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ANTIFUNGAL SUSCEPTIBILITY AND TEST FOR CURE OF *CANDIDA SPECIES* AMONG VULVOVAGINAL CANDIDIASIS PATIENTS IN A SECONDARY CARE HOSPITAL, NIGERIA

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ABSTRACT

Background: Antimicrobial resistance among *Candida species* is an intense public health concern. The aim of the study was to determine the antifungal susceptibility pattern and test for cure of *Candida species* among women of child bearing age who visited the General Hospital Onitsha, Nigeria with symptoms suggestive of Vulvovaginal Candidiasis (VVC).

Materials and Methods: Eight hundred and seventy six female patients participated in the study of which high vaginal swabs were collected and evaluated mycological by standard microbiological methods: microscopic examination and culture using sabouraud dextrose agar (SDA). Susceptibility of isolates to 4 antifungal agents was tested using agar dilution method. Clinico-mycological evaluation was also performed among the patients.

Result: Higher minimum inhibitory concentration (MIC) to azole antifungals was observed predominantly among non-albicans *Candida species* increasingly involved in VVC. The rate of mycological resolution was higher than symptomatic relief at 2 weeks after treatment with antifungal drug.

Conclusion: Efficacious treatment of VVC requires an adequate knowledge of the causative agents and more importantly the antimicrobial to which they exhibit high susceptibility.

Keywords: Vulvovaginal Candidiasis, Clinico- mycology, Antimicrobial resistance, *Candida species*

SUSCEPTIBILITÉ ANTIFONIQUE ET TEST POUR LA CURE D'ESPÈCES DE CANDIDA ENTRE LES PATIENTS DE CANDIDASES VULVOVAGINALES DANS UN HÔPITAL DE SOINS SECONDAIRES, NIGERIA

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ABSTRAIT

Contexte: La résistance aux antimicrobiens chez les espèces de Candida est un problème de santé publique intense. L'objectif de l'étude était de déterminer le schéma de susceptibilité aux antifongiques et le test de guérison des espèces de Candida parmi les femmes en âge de procréer qui ont visité l'hôpital général de Onitsha, au Nigeria, avec des symptômes suggérant une candidose vulvovaginale (VVC).

Matériaux et méthodes: huit cent soixante-seize six patientes ont participé à l'étude des prélèvements vaginaux élevés collectés et évalués par mycologie par méthodes microbiologiques standard: examen microscopique et culture à l'aide de la gélose sabouraud dextrose (SDA). La susceptibilité des isolats à 4 agents antifongiques a été testée en utilisant une méthode de dilution en agar. Une évaluation clinico-mycologique a également été réalisée chez les patients.

Résultat: une concentration minimale minimale d'inhibition (MIC) en anatoxines azoliques a été observée principalement chez les espèces non-albicans Candida de plus en plus impliquées dans VVC. Le taux de résolution mycologique était plus élevé que le soulagement symptomatique à 2 semaines après le traitement par un médicament antifongique.

Conclusion: Un traitement efficace de la VVC nécessite une connaissance adéquate des agents causaux et, plus important encore, des antimicrobiens auxquels ils présentent une forte susceptibilité.

Mots-clés: Candidiase Vulvovaginale, Clinico-mycologie, Résistance Antimicrobienne, Espèces Candida

INTRODUCTION

Antimicrobial resistance among *Candida species* involved in Vulvovaginal candidiasis (VVC) continues to thrive as a serious public health concern. Vulvovaginal candidiasis is a conventional gynecological opportunistic mycological infection caused by *Candida* species in the lower genital tract among females globally [1,2]. It has been ascertained that approximately 75 % of sexually active females have a minimum of one-time experienced symptomatic VVC [3].

The rise in incidence of fungal infection has resulted in an extensive use of antifungals [4] and the treatment is often carried out entirely on pragmatic basis, because vaginal cultures are not routinely taken, and susceptibility testing is scarcely done. Successful treatment of infections requires adequate information of the specific causative agent(s) and the drugs to which they are susceptible. Information available in the literature suggests that data on etiologic pattern in VVC and susceptibility to antifungal are lacking in Anambra State of Nigeria, particularly Onitsha, which is the commercial mainstay of the state.

Antifungal susceptibility testing provides an evaluation of antifungal efficacy, guaranteeing good treatment outcome, limiting the development of drug resistance and therapeutic ability of unendorsed composites [5-7].

MATERIALS AND METHODS

Study Population

The present study took place between 2008 and 2009 at an Obstetrics and Gynecology Department at General Hospital Onitsha, Nigeria. A total of 876 women of different age groups were included in this study.

Ethical Consideration

Ethical approval of the study was sought from the Nnamdi Azikiwe University Teaching Hospital Institutional Review and Ethics Committee in Nnewi, Nigeria. Written consent was sought from the study subjects before inclusion into the study.

Collection of Clinical Samples

The sample: high vaginal swab was obtained for the study. The samples were collected by the clinician from the posterior fornix of the vagina with sterile Dacron cotton swab stick after dilation using a sterile speculum. Two swab samples were collected per subject.

Microscopic Examination

The first vaginal swab was rolled in drop of potassium hydroxide (KOH) then covered with cover slip. The slide was mounted on a microscope and examined using 10x and 40x objective.

Isolation and Identification

The second vaginal swab was inoculated onto Sabouraud dextrose agar (SDA) and incubated at 37^0C and examined for growth daily for 4 days.

Clinico-mycological Evaluation

The patients with *Candida* positive cultures were asked to come back to the hospital after two weeks of treatment with antifungal drugs recommended to them. At the follow-up visit, the same questionnaire was administered and another vaginal swab was collected and processed as done previously. Clinico-mycological evaluation was then carried out using the following rating scale:

A. Symptom absent, culture negative = very good = 3 point score

B. Symptom present, culture negative = Good = 2 point score

C. Symptom absent, culture positive = fair = 1 point score

D. Symptom present, culture positive = poor = 0 point score

Antifungal Susceptibility Testing

Susceptibility of the isolates to antifungal drugs was carried out by agar dilution technique [8] using the following antifungal drugs: Fluconazole powder (Medich Plc, England); Itraconazole powder (Hanmi Pherm, Kore); Miconazole powder (Janssen Pharmaceutical Beerse Belgium); Clotrimazole powder (Symnedic Laboratories) as follows:

i. Preparation of stock solution of antifungal drugs:

Stock solution of each of the drugs was prepared by dissolving 25.6mg of the powder in 100ml of sterile 1: 9 dilution (in distilled water) of Dimethyl sulphoxide, to give 100ml of 256 µg/ml. All the stock solutions were sterilized by tyndalization.

ii Agar Dilution Technique:

Double strength SDA was prepared and dispensed into McCartney bottles in 10ml amounts and sterilized by autoclaving at 121^0C for 15 min, and allowed to cool to about 45^0C. Two fold serial dilution of each of the drugs was subsequently made, in the molten agar, according to the protocol in table 1. The dilutions were up to 15th dilution (i.e. 0.008 - 128µg/ml). The content of each McCartney bottle was thoroughly mixed and poured into plate and was allowed to set.

Table 3: PROTOCOL FOR THE DILUTION OF ANTIFUNGAL DRUGS IN CULTURAL MEDIA

	Tube No	Vol. of Medium	Vol. of Drug Soln*	Vol. of Water	Final Conc.
A	1.	10 ml	10 ml	0.000ml	128 µg/ml
	2.	10ml	5ml	5.00ml	64 µg/ml
	3.	10 ml	2.5ml	7.500ml	32 µg/ml
	4.	10 ml	1.25ml	8.750ml	16 µg/ml
	5.	10 ml	0.625ml	9.375 ml	8 µg/ml
	6.	10 ml	0.3125ml	9.688ml	4 µg/ml
B	7.	10ml	10ml	0.00ml	2 µg/ml
	8.	10ml	5ml	5.00ml	1 µg/ml
	9.	10ml	2.5ml	7.500ml	0.5 µg/ml
	10.	10ml	1.25ml	8.750ml	0.25 µg/ml
	11.	10ml	0.62ml	9.375ml	0.125 µg/ml
	12.	10ml	0.3125ml	9.688ml	0.0625 µg/ml
C	13.	10ml	10ml	0.00ml	0.032 µg/ml
	14.	10ml	5ml	5.00ml	0.016 µg/ml
	15.	10ml	2.5ml	7.500ml	0.008 µg/ml

*** Stock solutions used were: Stock solution A(Conc = 256 µg/ml) – for dilution 1-6; Stock solution B (Conc = 4 µg/ml) – for dilution 7-12; Stock solution C (Conc = 0.0625) – for dilution 13-15**

Iii: Preparation and Standardization of Inoculums:

A total of 60 Candida isolates representing all the species identified were used for the test, as follows:

Candida albicans	n=39
Candida glabrata	n=8
Candida tropicalis	n=4
Candida krusei	n=5
Candida dubliniensis	n=4

Candida albicans ATCC 10231 (control organism).

Prior to testing, all isolates were sub-cultured on SDA plates and incubated overnight at 37^0C to ensure purity and viability. Approximately five isolated colonies were picked and then suspended in sterile saline and homogenized; the turbidity of the suspension was adjusted to match that of a Mac Farland 0.5 turbidity standard using a spectrophotometer.

iv. Inoculation of Plates:

The surface of the prepared SDA plates were dried at 37^0C in the oven and each plate was divided into four segments. Each of the segments was inoculated with a loopful (0.01ml) of the standardized suspension of the organism. The organism was also inoculated into control plates (drug free).

v. Determination of MIC:

After 24hrs incubation at 37^0C, the amount of growth in the plates containing the different concentrations of antifungal agents was compared with the amount of growth in the drug- free growth control plates. The MIC was read as the lowest concentration of antifungal that totally inhibited the growth of the organism.

INTERPRETATIVE CRITERIA

The interpretation of the results was based on the break points [9] established by the National Committee for Clinical Laboratory Standards (NCCLS) for different antifungal drugs against *Candida* species [Table 2].

TABLE 2: MIC BREAKPOINTS FOR SUSCEPTIBILITY OF CANDIDA TO ANTIFUNGAL DRUGS

Interpretation of MIC Values

Drug	Susceptible	Intermediate(SDD)	Resistant
Fluconazole	**≤ 8µg/ml**	**16-32µg/ml**	**>64µg/ml**
Miconazole/ Cotrimazole	**≤ 0.5µg/ml**	**1-4 µg/ml**	**>8µg/ml**
Itraconazole	**≤ 0.125µg/ml**	**0.25-0.5 µg/ml**	**> 1µg/ml**

Key : ***S – D D = Dose dependent susceptibility***

STATISTICAL ANALYSIS

Statistical analysis: Chi square test, One way Analysis of Variance(ANOVA) and t- test conclude and validate the results at 0.05 level of significance.

RESULT

Yeasts cells were seen as a small, oval cell, measuring 2-4 in diameter that were budding or single. Following clinico-mycological evaluation of the patients, 73%, 67%, 50% and 50% of patients on Fluconazole, Miconazole, Clotrimazole and Itraconazole respectively were symptomatically relieved while 91%, 89%, 88% and 17% of patients on Fluconazole, Miconazole, Clotrimazole and Itraconazole respectively were mycologically relieved at 2 weeks after treatment [Table 3]. The rate of mycological resolution was higher than symptomatic relief at 2 weeks after treatment with antifungal drug [Table 4].

TABLE 3: CLINICO -MYCOLOGICALLY EVALUATION AT 2 WEEKS AFTER TREATMENT WITH ANTIFUNGAL DRUGS.

		A	B	C	D	
Drug	Number Admitted	Very Good	Good	Fair	Poor	Score
Fluconazole	11	7	3	1	0	28
Miconazole	9	5	3	1	0	22
Clotrimazole	8	3	4	1	1	18
Itraconazole	14	6	4	1	3	17

The following rating scales were used: A. Symptom absent, culture negative = Very good =3 point score; B. Symptom present, culture negative = Good = 2 point score; C. Symptom absent, culture positive = Fair = 1 point score; D. Symptom present, culture positive = Poor= Zero point score

TABLE 4: SYMPTOMATIC RELIEF AND MYCOLOGICAL CURE RATES AT 2WEEKAFTER TREATMENT WITH ANTIFUNGAL DRUGS

Drug	Symptomatic relief	Mycological cure
Fluconazole	8 (73%)	10 (91%)
Miconazole	6 (67%)	8 (89%)
Clotrimazole	4 (50%)	7 (88%)
Itraconazole	7 (50%)	10 (71%)

Mycological vs symptomatic relief at 2 weeks (t = 6.91 ;< 0.05)

The minimum inhibitory concentration (MIC) of Antifungal agents against *Candida species* isolated from women with vulvovaginal candidiasis was done using the interpretative criteria on Table 1. The susceptibility testing of 60 Candida isolates (representing different species) to fluconazole (*C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. krusei* and *C. glabrata*) showed varied degree of susceptibility. Of the 60 isolates, 52 (86.7%) were susceptible (MIC< 8 mg/ml) to fluconazole whereas, 8 (13.3%) isolates were intermediate with MIC 16-32 mg/ml. There was no record of resistance with fluconazole in this study [Table 5]. Of the 8 isolates with elevated MIC, 4 isolates were *Candida glabrata*, 1 isolate was *Candida tropicalis*, 2 isolates were *Candida krusei* and 1isolate *Candida albicans*. All the 60 C*andida* isolates (100%) had very low MIC to Miconazole and Cotrimazole (< 0.5 µg/m1) [Table 6]. On the contrary, only 26 (43%) of the isolates were susceptible to Itraconazole (MIC <0.125 µg/m1), whereas 21 (35%) were resistant (MIC>1µg/m1), while 13 (22%) showed dose dependent susceptibility to the drug. Among all the species, the most resistant was the *C. tropicalis* which recorded 100% resistance to Itraconazole, followed by *C. krusei* which has about 40% resistance. [Table 7].

TABLE 5: MINIMUM INHIBITORY CONCENTRATION OF FLUCONAZOLE ON CANDIDA SPECIES ISOLATED FROM VULVOVAGINAL CANDIDIASIS PATIENTS IN ANAMBRA STATE

	MIC (µg/ml)		
Candida species	**≤ 8 µg /ml (Susceptible)**	**16-32 µg/ml (Intermediate)**	**≥ 64µg/ml (Resistance)**
Candida albicans	38/39	1/39	0/39
Candida tropicalis	¾	¼	0/4
Candida dubliniensis	4/4	0/4	0/4
Candida krusei	3/5	2/5	0/4
Candida glabrata	4/8	4/8	0/4
Total (%)	52/60 (86.7%)	8/60 (13.3%)	0/60 (0%)

TABLE 6: MIC OF MICRONAZOLE / CLOTRIMAZOLE AMONG CANDIDA SPECIES

	MIC (µg/ml)	
Candida species	**≤ 0.5 µg /ml (Susceptible)**	**≥ 8µg/ml (Resistance)**
Candida albicans	39/39	0/39
Candida tropicalis	4/4	0/4
Candida dubliniensis	4/4	0/4
Candida krusei	5/5	0/5
Candida glabrata	8/8	0/8
Total (%)	60/60 (100%)	0/60 (0%)

TABLE 7: MINIMUM INHIBITORY CONCENTRATION OF ITRACONAZOLE ON CANDIDA SPECIES ISOLATED FROM VULVOVAGINAL CANDIDASIS PATIENTS IN ONITSHA, ANAMBRA STATE

	MIC (µg/ml)		
Candida species	**≤0.125 µg /ml (Susceptible)**	**0.25-0.5 µg/ml (Intermediate)**	**≥ 1µg/ml (Resistance)**
Candida albicans	19/39	8/39	12/39
Candida tropicalis	0/4	0/4	4/4
Candida dubliniensis	0/4	1/4	1/4
Candida krusei	2/5	1/5	2/5
Candida glabrata	2/8	3/8	2/8
Total (%)	26/60 (43%)	13/60 (22%)	21/60(35%)

DISCUSSION

The study compares the antifungal susceptibility patterns of 4 antifungal drugs against isolated *Candida species*. The antimicrobial susceptibility testing in this study revealed that most non albicans were resistant to Itraconazole. None of the candida isolates tested were resistant to Miconazole, Clotrimazole and Fluconazole, although susceptibility of 14% of the isolates to fluconazole was dose dependent (S-DD), and majority 7/8 (88%) of these (S-DD) were non-albicans Candida (*Candida glabrata* and *Candida krusei)*. The extensive use of Fluconazole leads to a shift in the causative agents of Candida infections to non-albicans species such as *C.glabrata, C.krusei* and *C.tropicalis* [10]. Although the efficacy of the drugs could not be established on the basis of the invitro susceptibility report since there was a degree of variability in the MIC values within the class of antifungals. Fluconazole, Miconazole and Clotrimazole appears to be the best choice, while Itraconazole due to its lower efficacy against non-albicans candida would be a poor choice in the blind treatment of vulvovaginal candidiasis in this locality as non-albicans are increasingly important participant in vulvovaginal candidiasis in this locality.

A recent study by Kiguli et al.,[11] described C. krusei with a high resistance of 71.43% to fluconazole whereas *C. glabrata*, and *C. krusei* exhibited 100% resistance and *C. albicans exhibiting 20.59%* to itraconazole. Resistance to clotrimazole was observed in 36.67% and 0.61% of *C. glabrata* and *C. albicans* respectively. Resistance to clotrimazole and fluconazole does not correlate with our study that revealed 0% resistance respectively by all *Candida species*. While, resistance to itraconazole by *C.glabrata, C.krusei* and *C.albicans* partially agrees with reports from our study which revealed 20%, 40% and 30.8% resistance to itraconazole by *C.glabrata, C.krusei* and *C.albicans* respectively.

Dharmik *et al*., [12] revealed that fluconazole was highly effective against *Candida Species* (97.2 %) while, the highest resistance was observed in the case of miconazole (63 %). This is contrary to our study that observed highest resistance to itraconazole (35%).

Resistance to itraconazole was observed in 16.2% (MIC ≥ 1 µg/ml). Among different species, elevated fluconazole MICs (≥16 µg/ml) were only observed in *C. glabrata* (15.2% resistant [R], 51.8% susceptible-dose dependent [S-DD]), and *C. krusei* (50% S-DD, 41.7% R, considered intrinsically fluconazole resistant). Resistance to itraconazole was observed among *C. glabrata* (74.1%) and *C. krusei* (58.3%). These results by Richter *et al*., [13] support that of this study which showed resistance to itraconazole ((MIC ≥ 1 µg/ml) among *C. glabrata* (20%) and *C. krusei* (40%). Furthermore, supports elevated fluconazole MICs (16-32 µg/ml) observed more frequently among *C.glabrata*, *C.krusei, C.tropicalis and C.albicans*. Thus, fluconazole resistance among vaginal *Candida albicans* isolates is emerging.

Comparison of symptomatic relief and mycological cure rate at second week after treatment with Fluconazole, Miconazole, Clotrimazole and Itraconazole shows that the rate of mycological cure was significantly more than the symptomatic relief ($t=6.91$; $p < 0.05$). The time required by the body to recover after the annihilation of the infecting organism could be a contributing factor. Hence, the use of azoles for empirical therapy of uncomplicated Vulvovaginal candidiasis is recommended.

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