fusarium equiseti* (1, 5.88%), *Fusarium luffae* (1, 5.88%), and *Sarocladium kiliense* (1, 5.88%) were obtained (Fig. 1).

Molecular Identification of the Moulds

The amplicon size of the isolates ranged between 600 bp and 700 bp (Fig. 2). DNA BLASTn analysis following sequencing showed that the isolates obtained in this study had 84.76% to 100% similarity with earlier GenBank® deposits (Table 1). The accession numbers assigned to the isolates are listed in Table 1.

The evolutionary history inferred using a neighbour-joining tree revealed that the 34 strains were mainly clustered into two distinct clades. *Candida tropicalis* MH748676 was used as the outgroup species.

Two isolates in this work, *Fusarium equiseti* OR3506030 and *F. luffae* OR350631, shared a common node, whereas *Sarocladium kiliense* OR350829 shared a common ancestor with PP590249, MK426286, and PQ114388. Surprisingly, *Cladosporium tenuissinum* OR350627 was closely related to *Aspergillus flavus* PP724390 and clustered with MN964070, MN964067, MK299130, JX852604 and OR350624 (also isolated from this work). *Aspergillus aculeatus* PP854644 and *Aspergillus clavatophorus* OR350624 shared a common node with MW254950 and OP479986, respectively. *Aspergillus niger* OR743920 clustered with OR195801 and OR195801 (Fig. 3).

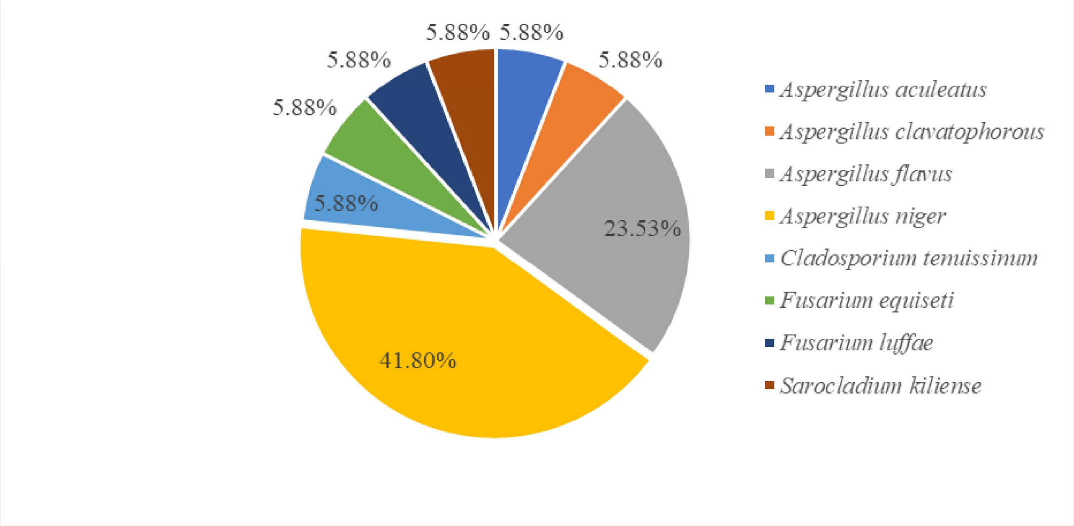

Fig. 1 Pie chart showing the frequency of fungal species identified

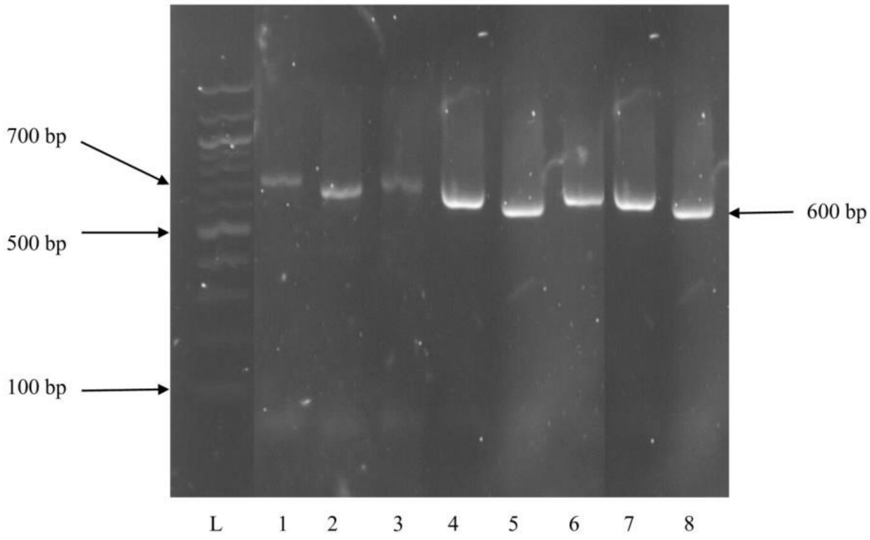

Fig. 2 Gel image of representative fungal isolates. L – DNA ladder; 1 – *A. niger*; 2- A. *flavus*; 3 - *A. clavatophorus*; 4 – *A. aculeatus*; 5 – *Cladosporium tenuissimum*; 6 – *Fusarium equiseti*; 7 – *Fusarium luffae*; 8 – *Sarocladium kiliense*

Table 1: Summary of BLASTn result

Presumptive identity	BLASTn identity of the sample	Percentage (%) identity	Accession number
Aspergillus aculeatus	MT007537	100.00	PP854544
Aspergillus flavus	MK091395	99.07	OR350624
Aspergillus clavatophorus	OP479986	84.76	OR350625
Aspergillus niger	MW255064	92.47	OR743920
Cladosporium tenuissimum	OW983641	99.81	OR350627
Fusarium equiseti	MN589627	99.42	OR350630
Fusarium luffae	OP642372	99.04	OR350631
Sarocladium kiliense	MK426286	99.82	OR350629

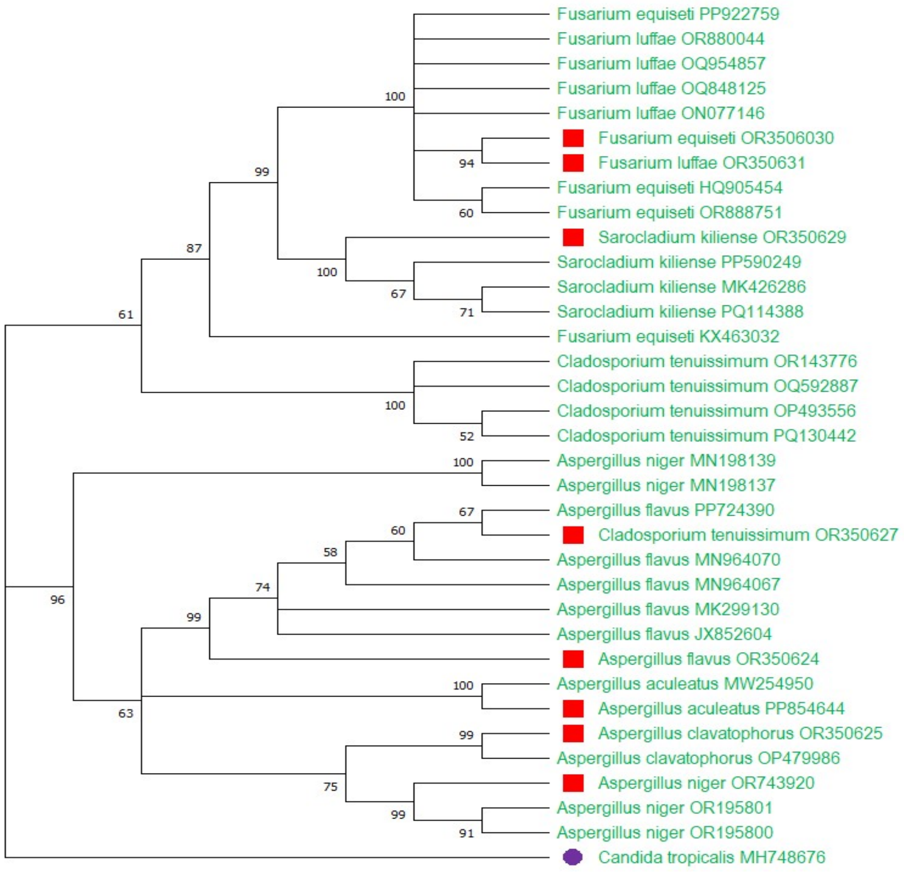

Fig. 3 Evolutionary trees showing the relationship between moulds from chronic wounds with selected sequences from GenBank®.

Antifungal Resistance Profile of the Moulds

The percentage resistance of the isolates to the test antifungals ranges from 37.5% - 87.5%. The chronic wound samples showed resistance to flucytosine (87.5%), fluconazole (87.5%), ketoconazole (62.5%), and amphotericin B (37.5%). The susceptibility of the isolates to the antifungal agents is in descending order: amphotericin B (62.5%), ketoconazole (25.0%), fluconazole (12.5%), and flucytosine (6.25%) (Fig. 4).

Specifically, 100% resistance was observed in *A. aculeatus* (n = 1), *F. equiseti* (n = 1) and *Fusarium luffae* (n = 1) to the four antifungals, and to 3 drugs, ketoconazole, fluconazole and flucytosine, in *A. clavatophorus* (n = 1) and *Sarocladium kiliense* (n = 1). The resistance observed in *A. flavus* (n = 4) *and A. niger* (n = 7) was as follows: ketoconazole (25%; 75%), fluconazole (100%; 86%), flucytosine (100%; 86%), and amphotericin B (25%; 29%), respectively. Whereas *Cladosporium tenuissinum* (n = 1) was 100% susceptible to all the drugs except amphotericin B. High susceptibility to amphotericin B was recorded for *A. clavatophorus* and *S. kiliense* (100%), *A. flavus* (75%) and *A. niger* (71%) (Table 2).

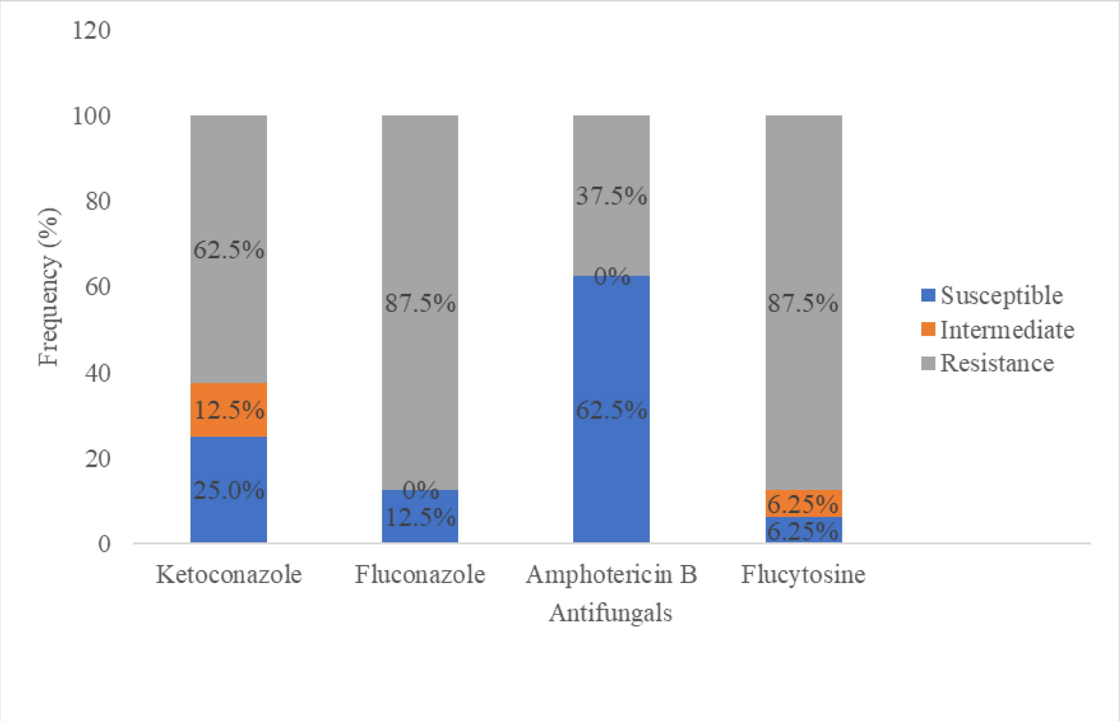

Fig. 4 Antifungal resistance pattern in moulds obtained from chronic wounds

Table 2 Resistance pattern of fungal species to the test antifungals

Fungal species	Percentage (%)											
	Ketoconazole			Fluconazole			Flucytosine			Amphotericin B		
	R	I	S	R	I	S	R	I	S	R	I	S
Aspergillus aculeatus	100	0	0	100	0	0	100	0	0	100	0	0
Aspergillus clavatophorus	100	0	0	100	0	0	100	0	0	0	0	100
Aspergillus flavus	25	50	25	100	0	0	100	0	0	25	0	75
Aspergillus niger	71	0	29	86	0	14	86	0	14	29	0	71
Cladosporium tenuissinum	0	0	100	0	0	100	0	0	100	100	0	0
Fusarium equiseti	100	0	0	100	0	0	100	0	0	100	0	0
Fusarium luffae	100	0	0	100	0	0	100	0	0	100	0	0
Sarocladium kiliense	100	0	0	100	0	0	100	0	0	0	0	100

R -Resistant; I – Intermediate; S - Susceptible

Antifungal Resistance Phenotypes of the Mould Isolates

Table 3 shows the antifungal-resistant phenotypes of the isolated moulds. Only 1 isolate each was resistant to 1 antifungal agent i.e. Flucytosine (AFY) and Fluconazole (FLU), respectively. A total of three isolates were resistant to two agents each (2 were resistant to AFY-FLU, and 1 to Ketoconazole (KCA)-FLU, respectively). The remaining 10 isolates exhibited resistance to ≥ 3 drugs, revealing three different phenotypes – AFY-FLU-Amphotericin B(AMB), KCA-AFY-FLU and KCA-AFY-FLU-AMB, being the highest MARP (4) obtained.

Table 3: Pattern of antifungal resistant phenotype of the chronic wounds

Number of moulds	No of antifungal drugs the isolates were resistant to	Resistance Profile	Frequency
(N = 16)	1	AFY	2
	1	FLU	1
	2	AFY-FLU	2
	2	KCA-FLU	1
	*3	AFY-FLU-AMB	1
	*3	KCA-AFY-FLU	4
	*4	KCA-AFY-FLU-AMB	5

*Multiple antifungal-resistant phenotypes

KET - Ketoconazole; FLU - Fluconazole; AFY – Flucytosine; AMB - Amphotericin B

Discussion

Fungi are an underestimated cause of human infections associated with about 1.5 million deaths and 1.7 billion superficial infections annually (Brown et al., 2013). Thus, accurate identification of fungi is crucial in preventing disease progression, reducing treatment complexities, and providing more successful therapeutic interventions (Fang et al., 2023). In this study, *Aspergillus clavatophorous*, *Aspergillus flavus, Aspergillus fumigatus*, *Aspergillus niger*, *Cladosporium tenuissinum, Fusarium equiseti,* and *Sarocladium kiliense* were identified. Previous study isolated *Aspergillus niger*, *Aspergillus terreus*, *Bipolaris sorokiniana*, *Cladosporium herbarum*, *Curvularia lunata*, *Engyodontium album*, *Exserohilum rostratum*, *Fusarium equiseti*, *Fusarium sporotrichioides*, *Fusarium moniliforme*, *Keissleriella cladophila*, *Trichophyton mentagrophytes*, *Penicillium janthinellum*, *Phoma betae*, *Phoma rabiei*, *Rhizopus oryzae*, and *Ulocladium botrytis* from wound samples (Dowd et al., 2011). The same node shared by *Fusarium equiseti* OR3506030 and *F. luffae* OR350631 in the phylogeny is probably because both are from the same source and have adapted to the environment, leading to convergent evolution (Xu et al., 2020). The different clusters noted suggest unique traits or characteristics such as metabolic pathways, virulence factors or antibiotic resistance genes shared by the organisms

The predominance of the genus *Aspergillus* and the species *Aspergillus niger* in this work is in line with previous studies (Kalan et al., 2016; Compton, 2013; Kalan & Grice, 2018), but disagrees with with Ge & Wang (2023), who reported in their study that *Trichophyton rubrum* was the main filamentous fungi involved in chronic wound infections. The occurrence of fungi in chronic wounds suggests their contribution to the bioburden of wounds (Fang et al., 2023). Specifically, wounds get infected by host cutaneous, commensal or environmental fungi and evolve into local infections, resulting in fungemia as well as invasive fungal disease.

Fungal infections are a serious public health concern. They are important in the morbidity and mortality of immunodeficient individuals and are mostly resistant to conventional treatments (Choi et al., 2021). The current work reveals 37.5% to 87.5% resistance among the isolates investigated. Although there is paucity of investigation on drug resistance of filamentous fungi of chronic wound origin, the high resistance to fluconazole, flucytosine (87.5%) and ketoconazole (62.5%) observed in this work portends serious treatment challenges, increased risk of complications, morbidity, and healthcare cost, and potential spread of resistant moulds to other patients, health workers, or environment, posing infection control challenges (Sydnor & Perl, 2011). Generally, azole resistance occurs through mutations in the drug target (ERG11) or target overexpression (Lee et al., 2023), while a reduction in flucytosine uptake by fungal cells (altered permease) and decreased synthesis of the active nucleotide metabolites (decreased deaminase and other enzyme activities) result in flucytosine resistance (Chandra & Ghannoum, 2017). The low resistance recorded for amphotericin B suggests its use as the drug of choice in the treatment of fungal infection of chronic wounds, particularly *A. clavatophorus*, *A. flavus*, *A. niger* and *S. kiliense*. This corroborates the findings of Sanchez et al. (2014).

Drug-resistant fungi, especially the multidrug-resistant species, are an emerging challenge threatening the limited antifungal armamentarium (Arastehfar et al., 2020). Majorly, multidrug resistance in fungi is caused by overuse and misuse of drugs, genetic mutations, and environmental factors (Cui et al., 2022). This study reveals 10 of 16 isolates resistant to 3 or more antifungals. This implies reduced effectiveness of available drugs, increased mortality, particularly in immunocompromised individuals, delayed diagnosis, increased risk of invasive fungal infections, transmission of resistant strains and economic burdens (Vitiello et al., 2023).

Conclusion and Recommendation

The study revealed that filamentous fungi are implicated in chronic wound infections; thus, disregarding their involvement in clinical management may result in suboptimal therapy. High prevalence of drug resistance towards standard antifungals and the multiple antifungal-resistant phenotypes was observed. This suggests poor treatment outcomes with associated economic burdens from fungal infection of chronic wounds. Amphotericin B may serve as the choice drug for treatment of chronic wound infection. Thus, it is recommended that fungal identification, antifungal susceptibility testing, and antifungal stewardship programmes and surveillance practices in the healthcare sector should be encouraged to curtail the emergence of drug-resistant fungi. Further studies focusing on the development of novel antifungal drugs, preferably from natural sources with no or reduced side effects, should be developed for effective infection management.

Data availability

All data generated or analysed during this study are included in this published article.

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Author's contribution statement

Mobolaji A. Titilawo: Investigation, data analysis and writing of the manuscript; Folasade M. Adeyemi: Conceptualization and reviewing of manuscript; Abideen A. Wahab: Conceptualization; Olaoniye H. Ajigbewu: Conceptualization, Investigation and reviewing of manuscript; Fiyinfolu A. Ajigbewu: Investigation; Deborah I. Oyetunbi: Investigation and writing of the manuscript; Chisom D. Prosper: Investigation.

Competing interest

The authors declare that there are no conflicts of interest.

Ethics approval

Approval for this study was granted by the Ethics Review Committee of UNIOSUN Teaching Hospital, Osogbo, Southwest Nigeria (UTH/REC/2023/01/30/739).

Consent to participate

Informed consent (oral and written) was obtained from all individual participants, or the parent in the case of children under 18 years.

Consent to publish

The authors confirm that human research participants provided informed consent for publication of the obtained data.

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