Onychomycosis in Diabetes Mellitus Patients: In Vitro Susceptibility Testing of Four Antifungal Drugs Against Fungal Isolates

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ABSTRACT

Introduction: Despite recent advances in the development of antifungal drugs, onychomycosis is still difficult to treat. It negatively affects the quality of life of affected patients. Antifungal susceptibility testing may provide insight into the use of appropriate antifungal medications, thus improving patients' care.

Objectives: The study aimed to compare the in-vitro susceptibility of fungal isolates' sensitivity patterns against four antifungal drugs among diabetic and non-diabetic patients with onychomycosis.

Methodology: This cross-sectional study was conducted at the Sub-Department of Dermatology, University of Nigeria Teaching Hospital, (UNTH) Ituku-Ozalla, Enugu, Nigeria. Single colony isolates from clinically suspected cases of onychomycosis among diabetics and non-diabetics were used for the antifungal susceptibility. The inoculum was streaked on Muller Hinton 2% glucose/ methylene blue agar and four commercially prepared available drugs (HIMEDIA, India) namely Fluconazole (25 µg), Itraconazole (30 µg), Terbinafine (e-test 0.002-32 µg) and Voriconazole (1 µg) were applied. The data was analyzed using the IBM Statistical Package for Social Science (version 25).

Results: A total of 276 isolates but single colonies were 217: non-dermatophyte moulds 94, dermatophytes 84, and candida species 39, and were tested against the four antifungal discs. Voriconazole had the highest diameter of inhibition against the three classes of pathogenic fungi among the DM participants (73.9%-100%) and was found to be highly statistically significant (P-value < 0.002).

CONCLUSION: Because of this, voriconazole may be recommended for diabetics with recalcitrant cases, while itraconazole, terbinafine, and fluconazole may be used as empirical treatment of dermatophytes, non-dermatophyte moulds, and yeasts.

KEYWORDS: Onychomycosis, Diabetes Mellitus, Antifungal Susceptibility Testing.

Onychomycose chez les patients diabétiques : Etude de la sensibilité in-vitro de quatre médicaments antifongiques contre les isolats fongiques.

Contexte : Malgré les progrès récents dans le développement de médicaments antifongiques, l'onychomycose reste encore difficile à traiter. Cela affecte négativement la qualité de vie des patients concernés. Les tests de sensibilité aux antifongiques peuvent donner un aperçu de l'utilisation de médicaments antifongiques appropriés, améliorant ainsi les soins aux patients.

Objectifs : L'étude visait à comparer la sensibilité in vitro des profils de sensibilité des isolats fongiques à quatre médicaments antifongiques chez des patients diabétiques et non diabétiques atteints d'onychomycose.

Méthodologie : Cette étude transversale a été menée au sous-département de dermatologie de l'hôpital universitaire de l'Université du Nigéria (UNTH) Ituku-Ozalla, Enugu, Nigéria. Des isolats de colonies uniques provenant de cas cliniquement suspectés d'onychomycose chez des diabétiques et des non-diabétiques ont été utilisés pour évaluer la sensibilité aux antifongiques. L'inoculum a été étalé sur une gélose Muller Hinton à 2 % de glucose/bleu de méthylène et quatre médicaments disponibles dans le commerce (HIMEDIA, Inde), à savoir le fluconazole (25 µg), l'itraconazole (30 µg), la terbinafine (e-test 0,002-32µg) et le voriconazole (1 µg) ont été appliqués. Les données ont été analysées à l'aide d'IBM Statistical Package for Social Science (version 25).

Résultats : Un total de 276 isolats mais 217 colonies uniques : 94 moisissures non-dermatophytes, 84 dermatophytes et 39 espèces de candida, ont été testés sur les quatre disques antifongiques. Le voriconazole présentait le diamètre d'inhibition le plus élevé contre les trois classes de champignons pathogènes parmi les participants atteints de diabète (73,9 % à 100 %) et s'est avéré hautement

statistiquement significatif (valeur P < 0,002).

Conclusion : Pour cette raison, le voriconazole peut être recommandé aux diabétiques présentant des cas récalcitrants, tandis que l'itraconazole, la terbinafine et le fluconazole peuvent être utilisés comme traitement empirique des dermatophytes, des moisissures non-dermatophytes et des levures.

Mots-clés : Onychomycose, diabète, tests de sensibilité aux antifongiques.

Introduction

Onychomycosis is a term that describes a fungal infection of the nail unit in which dermatophytes, yeasts, and non-dermatophyte moulds have been implicated as the aetiological agents.[1] More than 70% of culture-positive onychomycosis are caused by anthropophilic dermatophytes predominantly *Trichophyton rubrum*, while non-dermatophytes are responsible for approximately 20% of fungal infections and yeasts account for 10%-20% of cases.[2,3]

Resistance to antifungal drugs is an increasing health challenge, which may be responsible for treatment failure, fueled by injudicious use of various antifungal drugs, phenotypic and genetic alterations, and the presence of immunosuppressive states like diabetes mellitus (DM).[4,5] The need for targeted antifungal therapy for fungal organisms causing onychomycosis is of utmost importance,[5] especially in persons living with DM who are at risk of DM foot syndrome.[6] Therefore, it is vital to evaluate the antifungal susceptibility pattern of fungal organisms causing onychomycosis to available antifungal medications using a simple, standard, reproducible in-vitro assay. This will help select effective antifungal agents for the treatment of onychomycosis. Data regarding the sensitivity pattern of various antifungals especially among DM patients is scarce, especially in Nigeria. Hence, this study was to determine the in-vitro susceptibility of fungi causing onychomycosis in DM participants to terbinafine, fluconazole, itraconazole, and voriconazole. This susceptibility pattern was compared with that in non-diabetics.

Materials and Methods

This was a cross-sectional and descriptive study conducted at the Sub-Department of Dermatology, the University of Nigeria Teaching Hospital, (UNTH) Ituku-Ozalla, Enugu, Nigeria from October 2020 to March 2021. The sample population was diabetes mellitus patients and non-diabetics (controls) suspected to have onychomycosis. The inclusion criteria were known diabetes mellitus patients, normal fasting blood glucose or random blood glucose (for controls), clinically suspected onychomycosis, and informed consent. Exclusion criteria include patients with other immuno-suppressive states, pregnancy, and those who had taken antifungal medications within the prior month.

One hundred and fifty-one (151) nail clippings from 76 known diabetes mellitus patients and 75 non-diabetic patients (controls) were sampled. The study participants were recruited from the diabetic and dermatology (controls) clinics. Further processing and antifungal sensitivity study was done in the Sub-Department of Dermatology research laboratory. Nail clippings from each participant were cultured on both the selective media such as Sabouraud Dextrose Agar (SDA) and Dermatophytes Test Media with supplements (HIMEDIA, India) for the identification of the species.

Isolation and identification of the fungal organisms were based on the macroscopic observation of fungal colonies and lactophenol cotton blue mount microscopic examination. All yeast species on SDA were subcultured onto HiChrome agar Candida (HIMEDIA, India) for candida species identification. Only single colony isolates and four commercially prepared available drugs (HIMEDIA, India) namely Fluconazole (25 µg), Itraconazole (30 µg), Terbinafine (e-test 0.002-32 µg), and Voriconazole (1 µg) were used for antifungal susceptibility testing.

Disk Diffusion Assay: From identified fungal cultures, each inoculum from distinct colonies were harvested and suspended in 5ml of sterile 0.9% saline and the resulting suspensions were spun. Dense inoculum suspensions of conidia and hyphae elements were transferred to sterile test tubes and allowed to sediment for 30 minutes. After the settlement of heavy particles, each upper homogeneous suspension was transferred to another sterile tube and adjusted to 0.5 McFarland turbidity.[4]

Then a sterile non-toxic cotton swab was dipped into the standardized inoculum and used to streak the entire surface of the sterile Muller Hinton 2% glucose/ methylene blue agar on the petri dish, turning the dish at 60° angle between each streaking.

The inoculum was allowed to dry for 5-15 minutes before the commercially prepared antifungal disks and e-strips were applied. The plates were inverted and incubated, then examined after 2-5 days for zones of inhibition. After the colonies grew, the inhibition zones around the disks were measured in millimeters (mm) and recorded. Criteria for classification of susceptibility as sensitive, intermediate, and resistant were reported according to Pakshir *et al*.[7] The data were analyzed using the IBM Statistical Package for Social Sciences (SPSS version 25).

Results

The study was conducted on 151 nail clippings: 143 toenails and 8 fingernails and a total of 276 fungi isolates were isolated. Figures 1, 2, and 3 showed the spectrum of dermatophytes, non-dermatophyte moulds (NDM), and yeast species isolated from positive cultures and by lactophenol cotton blue mount microscopy. Aspergillus species [*A. niger* and *A. fumigatus*] (44.9%), *Trichophyton soudanense* (which is classified as part of T. *rubrum* of African origin complex found mainly in Sub-Saharan Africa)[8] (30%), and *Candida albicans* (4.7%) were the predominant NDM, dermatophyte, and yeast respectively.

Single colonies were 217: non-dermatophyte moulds 94, dermatophytes 84, and candida species 39. The frequencies of single colonies isolated in both the DM participants and non-DM group are shown in Table 1.

The antifungal susceptibility testing was conducted only on the single colonies isolated. The susceptibility patterns of antifungal drugs against different classes of onychomycosis-causing fungi in both the DM and non-DM groups are shown in Tables 2 and 3, respectively.

Voriconazole had the highest diameter of inhibition against the three classes of pathogenic fungi among the DM participants (73.9%-100%). The dermatophytes and candida species were more resistant to terbinafine (69.6% and 82.3% respectively). All the candida species isolated were susceptible to fluconazole and voriconazole. The sensitivity of voriconazole to the three classes of pathogenic fungal organisms isolated namely dermatophytes, non-dermatophytes, and candida species in the diabetic group was highly statistically significant (P value < 0.002) as shown in Table 2.

Table 3 revealed the antifungal activity against the dermatophytes, non-dermatophytes, and candida species among the comparative group. Itraconazole (86.5%) was the most sensitive antifungal against dermatophytes, followed by voriconazole (83.8%), while voriconazole had the highest inhibition zone diameter (IZD) against non-dermatophytes (91.4%). For the Candida species, itraconazole and voriconazole were the most sensitive (81.8%). The sensitivity of itraconazole and voriconazole to dermatophytes and candida species was highly statistically significant (P value < 0.00001). Similarly, the sensitivity of voriconazole to non-dermatophytes was found to be statistically significant, (χ^2=8.0769, P value = 0.04448).

The highest resistance of the dermatophytes was to terbinafine in both the DM participants (69.6%) and the comparative group (64.9%). Similarly, candida species had the most resistance to terbinafine in both the DM participants (82.3%) and controls (81.8%) as shown in Figure 4. Intermediate activity/response was observed only in the DM group.

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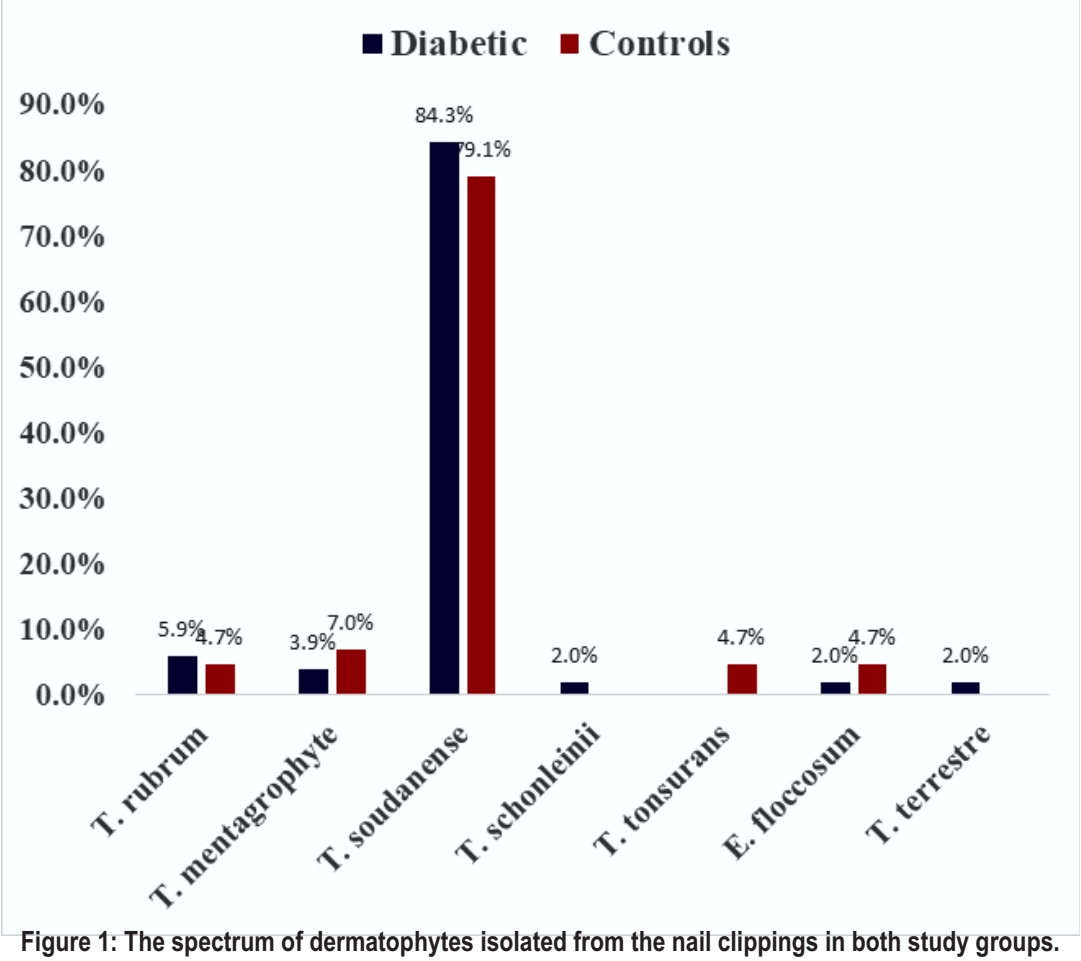

Figure 1: The spectrum of dermatophytes isolated from the nail clippings in both study groups.

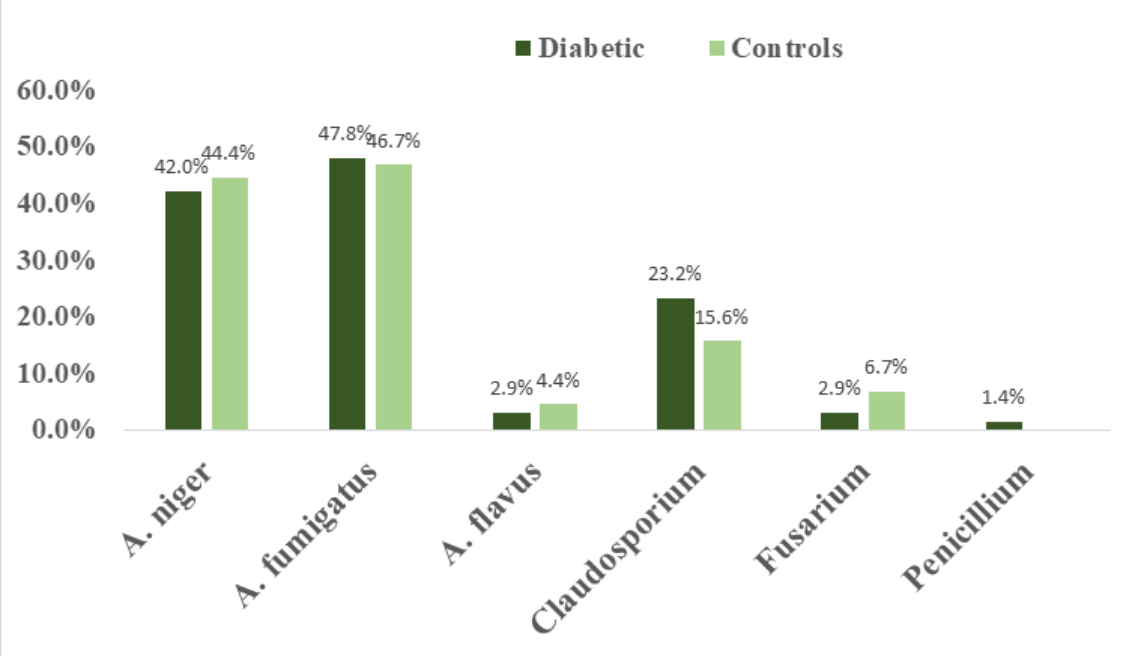

Figure 2: The spectrum of non-dermatophytes isolated from the nail clippings in both study groups.

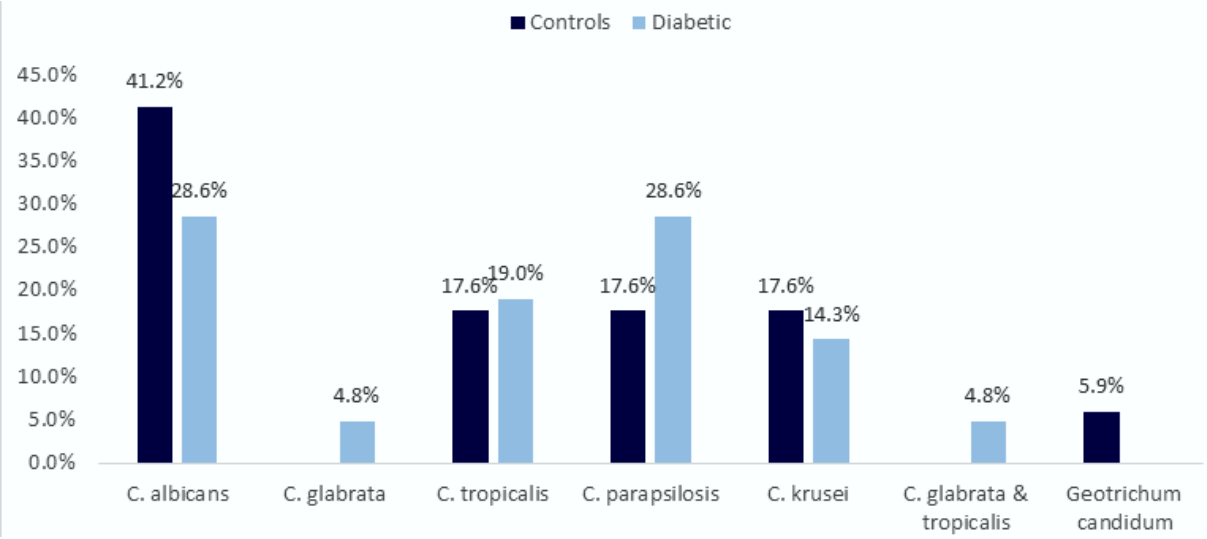

Figure 3: The spectrum of candida species isolated in both study groups.

Table 1. The spectrum of fungi used for antifungal susceptibility testing in both DM and non-DM isolates.

Single colonies (217)	DM N= 123 n (%)	Control N=94 n (%)
Dermatophytes (84)	47(38.2)	37 (39.4)
Non-Dermatophytes (94)	59 (48)	35 (37.2)
Candida species (39)	17 (13.8)	22 (23.4)

Table 2. Susceptibility patterns of common pathogenic fungi isolated from DM participants to antifungals.

		Antifungal activity observed in diabetic participants				
		Resistant	Sensitive	Intermediate	χ^2	P value
Pathogenic Fungi	**Antifungal**	n (%)	n (%)	n (%)		
Dermatophytes (47)	Terbinafine	33 (69.6)	14 (30.4)	0 (0.0)	28.2523	<0.0001
	Fluconazole	10 (21.3)	31 (65.9)	6 (12.8)		
	Itraconazole	16(34.8)	27 (56.5)	4 (8.7)		
	Voriconazole	10 (21.7)	35 (73.9)	2 (4.4)		
Non-Dermatophytes (59)	Terbinafine	31 (52.5)	28 (47.5)	0 (0.0)	19.7462	0.00192
	Fluconazole	35 (59.3)	21 (35.6)	3 (5.1)		
	Itraconazole	24 (41.2)	24 (41.2)	11 (18.6)		
	Voriconazole	10 (16.9)	49 (83.1)	0 (0.0)		
Yeasts (17)	Terbinafine	14 (83.3)	3 (16.7)	0 (0.0)	35.2154	0.0001
	Fluconazole	0 (0.0)	17 (100.0)	0 (0.0)		
	Itraconazole	11 (64.7)	9 (35.3)	0 (0.0)		
	Voriconazole	0 (0.0)	17 (100.0)	0 (0.0)		

Table 3: Susceptibility of common pathogenic fungi in the non-diabetic (control) group to antifungals.

		Antifungal activity observed in non-diabetic participants.				
		Resistant	Sensitive	Intermediate	χ^2	P value
Pathogenic Fungi	**Antifungal**	n (%)	n (%)	n (%)		
Dermatophytes (37)	Terbinafine	24 (64.9)	14 (35.1)	0 (0.0)	29.4723	<0.00001
	Fluconazole	10 (27.2)	27 (73.0)	0(0.0)		
	Itraconazole	5 (13.5)	32 (86.5)	0 (0.0)		
	Voriconazole	6 (16.2)	31 (83.8)	0 (0.0)		
Non-Dermatophytes (35)	Terbinafine	10 (28.6)	25 (71.4)	0 (0.0)	8.0769	0.04448
	Fluconazole	10 (28.6)	25 (71.4)	0 (0.0)		
	Itraconazole	13 (37.1)	22 (62.9)	0 (0.0)		
	Voriconazole	3 (8.6)	32 (91.4)	0 (0.0)		
Yeasts (22)	Terbinafine	18 (81.8)	4 (18.2)	0 (0.0)	35.2154	0.0001
	Fluconazole	7 (31.2)	15(68.2)	0 (0.0)		
	Itraconazole	4 (18.2)	18 (81.8)	0 (0.0)		
	Voriconazole	4 (18.2)	18 (81.8)	0 (0.0)		

Figure 4: Antifungal susceptibility testing using antifungal discs (Fluconazole[FLC], Itraconazole [IT], Voriconazole [VRC]) and terbinafine e-strip [Ezy TRB] on Muller-Hinton agar with 2% glucose and methylene blue.

Discussion

A standardized disk diffusion-based assay for determining the antifungal susceptibility testing of pathogenic fungi causing onychomycosis are desirable and have advantages. In developing countries especially, disk diffusion assays will aid antifungal susceptibility testing as several studies show that this assay is not only reproducible and accurate but also economical and very easy to perform.[9] There is a need for accurate, reproducible, and predictive susceptibility testing of fungal isolates to help inform clinical choice. The standard disk diffusion assay can be adapted for the assessment of dermatophytes, non-dermatophytes, and yeasts resistance against antifungal drugs.

In this study, the main specific fungal species identified were *Trichophyton soudanense:* a dermatophyte, followed by the *Aspergillus spp*. (*A.*

fumigatus, A. flavus and *A. niger)* and *Candida albicans*. The least common isolated fungal species (<2%) were *T. rubrum, T. mentagrophyte, Fusarium spp., Candida tropicalis, Candida krusei, Candida parapsilosis, Epidermophyton floccosum and Penicillium.* This finding of *T. soudanense* as the main onychomycosis-causing dermatophyte has been reported mainly in Africa, especially the West African Sub-region,[8] and is similar and comparable to the findings of studies by Sylla *et al* conducted in Senegal, West Africa, and Afene *et al* conducted Gabon, which are all in the Sub-Saharan African region.[10,11] However, *T. rubrum* was the major fungi isolated in several studies in North America, Europe, and many other parts of the world, including Africa.[12-17] The geographical distribution of aetiologic fungi explains the different profiles of species based on the study area.[18-20]

Antifungal susceptibility testing results in our study revealed that voriconazole had the highest activity (highest inhibition zone diameter) against the three pathogenic groups: non-dermatophytes, dermatophytes, and yeasts in both the diabetic subjects (82.4%, 73.9%, and 100%) and controls (90.9%, 81.8% and 80%) using the Agar Based Disk Diffusion (ABDD) method. This may be because voriconazole is a third-generation azole antifungal that has a fluoropyrimidine ring instead of a triazole ring seen in the second-generation azole family (fluconazole and itraconazole).[21] This structural difference makes the compound 10-30 folds more potent against non-dermatophytes and candida organisms.[22] It is also not commonly prescribed and available in our study setting and hence the fungal organisms are not likely to have developed resistance against voriconazole.

Terbinafine, followed by itraconazole, had the poorest antifungal activity against dermatophytes and candida in our study. This high resistance is comparable to the findings of Prabhat *et al*[23] and that of a multicentre study conducted by Yamada *et al*, where isolates tested had more resistance to terbinafine.[24] The study also determined the mechanism of resistance and discovered that all the resistant isolates had single-point mutations on the squalene epoxidase gene alleles.[25] Surprisingly, terbinafine had more activity against non-dermatophytes in the control group when compared to the diabetic participants in this study. On the contrary, several studies conducted in Iran, India, and Nepal reported terbinafine (though voriconazole was not among the antifungals tested) to be the most sensitive antifungal against dermatophytes and non-dermatophytes by employing ABDD and/or dilution methods.[7,26,27]

Itraconazole and fluconazole had some activity against dermatophytes and non-dermatophytes in our study. Notably, Itraconazole was recorded to have the highest activity against dermatophytes in some studies.[28] In contrast, Eba *et al* in Cameroun found all dermatophytes tested to be resistant to Itraconazole.[17] Candida species were most susceptible to fluconazole and voriconazole in the diabetic group and itraconazole and voriconazole in the control group in this study, following other studies in India and Spain.[29,30] However, fluconazole was noted in an isolated uncontrolled study among diabetics in Iran to have the least inhibition zone diameter against candida spp.[31] The sensitivity of voriconazole to the different classes of pathogenic fungi in both study groups was highly statistically significant.

The pattern of resistance seen among the tested antifungals in this study may be due to easy availability, and frequent empirical or indiscriminate use of antifungals (both topical and systemic) in the treatment of fungal infections. Moreover, these infections may occur more among diabetics because of their immunocompromised state and thus they become more at risk of developing resistance. Resistance to voriconazole was the least, probably because of the non-availability of this drug in the Southeastern region of Nigeria.

Some differences in the antifungal susceptibility patterns were observed between the DM group and controls (non-diabetics) in our study; resistance of non-dermatophytes to the four antifungals was higher in the diabetics than in the controls. The observed differences may be due to changes in virulence, the presence of immunosuppressed states like DM, abuse of antifungals as an over-the-counter medication in the study setting, and species-specific susceptibility against antifungal drugs.[32]

Conclusion

Overall, the maximum sensitivity of the isolated fungal organisms was Voriconazole> Itraconazole > Fluconazole > Terbinafine, and resistance was observed more in the diabetics, especially to terbinafine. Treatment may be based on antifungal sensitivity testing in refractory cases among people living with Diabetes Mellitus.

The disc diffusion method is a simple and valuable method for the in-vitro evaluation of antifungal susceptibility of fungi causing onychomycosis and this could play a key role in decision-making for the choice of antifungal medications.

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