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In Vitro Antifungal Susceptibility Profiles of Dermatophytes Isolates from Tinea Capitis in Northwest, Nigeria

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ABSTRACT

Background: The fungal infection of tinea capitis is a common mycosis that affects the scalp superficially, especially in children. Oral treatment of this infection remains the preferred treatment process in clinical dermatology. Many antifungals available for dermatophyte treatment lead to treatment failure. Determination of antifungal susceptibility of dermatophytes *in-vitro* has been reported to be important to curb dermatophyte infections using effective antifungal drugs. The aim of this study was to investigate and determine *in vitro* minimum inhibitory concentration (MIC) of amphotericin B, ketoconazole, griseofulvin, terbinafine, and fluconazole against dermatophyte clinical isolates using agar dilution method.

Materials and Methods: In this study, *in vitro* susceptibilities of 32 dermatophyte clinical isolates collected from primary school pupils in Sokoto metropolis were investigated to five antifungals (fluconazole, terbinafine, ketoconzole, amphotericin B, and griseofulvin) using the CLSI agar dilution method.

Findings: The results obtained revealed that griseofulvin and terbinafine were the most potent antifungal agents among those tested.

Conclusion: Agar dilution method could be an alternative method for MIC-determination of antifungal drugs against dermatophyte species, since it is cost effective and affordable with consistent results, especially in developing countries.

Keywords: Dermatophytes, Tinea capitis, Antifungal susceptibility testing, Nigeria

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Introduction

Tinea capitis (scalp dermatophytosis) is an inflammatory dermatophytosis of the scalp, which is a chronic and highly contagious infection distributed worldwide. This disease is the most common superficial mycosis that infects children between the ages of 4 to 14 years and regarded as a major public health concern [1].

Treatment of such an infection mostly requires oral administration of antifungal drugs to penetrate the hair shaft [2]. Most treatments of superficial dermatophytosis are solely based on the use of topical and systemic antifungal agents. Recently, safe, affordable, and highly effective antifungal agents have been introduced into clinical practice [1]. Antifungal drugs used in the treatment of superficial mycosis have advantages and disadvantages in the field of epidemiology with increased drug resistance and recalcitrance without a similar study in this domain. With the introduction of griseofulvin in 1958, anthropophilic agents of tinea capitis, including *Trichophyton* spp. and *Microsporum* spp., were almost eradicated in most parts of the world [3]. Currently, tinea capitis is prevalent mainly in African countries (Nigeria and Ethiopia) and western China and in general in geographical regions where lifestyles are mostly associated with malnutrition and poverty. This infection has also been reported occasionally in Nigeria [4, 5]. A prevalence rate of 75.7%, resistance of 21.2–100 %, and sensitivity of 78.8-100% have been reported in north central Nigeria for *T. rubrum* as the most prevalent dermatophyte [6], while *T. verrucosum* has been shown to be the most prevalent isolate in Ethiopia [7].

Due to the increasing incidence of resistance, in vitro antifungal susceptibility tests are frequently used to determine the drug of choice among the available antifungal agents [8]. Despite good in vitro activity of TRB (terbinafine) against several anthropophilic and zoophilic species, several recent surveys have reported the growing incidence of TRB resistance among dermatophytes. It seems that the global spread of terbinafine-resistant *Trichophyton* strains with point mutations in the squalene epoxidase (SQLE) gene is a great concern. Resistant isolates have also been reported recently in Asia and Europe. Therefore, the following reference has missed its credibility because of fully resistant isolates reported to terbinafine [9]. In vitro activity of TRB has been reported against some dermatophyte species such as *T. rubrum, T. mentagrophytes/interdigitale, T. schoenleini,* and *Epidermophyton floccosum* [10].

In order to determine the ability of a given antifungal agent to eliminate dermatophytes causing tinea infection and to help manage patients, determination of *in vitro* antifungal susceptibility of dermatophytes would be helpful in understanding an unsuccessful or successful treatment [11]. However, not all species have the same susceptibility pattern, and it may be necessary to perform *in vitro* susceptibility testing to select and monitor antifungal therapy [12].

Some methods used for antifungal sensitivity testing include: National Committee for Clinical Laboratory Standards NCCLS – the new name of Clinical Laboratory Standards Institute (CLSI) broth based methodology (M 27-A), CLSI methodology for moulds, E-test agar based testing methods, flow cytometry, and the use of viability dyes. These methods are time consuming and labour intensive; hence, a more economical method such as agar dilution have been described [13].

Objectives: The present study focused on *in vitro* agar dilution method to determine minimum inhibitory concentration (MIC) of amphotericin B, ketoconazole, griseofulvin, terbinafine, and fluconazole against dermatophyte clinical isolates.

Material and Methods

Fungal strains: In this study, *T. rubrum*

ATCC 28188 was included for *in-vitro* susceptibility testing based on the guidelines of CLSI, which state ideal reference strains for quality control of all antifungal drugs. A total of 32 clinical *dermatophyte* strains (Accession Number MT 893932 – MT 893963) were isolated from primary school pupils suspected with tinea capitis infection in Sokoto State metropolis, Nigeria from May to December 2020. An informed consent form was signed and approved by each of the participants. All isolates were cultured on Sabouraud dextrose agar (Oxoid, Basingstoke, U.K.) at 25 °C for 5 to 21 days and identified using sequence – based analysis of *28S rRNA* sequencing. The inocula (*T. eriotrephon*=10, *T. bullosum*=2, *T. simii*=12, *T. benhamiae*=1, *T. rubrum*=1, *T. tonsurans*=3, *Microsporum audouinii*=2, *Ctenomyces serratus*=1, and a reference strain of *T. rubrum* 28188) were then prepared spectrophotometrically using a spectrophotometer (Milton Roy company, Madrid Spain) and further diluted with normal saline in order to obtain a final inoculum concentration of 1×10^5 CFU/mL.

Preparation of stock solutions of standard antifungal agents*:* The *in vitro* susceptibilities of 32 dermatophyte clinical isolates to five antifungals were tested using CLSI agar dilution method guidelines according to Dambuza et al. (2017) [14]. Stock solutions of reference antifungal agents, including fluconazole, terbinafine, ketoconazole, amphotericin B, and griseofulvin (Sigma Aldrich, U.S.A), were prepared by dissolving appropriate quantity of the antifungal agents in 10% v/v dimethylsulfoxide (DMSO) (Sigma Aldrich, U.S.A.), water, and ethanol (Sigma Aldrich, U.S.A.), which were then diluted to the required concentrations using Sabouraud liquid medium (broth) (Oxoid, Basingstoke, U.K.). Final concentrations of the antifungal agents were as follows: amphotericin B: 0.125-32 µg/mL, ketoconazole: 0.875-28 µg/mL, fluconazole: 1.68-53.76 µg/mL, griseofulvin: 0.25-16 µg/mL, and terbinafine: 0.25-8 µg/mL.

Determination of MIC endpoints: The MIC (the lowest concentration of drug preventing the growth of microscopically visible colonies on drug-containing plates when there was visible growth on drug-free plates) readings were performed at the end of 48 hours of incubation, when growth appeared on the control plate, the time period was followed according to CLSI which recommended for 72 hours [5]. The criteria for susceptibility/resistance testing of antifungal drugs using the peak plasma concentration were followed as previously described [15]. Thus, isolates that were sensitive to less than or equal to 4.00 µg/mL of amphotericin B, 3.50 of µg/mL of ketoconazole, 6.72 µg/mL of fluconazole, 2.00 µg/mL of griseofulvin, and 1.00 µg/mL

Table 1) Criteria for susceptibility/resistance to antifungal drugs

Antifungal Agent	Susceptible	Resistant
	µg/mL	µg/mL
Amphotericin B	≤ 4.00	> 4.00
Fluconazole	≤ 6.72	> 6.72
Griseofulvin	≤ 2.00	> 2.00
Terbinafine	≤ 1.00	> 1.00
Ketoconazole	≤ 3.50	> 3.50

of terbinafine were considered as susceptible (Table 1). All experiments on each strain were performed using three independent replicates.

Determination of Minimum fungicidal concentration: The filter paper discs showing no visible growth in determining minimum inhibitory concentration were aseptically removed with the aid of a sterile forceps and transferred into 5.0 mL of sterile Sabouraud dextrose liquid medium containing 0.050% v/v glycerol and incubated at 30 °C for 48 hrs. Minimum fungicidal concentrations (MFC) were determined as the lowest concentration resulting in no growth in subculture [16].

Statistical analysis: The analysis of the qualitative data was conducted by means of Chi-square and Fisher's exact tests. A *p*-value less than .05 was considered statistically significant. Data analysis was performed in SPSS software (Version 12).

Findings

Antifungal susceptibility testing of Dermatophytes: Antifungal susceptibilities, involving MIC and MFC, of 32 clinical isolates of dermatophytes are shown in Tables 2 and 3. The MICs of griseofulvin (GF), ketoconazole (KTC), terbinafine (TBF), amphotericin B (AMB), and fluconazole (FLU) were determined for *T. eriotrephon* (10), *T. bullosum* (2), *T. simii* (12), *T. benhamiae* (1), *T. rubrum* (1), *T. tonsurans* (3), *M. audouinii* (2), *C. serratus* (1), *T. rubrum* 28188 (1), and others (8). As shown in Table 2, the MICs of AMB, KTC, FLU, GF, and TBF for most dermatophytes ranged between 0.125 – > 32, 0.875 – >28, 1.68 – > 53.76, 0.25 – ≥16, and 0.25 - 8 µg/mL, respectively. The most frequent MICs of these antifungals against the isolates were > 32, 0.875 and 1.75, 3.36 and >53.76, 0.5, and 0.25 µg/mL, respectively. Additionally, the MFCs were 0.25 – >32, 0.875 – 28, 3.36 – >53.76, 0.25 – 16, and 0.5 - 8 µg/mL for AMB, KTC, FLU, GF, and TBF, respectively as shown in Table 3. *C. serratus* was only susceptible to KTC and TBF at higher MIC values of 14 and 4 *µg*/mL, respectively.

The MICs of AMB, KTC, FLU, GF, and TBF against all the isolates ranged from 0.5-

Table 2) MIC ranges of different antifungals against dermatophytes

Antifungal Agents (µg/mL)	AMB	KTC	FLU	GF	TBF
T. eriotrephon (n = 10)	(0.25 - > 32)	(0.875 - 28)	(1.68 - > 53.76)	(0.25 – 1)	(0.25-0.5)
T. bullosum (n = 2)	(0.125 - > 32)	(1.75 - 7)	(1.68 - 3.63)	(0.25 – 0.5)	(0.25)
T. simii (n = 12)	(0.125 - > 32)	(1.75 - 7)	(1.68 - 6.72)	(0.25 – 0.5)	(0.25 - 4)
T. benhamiae (n = 1)	(0.5)	(0.875)	(3.36)	(0.5)	(0.25)
T. rubrum (n = 1)	(0.25)	(0.875)	(> 53.76)	(0.5)	(0.5)
T. tonsurans (n = 3)	(0.25 - > 32)	(1.75 - > 28)	(3.36 - > 53.76)	(0.25 – 16)	(0.25-8)
M. audouinii (n = 2)	(4)	(3.5 - 7)	(3.36 -6.72)	(0.5)	(0.25-0.5)
C. serratus (n = 1)	(> 32)	(14)	(> 53.76)	(> 16)	(4)
Others (n = 8)	(> 32)	(0.875 - >28)	(1.68 - >53.76)	(0.25 -0.5)	(0.25 - 4)
T. rubrum (ATCC 28188)	(0.25)	(1.75)	(3.36)	(0.5)	(0.5)

Table 3) MFC ranges of different antifungals against dermatophytes

Antifungal Agents (µg/mL)	AMB	KTC	FLU	GF	TBF
T. eriotrephon (n = 10)	(0.5 - > 32)	(1.75 - > 28)	(3.36 - > 53.76)	(0.5 – 2)	(0.5-1)
T. bullosum (n = 2)	(0.25 -32)	(3.5 - 14)	(3.63-6.72)	(0.5 – 1)	(0.25)
T. simii (n = 12)	(0.25 - > 32)	(1.75 - 14)	(3.36 - > 53.76)	(0.25 – 1)	(0.5 - 8)
T. benhamiae (n = 1)	(1)	(0.875)	(6.72)	(1)	(0.5)
T. rubrum (n = 1)	(0.5)	(1.75)	(> 53.76)	(1)	(1)
T. tonsurans (n = 3)	(0.5 - 1)	(3.5)	(6.72 - 53.76)	(0.5 – 16)	(0.5-1)
M. audouinii (n = 2)	(8)	(3.5 - 14)	(6.72-13.44)	(1)	(0.5-1)
C. serratus (n = 1)	(> 32)	(28)	(> 53.76)	(> 16)	(8)
Others (n = 8)	(0.5- > 32)	(1.75 -28)	(3.36 - >53.76)	(0.5-1)	(0.5 - 4)
T. rubrum (ATCC 28188)	(0.25)	(1.75)	(3.36)	(0.5)	(0.5)

>32, 1.75- >28, 3.36- >53.76, 0.5–16, and 0.5-8 µg/mL, respectively; thus, 34 (85%), 23 (57.5%), 33 (82.5%), 38 (95 %), and 35 (87.5 %) isolates of dermatophytes were susceptible to AMB, KTC, FLU, GF, and TBF, respectively. Terbinafine and griseofulvin showed lower MIC values compared to fluconazole, ketoconazole, and amphotericin B; thus, they were the most potent agents against all the dermatophyte isolates.

The antifungal resistance pattern of dermatophyte isolates is shown in Table 4. According to the results, 10, 40, and 20% of *T. eriotrephon* isolates were resistant to amphotericin B, ketoconazole, and fluconazole, respectively. Also, 50% of *T. bullosum* strains were resistant to both AMB and KTC. About 8, 41, and 15% of *T. simii* strains were resistant to AMB, KTC, and TBF, respectively. *T. benhamiae* and *T. rubrum* isolates were susceptible to all the antifungal agents, except *T. rubrum* to FLU. Only 50% of *M. audouinii* strains were resistant to KTC. About 33% of *T. tonsurans* isolates were resistant to AMB, KTC, GF, and TBF, while 66 % were resistant to FLU. *C. serratus* was resistant to all of the antifungal agents. Out of 8 species tested, 4, 2, 5, 7, and 6 cases were resistant to TBF, GF, FLU, KTC, and AMB, respectively. Table 4 shows that 6 (15%), 17 (42.5%), 7 (17.5%), 2 (5%), and 5 (12.5%) clinical isolates of dermatophytes were resistant to AMB, KTC, FLU, GF, and TBF, respectively. Some of these clinical isolates were multidrug resistant. As shown in the table, *T. tonsurans* and *C. serratus* were resistant to all the antifungal agents, and *T. eriotrephon* and *T. simii* were resistant to AMB and KTC. Only one isolate of *T. simii* was resistant to AMB, KTC, and TBF. This shows that most multidrug-resistant isolates were resistant to AMB and KTC.

Correlation of Antifungal Susceptibility Testing and gender of patients: The correlation of antifungal susceptibility testing of dermatophytes clinical isolates and gender showed that 20, 50, 55, 8, and 12.5% of dermatophytes isolates from males were resistant to AMB, KTC, FLU, GF, and TBF, respectively, while 17, 31, and 13% of the

Table 4) Resistance distribution of dermatophytes to different antifungals

	Resistance against (%)				
Antifungal Agents (µg/mL)	**AMB (>4)**	**KTC (> 3.5)**	**FLU (> 6.72)**	**GF (> 2)**	**TBF (>1)**
T. eriotrephon (n = 10)	1(10)	4 (40)	2 (20)	-	-
T. bullosum (*n* = 2)	1(50)	1 (50)	-	-	-
T. simii (*n* = 12)	1(8.3)	5 (41.7)	-	-	2 (15.4)
T. benhamiae (*n* = 1)	-	-	-	-	-
T. rubrum (*n* = 1)	-	-	1(100)	-	-
T. tonsurans (*n* = 3)	1(33.3)	1(33.3)	2 (66.7)	1(33.3)	1(33.3)
M. audouinii (*n* = 2)	-	1(50)	-	-	-
C. serratus (*n* = 1)	1(100)	1(100)	1(100)	1(100)	1(100)
Others (*n* = 8)	1(12.5)	4(50)	1(12.5)	-	1(12.5)

isolates from females were resistant to AMB, KTC, and FLU, respectively. This shows that resistance is markedly pronounced in males than in females with the highest percentage of resistance to ketoconazole. Less resistance to griseofulvin and terbinafine was observed in males but not in females (Table 5).

Discussion

In vitro analysis of antifungal activity of antifungal agents enables comparison between different antimycotics, which in turn may clarify the reasons for lack of clinical response and assist clinicians in choosing an effective therapy for their patients. However, it is important that the methodologies used for in vitro testing be standardized to facilitate the establishment of quality control parameters and interpretive breakpoints [17]. In this study, the MIC values of five antifungal agents (amphotericin B, fluconazole, griseofulvin, terbinafine, and ketoconazole) were investigated against different species of dermatophyte clinical specimens using the agar dilution method.

The most active agents against all dermatophytes species (*T. eriotrephon, T. bullosum, T. simii, T. benhamiae. T. rubrum, T. tonsurans, M. audouinii. C. serratus,* and others) were terbinafine with MIC ranges of 0.25–0.5, 0.25, 0.25–4, 0.25, 0.5, 0.25–8, 0.25–0.5, 4, and 0.25-4 µg/mL and griseofulvin with MIC ranges of 0.5–2, 0.5–1, 0.25–1, 1, 1, 0.5–16, 1, > 16, and 0.5-1 µg/mL, respectively. This finding is similar with the finding of another study by Badali et al. (2015) [18], evaluating the efficacy of nine antifungals (AMB, fluconazole, itraconazole, voriconazole, posaconazole, isavuconazole, caspofungin, anidulafungin, and terbinafine) and reporting terbinafine as the most effective drug. This finding is not similar with the finding of another study by Coelho et al. (2008) [19], comparing *in vitro* antifungal susceptibility of the microconidia of *T. rubrum* and *T. tonsurans* to five commonly used drugs (AMB, fluconazole, terbinafine, itraconazole, and griseofulvin) and reporting AMB as the most superior drug. Also, the current study findings are in

tandem with the findings of Aktas et al. (2014) [20], reporting AMB to be consistently better than ketoconazole and fluconazole against all the dermatophytes tested [21, 22]. A study in northern India also showed that there was a good response to griseofulvin therapy among the tinea capitis patients, which agrees with the present study finding. With the emergence of treatment failure with griseofulvin, allylamines became the preferred choice of treatment, which is in agreement with this study findings clearly showing less resistance to terbinafine; thus, terbinafine becomes an alternative in case of griseofulvin failure.

These two drugs (terbinafine and griseofulvin) had the least resistant isolates (five and two, respectively). Resistance to

Table 5) Correlation of antifungal susceptibility testing and gender of patients

Antifungal Agents (µg/mL)	AMB	KTC	FLU	GF	TBF
T. Eriotrephon (n = 10)					
Male (*n* = 6)	(0.5 - > 32)	(1.75 - 28)	(1.68 - > 53.76)	(0.25 – 1)	(0.25-0.5)
Female (*n* = 4)	(0.5 - 4)	(0.875 - 7)	(1.68 - 3.36)	(0.25 – 0.5)	(0.25-0.5)
T. bullosum (n = 2)					
Male (*n* = 2)	(0.125 - > 32)	(1.75 - 7)	(1.68 - 3.36)	(0.25 – 0.5)	(0.25)
T. simii (n = 12)					
Male (*n* = 6)	(0.125 - > 32)	(0.875 - 7)	(1.68 - 6.72)	(0.25 – 0.5)	(0.25-4)
Female (*n* = 6)	(0.125 - 4)	(1.75 - 7)	(1.68 - 6.72)	(0.25 – 0.5)	(0.25-0.5)
T. benhamiae (n = 1)					
Female (*n* = 1)	(0.5)	(0.875)	(3.36)	(0.5)	(0.25)
T. rubrum (n = 1)					
Male (*n* = 1)	(0.25)	(0.875)	(> 53.76)	(0.5)	(0.5)
T. tonsurans (n = 3)					
Male (*n* = 2)	(0.5 - > 32)	(1.75 - > 28)	(3.36 - > 53.76)	(0.5 – >16)	(0.25 - >8)
Female (*n* = 1)	(0.25)	(1.75)	(> 53.76)	(0.5)	(0.25)
M. audouinii (n = 2)					
Male (*n* = 1)	(4)	(3.5)	(3.36)	(0.5)	(0.5)
Female (*n* = 1)	(4)	(7)	(6.72)	(0.5)	(0.25)
C. serratus (n = 1)					
Male (*n* = 1)	(> 32)	(14)	(> 53.76)	(> 16)	(4)
Others (*n* = 8)					
Male (*n* = 5)	(0.125- 4)	(1.75 - >28)	(1.68 - >3.36)	(0.25 -0.5)	(0.25 - 0.5)
Female (*n* = 3)	(0.25-> 32)	(0.875 - 7)	(1.68 - >3.36)	(0.5)	(0.25 - 0.5)

terbinafine is not far-fetched as some studies reported such resistance. Some studies have reported documented cases of terbinafine-resistant *T. rubrum* [23, 24]. The least active agent was ketoconazole with MIC ranges of 0.875–28, 1.75–7, 1.75–7, 0.875, 0.875, 1.75- >28, 3.5–7, 14, and 0.875– >28 µg/mL against 17 resistant isolates.

With respect to terbinafine, all of *T. eriotrephon, T. bullosum, T. benhamiae. T. rubrum,* and *M. audouinii* isolates were inhibited at concentrations ranging from 0.25 to 0.5 µg/mL. Some isolates of *T. simii* (2), *T. tonsurans* (1), *C. serratus* (1), and others (1) showed different sensitivity at high ranges. The same sensitivity pattern was observed to griseofulvin for *T. eriotrephon, T. bullosum, T. simii, T. benhamiae. T. rubrum, M. audouinii,* and others, ranging from 0.5 to 0.1 µg/mL. *T. tonsurans* (1) and *C. serratus* (1) had high MIC values.

In this study, 17 (42.5%) isolates of dermatophytes tested by agar dilution, including *T. eriotrephon* (4), *T. bullosum* (1), *T. simii* (5), *T. tonsurans* (1), *M. audouinii* (1). *C. serratus* (1), and others (4), were resistant to ketoconazole with a MIC range of ≥ 3.5 µg/mL. Also, 6 (15 %) isolates of dermatophytes tested by agar dilution, including *T. eriotrephon* (1), *T. bullosum* (1), *T. simii* (1), *T. tonsurans* (1), *C. serratus* (1), and others (1), were resistant to AMB with a MIC range of ≥ 3.5 µg/mL. In addition, 7(17.5%) isolates, including *T. eriotrephon* (2), *T. rubrum* (1), *T. tonsurans* (2), *C. serratus* (1), and others (1), were resistant to FLU with a MIC range of ≥ 6.72 µg/mL.

Antifungal susceptibility testing is a dynamic field, especially in medical mycology. Development and standardization of antifungal susceptibility test have shown remarkable progress in this field [17], although the use of agar dilution method for dermatophytes susceptibility testing is much sufficient. Most studies have showed that agar dilution seems to be an alternative method to MIC-determination of antifungal drugs for dermatophytes, especially in underdeveloped countries, though it is a laborious methodology, but results could be obtained faster and more cost-effectively [25]. Examination of the resistance of dermatophytic clinical isolates to five antifungal agents showed that this resistance was prominent in males compared to their female counterpart. The study also found that none of the clinical isolates from female was resistant to terbinafine and griseofulvin. Mikaeili et al. (2019) [26]. reported that males responded better when treated with terbinafine and griseofulvin [27], which is inconsistent with this study findings showing that dermatophyte clinical isolates from female responded better to griseofulvin and terbinafine. Another study in Nigeria reported terbinafine as the most active antifungal agent against dermatophytes [28].

Study limitation: The sample collection from children was tasking and time-consuming, and internet service was also limited for efficient and effective write-ups.

Conclusion

In conclusion, our results revealed that griseofulvin and terbinafine were the most potent antifungals against dermatophytic fungi among systemic antifungals tested. It is worthy to note that more clinical data are needed to confirm if this potent efficacy *in vitro* is predictive of clinical outcome; however, at the interim, this finding will provide bases for choosing appropriate treatments for tinea infection in Nigeria hospitals.

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