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Antifungal Susceptibility Pattern of *Candida species* among Female Patients Attending Murtala Mohammed Specialist Hospital Kano, Nigeria

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Abstract: *Antifungal drugs such as Clotrimazole, Itraconazole, Ketoconazole and Fluconazole are the main drugs of choice being used in the treatment of candidiasis at contemporary level. The present study was conducted with the aim of isolating Candida species and determining their susceptibility pattern against selected antifungal drugs. High clinical vaginal swabs were the specimens used for isolation of Candida species. 150 specimens from both symptomatic and asymptomatic female patients of suspected vulvovaginal candidiasis (VVC) were collected and subjected to culture followed by microscopy analysis. 39 samples were found positive on culture out of which, only 24 were Gram positive for Candida species recording to about 61.54%. The disc diffusion method was employed for the determination of susceptibility of Candida species against the antifungal drugs. Highest susceptibility was observed with Fluconazole, followed by Ketoconazole, and then Itraconazole. The least sensitivity was observed with Clotrimazole, and the sensitivity was found to be more effective at 30 µm/disc concentration. Thus, the susceptibility of the fungal isolates was dose dependent. However, the susceptibility results showed that there is emerging fungal resistance to azoles, and Fluconazole is still the most effective and drug of choice in the management of vulvovaginal candidiasis.*

Keywords: *Candida species; Gram staining; Antifungal drugs; Susceptibility; Vaginal swab*

INTRODUCTION

Currently, the clinical spectrum of fungal infections is exhibiting a new but disturbing trend, with increasing prevalence and incidence rates, as well as resistance to commonly used antifungal drugs. Over the last decade, there also has been a rise in the number of candida infections that are attributable to non-*candida albicans* species (NACs). *Candida* infections are caused by an over proliferation of candida species. These are normally found in the normal micro biota of 3 different structures within the human body; the oral cavity, gastrointestinal tract and the vagina [1]. These yeasts are responsible for various clinical manifestations from *mucocutaneous* overgrowth to blood-stream infections [2].

Vaginal inflammation is the main reason for vaginal discharges [3]. Inflammatory responses in the vagina could arise due to variety of infections. It can be fungal infection due to *candida* species (candidiasis) and *Tricophyton* species in low percentage or parasitic due to *Trichomonus vaginalis* or bacterial infection [4, 5, 6, 7]. Vaginal c*andidiasis* is a common gynaecological finding among females worldwide [8, 9]. Reports have emerged indicating that well up to 75% of sexually active women have at some point experienced symptomatic vaginal *candidiasis* [10]. Risk factors associated with vaginal *candidiasis* include elevated estrogen, diabetes mellitus, use of antibiotics, and use of oral contraceptives, hormone replacement therapy and immune suppression among others [11, 12]. Several reports have recently indicated a close relationship between HIV and vulvoginal *candidiasis* in sub-saharan Africa [13]. In the Nigerian context, Nwosu in a study on patients with AIDS from 3 private medical laboratories reported vaginal *candidiasis* in 34.8% as the most common genital infection [14]. A limited number of antifungal agents including azoles (*imidazoles* and *triazoles*) and polyenes (*amphotericin B and nystatin*) are used for the treatment of *candida* vaginitis, and strategies and drug combinations may be useful to VVC therapy [11].

More than seventeen species are known to cause infections in humans; with over 90% of invasive infections being due to *C. Albicans, C. Glabrata, C. Parapsilosis, C, Tropicalis* and *C. Krusei* [15]. Each of these organisms has unique virulence potentials, antifungal susceptibility, and epidemiology. Increase in the number of immunocompromised individuals has lead to a rise in the use of intravenous catheters, total parenteral nutrition, invasive procedures, use of broad spectrum antibiotics cytotoxic therapies, and transplantation, all of which have directly lead to a rise in *candida* infections. Most recently, reports have surfaced of increased resistance to anti-fungal drugs by several yeasts, as well as a steep rise in *candida* infections (Ortega *et al.*, 2011).

In 1997, the National Committee for Clinical Laboratory Standards (NCCLS), published standard guidelines for antifungal susceptibility testing of *Candida* species to *amphotericin B, flucytosine, fluconazole, intraconazole*, and *ketoconazole*. Although the methods were standard, they were time consuming difficult to interpret, and appropriate only for testing limited organisms and drugs. Modifications to the methods and alternative approaches have been proposed to make the tests more convenient and efficient, applicable to a greater number of species, and appropriate for performing in the clinical laboratory [16]. Rapid, reliable identification of *Candida* to species level is now needed more than ever in susceptibility testing. *In vitro* testing has revealed that there are clear differences among the various *Candida* species in their susceptibility to specific drugs. Due to an increasing array of systemic antifungal agents, there is need for accurate, reproducible and predictive susceptibility testing of fungal isolates in order to help the clinician in decision making in term of choice of antifungal treatment [17].

The irrational and increased use of antifungals in recent years has resulted in the development of resistance to such drugs. The significant clinical implication of resistance has led to a heightened increase into its research from different angles [18]. The clinical consequence can be seen in failure of therapy in patients and also in the increased prevalence of *candida species* causing disease. Evidence of emerging azole resistance exists especially in patients empirically treated with fluconazole [19]. Thefore, routine susceptibility tests are essential for informing the physicians of the emergence of resistance strain to commonly used antifungal drugs and hence, the need to modify standard treatments. They also represent means of predicting therapeutic concentrations of antifungal drugs used to treat a variety of fungal infections [20]. Thus, guides the clinicians in selecting the best antifungal agent for an individual patient. Following the trend of resistance to commonly used antifungal agents, susceptibility testing will thus determine if resistance has emerged to these agents/drugs. Therefore, the present study was aimed at isolating *Candida species* and determining their susceptibility pattern against selected antifungal drugs.

MATERIALS AND METHODS

Study Design

The study is laboratory based and of cross-sectional design. Samples were collected at the Murtala Mohammed specialist hospital Kano, via high vaginal swab (HVS). All isolates of *Candida* were processed and their susceptibilities determined at the laboratory of the same hospital.

Study area and Sample collection

The study site is the laboratory of Murtala Mohammed Specialist Hospital Kano, Nigeria. High vaginal swabs were collected from patients reporting to the Microbiology Laboratory at Murtala Mohammed Specialist Hospital with request forms. High vaginal swabs were aseptically collected with the aid of sterile swab sticks as described by Van Dycke *et al.*, [21]. The samples of the vaginal fluid were obtained by scrapping the vaginal walls with a sterile cotton swab. A total of 150 samples both from symptomatic and asymptomatic female patients of suspected vulvovaginal candidiasis (VVC) were obtained, yielding only 39 positive isolates of candida. All 39 isolates were utilized in the study.

Culture technique

A culture of the yeast isolates (*Candida* species) was obtained using streaking method of inoculation as described by Cheesbrough [22] and Tortora *et al.*, [23]. The swab sticks containing the high vaginal swabs obtained from the hospital were streaked onto the plates of Saboraud Dextrose Agar (SDA) treated with antibiotic; Chloramphenicol to rid the medium of bacterial contaminants. The plates were incubated at 37 ^{o}C for 24 hours. Small amounts of the fungal colonies were smeared on glass slides and Gram stained for examination under the microscope.

Microscopy analysis

The swab samples obtained from the vaginal discharge were covered with a cover slip on a glass slide and microscopically examined using x400 magnifications for the examination of macro/ microconidia of yeast cells as demontrated by [24].

Bioassay procedure for susceptibility testing

Sensitivity disc of 6mm in diameter were punched out of Whatman's No. 1 filter paper and 100 disc were put in Bijou bottles, sterilized and kept for further use. 1mg (10,000µg/ml), 2mg (20,000µg/ml), 3mg (30,000µg/ml) of the antifungal agents (*Ketoconazole, Clotrimazole, Fluconazole* and *Itraconazole*) were prepared using sterile deionized distilled water as the diluent. These were dispensed in the Bijou bottles containing the filter paper disc and kept at 4^{o}c in the refrigerator for further analysis. The isolates positive for *candida* were subculture onto the prepared SDA and pour plating was done. The antifungal antibiotic discs were aseptically placed onto the inoculated culture media using sterile forceps and incubated at 37 ^{o}C for 24 hours. The zones of inhibition were finally recorded.

RESULTS

Based on cultural and morphological characteristic of 150 samples analyzed, 13 (26%) were found positive with mucoid and cream coloration, while 37 (74%) samples were negative showing no morphological characteristics of *Candida* as shown in Table 1.

Table 1: *Shows number and occurrence (%) of Candida isolates*

Isolates	Morphological characteristic Appearance	Colour	percentage occurrence (%)
39 (Positive)	Mucoid	Creamy	26
111 (Negative)	Powdered	Black	74
Total			100

Microscopic analysis of the 39 samples showing positive morphological characteristics of *Candida* revealed 24 Gram-positive for *Candida* species recording to about 61.54%, and 5 Gram-negative with a percentage of 38.50 % as shown in Table 2.

Table 2: *Shows Gram staining reaction of fungal isolates using microscopy*

Gram reaction	No. of samples observed	Percentage Occurrence (%)
Positive	24	61.54
Negative	15	38.50
Total	39	100

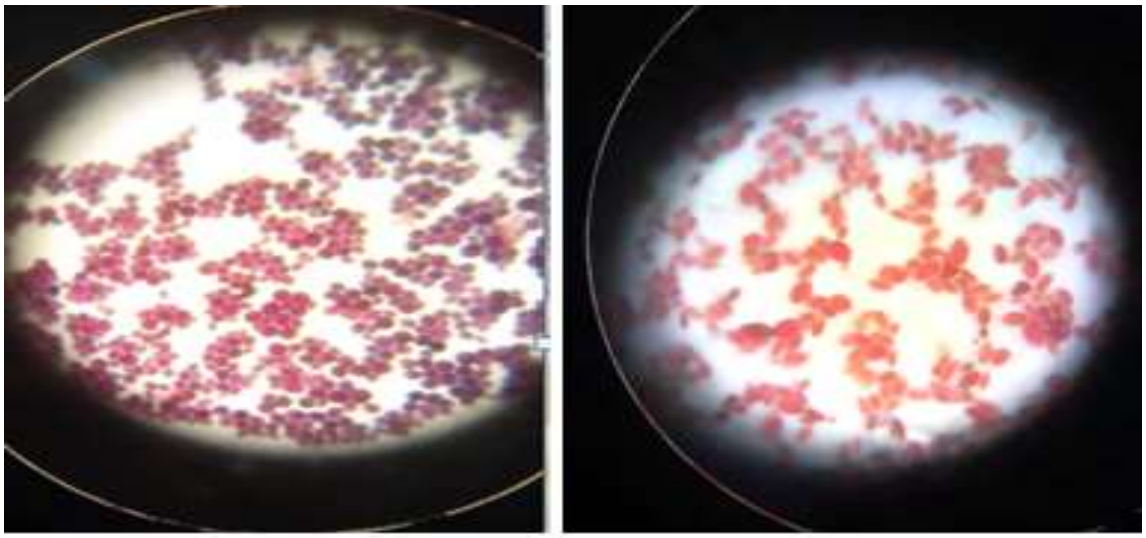

Figure 1: *Gram's staining observation under the microscope for Candida isolates identified*

Table 3: *Susceptibility pattern of Candida isolated to different antifungal drugs*

S/N	Antifungal Drug	No. of sample/ Percentage occurrence (%)							
		Susceptible		Intermediate		Resistant		Total	
1.	Ketoconazole	15	63%	6	25%	3	12%	24	100%
2.	Fluconazole	18	75%	0	0%	6	25%	24	100%
3.	Clotrimazole	6	25%	12	50%	6	25%	24	100%
4.	Intraconazole	15	63%	6	25%	3	13%	24	100%

The susceptibility pattern of *Candida* isolates to ketoconazole indicates 15 samples (63%) were highly susceptible, 6 (25%) intermediate, while 3 (12%) was resistant. For fluconazole, 18 samples (75%) were highly susceptible, none were intermediate, while 6 (25%) were resistant. For, clotrimazole, 6 samples (25%) were highly susceptible, 12 (50%) intermediate, while 6 (25%) were resistant. And for itraconazole, 15 samples (63%) were highly susceptible, 6 (25%) were intermediate, while 3 (13%) was resistant.

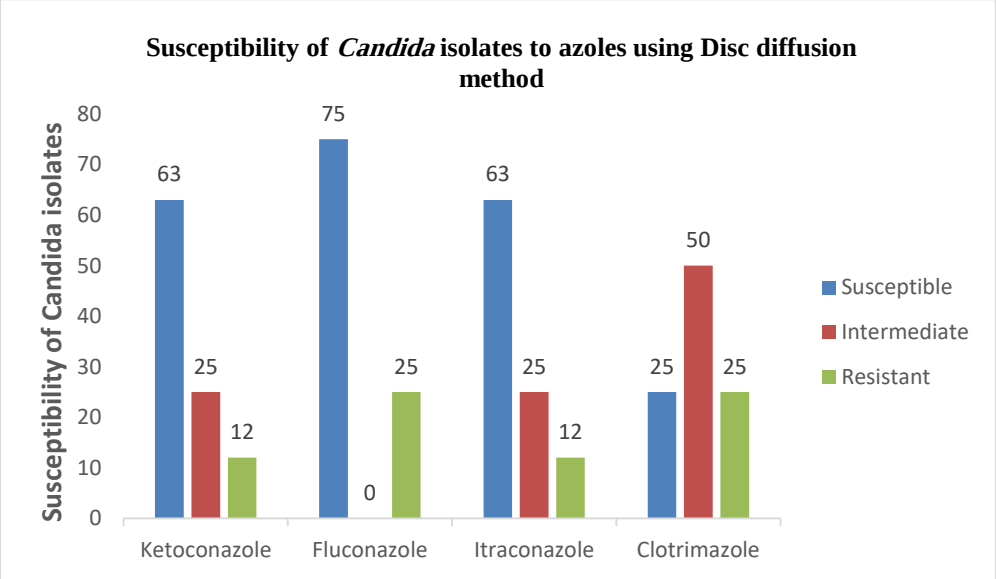

Figure 2: *Graphical representation of susceptibility profile of Candida isolates to azole antifungals.*

DISCUSSION

Between the ages of puberty and young adulthood, many factors may predispose a young female to viginal discomfort, of which VVC continues to be considered the most common cause of vaginitis amongs young women. Gram staining technique was carried out as a preliminary test before identification to ensure that the archived *candida* species were viable and free of contamination. Culture on SDA was conducted to obtain grown colonies that could be used for identification and later susceptibility testing. Imidazoles have been effective in the treatment of mycotic infections over the past years but emergence of resistance is continually increasing [25]. Thus, there is a big challenge in the future treatment of fungal infections which makes it necessary for constant monitoring of antifungal drugs to detect further emergence of resistance. Modifications to the NCCLS method in term of the drugs characteristics such as solubility, incubation time, buffer and glucose concentration, pH, inoculum size and endpoint determination has been shown to affect susceptibility results [26].

Culture on SDA is the gold standard method for the diagnosis of vulvovaginal candidiasis. Out of 150 samples obtained from both symptomatic and asymptomatic patients, 39 samples were found positive on culture out of which, only 24 were Gram positive for *Candida* species recording to about 61.54% which represents only 8% prevalence rate. However, this finding is not comparable with those of earlier studies which reported rates ranging from 20.8 to 23% in India, respectively [27, 28]. Moreover, in Edo state Nigeria, a prevalence of 49% was documented (Howard, 1999). While another study among female students of the University of Jos showed a prevalence of 59.5% [29]. Differences in the prevalence of VVC, as compared with other studies, could be attributed to differences in geographical location and identification technique [30, 31].

Susceptibility testing has shown that for clotrimazole, there was resistance in only 6(25%) isolates, 12(50%) isolates were classified as intermediate while 6(25%) were susceptible. No information was available to classify the cases as either recurrent or as previously exposed to candidiasis. In the case of fluconazole, there was resistance in 25% (n=2) of the isolates, 0% (n=0) was intermediate while 75% (n=6) were susceptible. In the case of Itraconazole, 12%(n=1) was resistant, 25%(n=2) were intermediate and 63% (n=5) were susceptible. Ketoconazole on the other hand showed susceptibility in 5(63%) isolates, intermediate in 2(25%) and showed 1(13%) resistance. Therefore, highest susceptibility was observed with Fluconazole, followed by Ketoconazole, and then Itraconazole. The least sensitivity was observed with Clotrimazole, and the sensitivity was found to be more effective at 30µm/disc concentration and thus, the susceptibility of the fungal isolates was dose dependent.

CONCLUSION

Based on the results obtained, 39 samples were found positive on culture out of which, only 24 were Gram positive for *Candida* species recording to about 61.54%. Highest susceptibility was observed with Fluconazole and the least was observed with Clotrimazole. Vulvovaginal candidiasis (VVC) presents both a diagnostic and therapeutic challenges, and inappropriate management may result in the progression to complicated vaginitis, resulting due to inadequate treatment. Enhancement of antifungal resistance may develop, leading to co-infections with microorganisms which could lead to infertility in females. Therefore, based on the results of emerging fungal resistance to azoles in this study, Fluconazole is still the most effective and drug of choice in the management of vulvovaginal candidiasis.

RECOMMENDATIONS

Further investigations on fungal resistance against various azoles using various susceptibility methods are highly recommended. Development of new drugs with different mechanisms of action from those of the azoles is very important to improve the treatment of the drugs. Expansions of the spectrum of activities of the current antifungal drugs to cover a wide spectrum of *candida* infection should be implemented.

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AUTHOR CONTRIBUTIONS

All authors contributed toward data analysis, drafting and critical revision of the paper and agreed to be accountable for all aspects of the research.

CONFLICTS OF INTEREST

None declared.

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