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***Corresponding author**
Ikoojo Charity Agada
E-mail
dr.ikoojo@gmail.com

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Antifungal Susceptibility of *Cryptococcus neoformans* Strains from Poultry in Federal Capital Territory, Nigeria

Ikoojo Charity Agada[1]*, Olatunde Kazeem Olabode[1], Samuel Mailafia[1], Salami Adeiza Charles[1], Martha Echoida-Ogbole[1], Agnes Oluwasheun Abioje[1], Emmanuel Ugochukwu Anaso[2]

[1]Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Abuja, Abuja, Nigeria
[2]Department of Animal Science, Faculty of Agriculture, Federal University of Agriculture, Mubi, Adamawa, Nigeria

Abstract

Cryptococcosis is a mycotic disease mostly caused by the encapsulated yeast *Cryptococcus neoformans* and *Cryptococcus gatti*. There is a global trend in antifungal resistance in both animal and human medicine. This study was undertaken to evaluate the antifungal susceptibility of *Cryptococcus neoformans* strains from poultry in the Federal Capital Territory (FCT), Nigeria. A total of 300 fecal samples from pigeons (n = 100), broiler (n=100), and indigenous chicken (n=100) were collected and analyzed using standard mycological techniques. Presumptive isolates were confirmed using polymerase chain reaction (PCR). PCR confirmed 15 (5%) *C. neoformans* isolates. Antifungal susceptibility testing of 15 *C. neoformans* strains isolated from pigeons (n = 3), broiler chickens (n = 3), and indigenous chickens (n = 9) were evaluated against 10 commonly prescribed antifungal agents using the Kirby-Bauer disc diffusion method. The result of the ASF showed varying degrees of susceptibility of *C. neoformans* strains to the azole group and other antifungal agents tested. The strains were susceptible to itraconazole (93.3 %), posaconazole (86.7%), voriconazole, and caspofungin (73.3%). The isolates were completely resistant to metronidazole and griseofulvin (100 %), ketoconazole (86,7%), and fluconazole (60%). Six resistance phenotypes were exhibited by the *C. neoformans* isolates against the ten antifungal agents tested. The common resistance pattern observed was fluconazole, ketoconazole, griseofulvin, and metronidazole, displayed by 46.6% (n = 7) of the isolates. Antifungal susceptibility testing showed variable susceptibility patterns among the *C. neoformans* strains, highlighting the importance of tailored therapeutic approaches. Furthermore, the emergence of antifungal resistance underscores the need for continued surveillance and development of novel therapeutic agents targeting *Cryptococcus* species as avian habitats serve as major reservoirs for human exposure.

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Introduction

Cryptococcosis is a mycotic disease that affects both healthy and immunocompromised humans and animals, caused by *Cryptococcus neoformans* and *Cryptococcus gattii* complexes [1]. *Cryptococcus neoformans* is a round encapsulated yeast, classified into four primary serotypes based on their capsular epitopes as *C. neoformans var. neoformans* (serotypes A and D) and *C. neoformans var. gattii* (serotypes B and C) [2]. The most common method of infection for *Cryptococcosis* is via inhalation of fungal spores, which causes respiratory infection. Studies have shown that humans may become infected with cryptococcosis through zoonotic transmission if they come into direct contact with infected animals or objects derived from animals [3]. An individual may develop neurocryptococcosis after inhaling the fungal spores [4]. According to global estimates of the disease burden, over 25,000 cases of Cryptococcosis are reported in Nigeria annually [5]. North-Central Nigeria accounts for 36% of hospital-based cases of *Cryptococcal* meningitis, which is associated with a high fatality rate [3]. Among AIDS patients, Cryptococcosis has been linked to significant rates of morbidity and mortality [6]. In immunocompromised individuals, it is the fourth most prevalent infection [7]. Every year, the disease affects about a million people worldwide, and patients who die within three months of being ill account for 400,000 of the deaths [8]. While ruminant infections usually result in mastitis, birds are asymptomatic carriers [9]. Dogs and cats may have systemic or localized Cryptococcosis [10].

Globally, there is a trend in antifungal resistance, with an increasing public health alarm. The growing prevalence of antifungal-resistant infection and the emergence of multidrug-resistant pathogens necessitated this study. Knowledge of antifungal resistance patterns is vital for the proper management of the disease. This study will help in understanding the antifungal susceptibility and resistance pattern of *C. neoformans* in the study area, providing new trends of resistance, if any, and the appropriate antifungal agents to be used in the treatment of cryptococcosis.

Materials and Methods

Cryptococcus neoformans strains

Fifteen (15) *Cryptococcus neoformans* strains previously isolated from pigeons (n = 3), broiler chickens (n = 3), and indigenous chickens (n = 9) were used for the study. The *C. neoformans* strains were isolated based on standard mycological methods such as cultural morphology on Sabouraud's dextrose agar (SDA) and Potato dextrose agar (PDA), microscopic examination using lactophenol stain, and confirmed positive using polymerase chain reaction (PCR) as described by Kidd et al [12]. *Cryptococcal* isolates were subjected to multiplex PCR, with modifications [13]. The primer sequence was STE20aDF (5'-TGGGCGTATCCCAACCTACGA-3') and STE20aDR (5'-TAACGA CTCCGGTGCCGTGAA-3'). The thermal cycling conditions were initial denaturation at 95°C for 1 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The expected amplicon size was 413 bp. All data were subjected to simple descriptive statistics, and the results were expressed and analyzed by percentages, ratios.

Antifungal susceptibility testing

The antifungal susceptibility of isolates was evaluated using the disk diffusion method as described by Kirby-Bauer method [14] and in accordance with the Clinical and Laboratory Standards Institute (2017). The inoculum was standardized to a 0.5 McFarland turbidity standard before dilution and use in antifungal susceptibility testing. The following antifungal agents and their concentrations: amphotericin B (AMB, 20µg), fluconazole (FLU, 25µg), itraconazole (ITC, 50µg), nystatin (NY, 100IU), posaconazole (PO, 5µg), caspofungin (CAS, 5µg), metronidazole (MTZ, 5µg), Voriconazole (V01, 1 µg), griseofulvin (AGF, 10µg), and ketoconazole (KCA, 10µg) were tested (Table 1). The growth inhibition zone diameter was measured to the nearest millimeter, and the results were interpreted according to Clinical and Laboratory Standards Institute (2017) guidelines.

Multiple drug resistance (MDR) and multiple antimicrobial resistance index (MARI) assays

Multiple drug resistance (MDR) was defined as antimicrobial resistance to two or more antifungal class, while multiple antimicrobial resistance index (MARI) was defined as the ability of the isolate to resist at least one antifungal medication across the all the antifungals used. The computation involved dividing the number of antifungals (a) that the tested isolates were resistant to by the total number of antifungals tested on the isolates (b) [15]. The formula used is:

Table 1 Antifungal agents tested and interpretative criteria used.

Antibiotic	Concentration (µg)	Sensitive	intermediate	Resistant
Amphotericin B	20	>15	10-14	<9
Caspofungin	5	≥11	-	≤10
Fluconazole	25	≥19	15-18	≤14
Griseofulvin	10	≥19	15-18	≤14
Itraconazole	50	>16	10-15	<9
Ketoconazole	10	≥30	23-29	≤22
Nystatin	100IU	≥25	17-24	<16
Posaconazole	5	≥17	14-16	≤13
Voriconazole	1	≥17	14-16	≤13
Metronidazole	5	≥21	17-20	<16

Source; CLSI, 2017

Table 2 Antifungal susceptibility of *C. neoformans* to ten antifungal agents.

Antifungal agents	Sensitivity	Intermediate	Resistance
fluconazole	2(13.3%)	4(26.7%)	9(60%)
Ketoconazole	0(0%)	2(13.3%)	13(86.7%)
Posaconazole	13(86.7%)	2(13.3%)	0(0%)
Metronidazole	0(0%)	0(0%)	15(100%)
Voriconazole	11(73.3%)	2(13.3%)	2(13.3%)
Itraconazole	14 (93.3%)	1(6.6%)	0(0%)
Caspofungin	11(73.3%)	0(0%)	4(26.7%)
Griseofulvin	0(0%)	0(0%)	15(100%)
nystatin	0 (0%)	14(93.3%)	1(6.7%)
Amphotericin B	1(6.7%)	10(66.7%)	4(26.7%)

Table 3 Antifungal resistance patterns displayed by *C. neoformans* isolates on different antifungal drugs.

Isolate number	Resistance pattern	Number of resistant antifungals
1	FLU, KCA, AGF, MTZ, CAS	5
2	FLU, KCA, AGF, MTZ	4
3	FLU, KCA, AGF, MTZ	4
4	AMB, KCA, AGF, MTZ	4
5	AGF, MTZ	2
6	AMB, KCA, NY, AGF, MTZ	5
7	FLU, KCA, AGF, MTZ	4
8	FLU, KCA, AGF, MTZ	4
9	FLU, KCA, AGF, MTZ	4
10	KCA, AGF, MTZ, CAS	4
11	AGF, MTZ	2
12	KCA, AGF, MTZ, ITC	4
13	FLU, KCA, AGF, MTZ, CAS	5
14	FLU, KCA, AGF, MTZ	4
15	FLU, KCA, AGF, MTZ, CAS	5

Key: Fluconazole (FLU, 25µg), Amphotericin B (AMB, 20µg), Ketoconazole (KCA, 10µg), Nystatin (NY, 100IU), Griseofulvin (AGF, 10µg), Posaconazole (POS, 5µg), Metronidazole (MTZ, 5µg), Voriconazole (VOI, 1µg), Caspofungin (CAS, 5µg), Itraconazole (ITC, 50µg) (Liofilchem, UK).

MARI = a/b

Where a is the total number of antifungals that the tested isolates were resistant to while b is the total number of antifungals that was tested.

MDR = a/n

Where a is the number of class of antifungals that the isolates where resistant to, while n is the total classes of antifungals tested.

Results and Discussion

The antifungal susceptibility testing results showed a varying degree of *C. neoformans* strains to the antifungal agents tested. The isolates were susceptible to itraconazole (93.3%), posaconazole (86.7%), voriconazole, and caspofungin (73.3%) each. While 13.3% and 6.7% were sensitive to fluconazole and amphotericin B, respectively. All

the isolates in this study (100%) were resistant to griseofulvin and metronidazole, and also resistant to ketoconazole (86.7%), fluconazole (60%), amphotericin B (26.7%), caspofungin (13.3%), 6.7% resistance to voriconazole and nystatin (Table 2). Six (6) resistance phenotypes were exhibited by the *C. neoformans* isolates against the ten antifungal agents tested (Table 3). The common resistance pattern observed is FLU, KCA, AGF, MTZ, displayed by 46.6 % (n = 7) of the isolates (Table 4).

Table 4 Multiple antifungal resistance index and multidrug resistance of *C. neoformans.*

Isolate number	MARI	No. of antifungal resistance categories (n=3)	MDR
1	0.5	5	1.7*
2	0.4	4	1.3*
3	0.4	4	1.3*
4	0.4	4	1.3*
5	0.2	2	0.7*
6	0.5	5	1.7*
7	0.4	4	1.3*
8	0.4	4	1.3*
9	0.4	4	1.3*
10	0.4	4	1.3*
11	0.2	2	0.7*
12	0.4	4	1.3*
13	0.5	5	1.7*
14	0.4	4	1.3*
15	0.5	5	1.7*

MARI: Multiantibiotic Index, MDR: Multidrug Resistance, * = significant

This study indicates that 86.7% of the *C. neoformans i*solates were susceptible to itraconazole and posaconazole, and 73.3% were susceptible to caspofungin and voriconazole. These findings are consistent with global patterns where newer azoles, such as itraconazole and posaconazole, generally show good efficacy against *C. neoformans* [15]. The variability in susceptibility noted in our study, particularly the 13.3% sensitivity to Fluconazole and 6.7% sensitivity to amphotericin B, reflects a growing concern about antifungal resistance. Azole resistance in *Cryptococcus* has been primarily associated with two major mechanisms: Overexpression of the efflux pumps and mutation in ERG11, which encodes the azole target enzyme, leading to reduced drug binding [16, 17]. This mechanism may occur independently or synergistically, contributing to multidrug resistance [18]. Fluconazole resistance in *C. neoformans* can be attributed to the widespread use of azole drugs in veterinary practice, exposing Cryptococcus to sublethal azole concentrations, creating selective pressure for a resistant strain [19]. Findings in this study are somewhat lower compared to studies in other parts of Africa and the world, where higher resistance rates to Fluconazole are reported [16], possibly reflecting regional variations in antifungal use and exposure. The 100% resistance to griseofulvin and metronidazole aligns with the general understanding that these agents are not effective against *C. neoformans*, as they are not typically used for treating *Cryptococcosis*. The high resistance to Ketoconazole (86.7%) in this study is notable and reflects a broader trend of reduced efficacy of older azoles against *C. neoformans* [16]. Intermediate resistance rates varied, with 93.3% intermediate for nystatin, 66.6% intermediate for Amphotericin B, and other agents showing varying intermediate levels. The intermediate susceptibility to Amphotericin B (66.6%) is concerning because this drug is often reported to rarely have resistance to the drug [20]. Amphotericin B is generally effective and has been prescribed as the primary antifungal drug for the management of Cryptococcal infections [21]. The resistance patterns observed in this study are consistent with a growing trend of antifungal resistance. Dongmo et al. [16] reported increasing resistance to older antifungals like Fluconazole and Ketoconazole. The increasing resistance to antifungals underscores the need for continuous monitoring and potentially re-evaluating treatment protocols to address emerging resistance patterns effectively. In addition, it emphasizes the importance of integrating environmental, veterinary, and clinical surveillance to mitigate the spread of resistance.

Conclusion

This study documents the first reports of the antifungal susceptibility profile of *C. neoformans* from poultry in the study area. The isolates in this study were most susceptible to Itraconazole (93.3 %), Posaconazole (86.7%), and Caspofungin (73.3 %). However, the isolates were completely resistant to Metronidazole and Ketoconazole (100%). The common resistance pattern observed is FLU, KCA, AGF, MTZ, displayed by 46.6% (n = 7) of the isolates. The emergence of antifungal resistance underscores the need for continued surveillance and development of novel therapeutic agents targeting *Cryptococcus* species.

Conflict of interest

The authors declare no conflict of interest.

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