ANTIFUNGAL SUSCEPTIBILITY PATTERN OF SOME CLINICAL ISOLATES OF *CANDIDDA ALBICANS*

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Summary

Antifungal susceptibility pattern of some clinical isolates of *Candida albicans* were investigated from 82 urine (25 from males, 57 from females) and 42 high vaginal swab (HVS) samples. All the urine samples from males did not yield any *C. albicans* isolates, 8 samples from female urine and 6 samples from high vaginal swab samples yielded isolates of *C. albicans.* Antifungal susceptibility tests of the isolates showed that only 3 (37.5%) of the isolates were susceptible to the antifungal agents tested with the overall of 78.57% of all the *candida albicans* isolates resistant to fluconazole, ketokunazole and nystatin. There is evidence that antifungal resistant strains of *C. albicans* exist in the environment, which therefore highlights the need to conduct a wider survey with the aim of determining rate of resistance for possible development of effective control strategies.

Introduction

Opportunistic fungal infections resistant to antifungal agents have been increasingly documented in recent years and their frequency will likely continue to increase (1,2). This phenomenon appears due largely to the extensive use of antifungal agents to treat fungal infections that typically occur in severely immunocompromised and/or critically ill patients. *Candida* spp., *Cryptococcus neoformans*, and *Aspergillus* spp. are among the leading fungi responsible for these invasive infections (3). While antifungal resistance has been described with each of these fungi, resistance among *Candida* constitutes by far the most significant problem (4). *Candida* yeasts are commonly present in humans, and their growth is normally limited by the human immune system and by other microorganisms, such as bacteria occupying the same locations (niches) in the human body (Mulley and Goroll, 2006). *Candidda albicans* (Phylum-Ascomycota, family-Saccharomycetaceae) though a commensal among the human mouth and gastrointestinal tract flora of 80% of individuals (5,6,7), causes a wide variety of clinical diseases (8). Candidiasis is a very common cause of vaginal irritation, or vaginitis, and can also occur on the male genitals (9). In immunocompromised patients, *Candida* infections can affect the esophagus with the potential of becoming systemic, causing a much more serious condition, a fungemia called candidemia (10). Oral infections by Candida species usually appear as thick white or cream-coloured deposits on mucosal membranes. The infected mucosa of the mouth may appear inflamed (red and possibly slightly raised). In babies the condition is termed thrush.

Children, mostly between the ages of three and nine years of age, can be affected by chronic mouth yeast infections, normally seen around the mouth as white patches. Adults may experience discomfort or burning. Risk groups include newborn babies, diabetics (with poorly controlled diabetes), as a side effect of medication, most commonly having taken antibiotics (11). Other factors include inhaled corticosteroids for treatment of lung conditions (e.g, Asthma) may also result in oral candidiasis, People with an immune deficiency (e.g. as a result of AIDS/HIV or chemotherapy treatment), women undergoing hormonal changes, like pregnancy or those on birth control pills, people with fresh oral piercings coming into regular contact with yeast, denture users and smokers.

Most candidial infections are treatable and result in minimal complications such as redness, itching and discomfort, though complication may be severe or fatal if left untreated in certain populations (8). In immunocompetent persons, candidiasis is usually a very localized infection of the skin or mucosal membranes, including the oral cavity (thrush), the pharynx or esophagus, the gastrointestinal tract, the urinary bladder, or the genitalia (vagina, penis) (10,12,13).

Systemic candidal and other fungal infections (fungemiasis) have emerged as important cause of morbidity and mortality in human immune compromised patients (such as AIDs, cancer, organ or bone marrow transplant patients) and addition, hospital related infections in patients not previously considered at risk (e.g patients in an intensive care unit) have become a cause of major health concern (14). In clinical settings, candidiasis is commonly treated with antimycotics - the antifungal drugs commonly used to treat candidiasis are topical clotrimazole, topical nystatin, fluconazole, and topical ketoconazole. In severe infections (generally in hospitalized patients), amphotericin B, caspofungin, or voriconazole are employed (15). However antifungal treatment has been undermined with rising incidences of antifungal resistance amongst *Candida* species. Candidiasis that fails to respond to treatment has been increasingly reported even among people with no prior exposure to flucunazole drugs. This is partly due to the widespread, long-term use of azoles for treating and preventing candidiasis (16). Other factors include treatment with anti-TB drugs, ciprofloxacine and CD4 cell counts below 50. Resistance to azole drugs was often acquired using amphotericin B. While potent and effective, amphotericin B is toxic, especially to the kidney. Newer versions such as ABLC, Ambisome and Ambelect have proven less toxic to the kidneys. Recent studies also showed that exposure to azole treatment decreases antifungal activity of amphotericin B.

Resistance to the newer systemic triazoles, itraconazole and fluconazole has been reported, in addition to resistance to the older azole, ketoconazole.. Currently, the most significant form of azole resistance is that seen between *Candida* and fluconazole. Both acquired resistance and intrinsic resistance are clinically relevant. Acquired resistance is most often seen with HIV-infected patients, where fluconazole-resistant oropharyngeal candidiasis (OPC) develops in patients with CD4+ T cell counts <100/mm and with a history of multiple episodes of OPC. Cross resistance to other azole antifungal agents is also a common phenomenon (17, 18, 19).

The importance of *C. albicans* in causing human diseases and the incidence of antifungal resistance requires that the organism be identified from clinical specimens and antifungal resistance pattern of such isolates investigated (20, 21). The recognition of antifungal resistance stresses the need for increased surveillance of the phenomenon among different isolates (21).

This work was therefore aimed at determining the antifungal susceptibility pattern of some clinical isolates of *Candida albicans.*

Materials and Methods

Sample Collection

Clinical urine and high vaginal swab (HVS) samples were collected from patients with complaints of urinary tract infections sent to the Microbiology Laboratory, Specialist Hospital Yola, Nigeria. The urine samples were collected in sterile bottles, while the HVS samples on cotton wool swabs. The samples were immediately transported to the Microbiology Laboratory of the Microbiology Department, Federal University of Technology Yola for microbiological investigation.

Media preparation

Sabouroud Dextrose Agar (SDA, Oxoid) was used for the purpose of this study. SDA was prepared according to manufacturers' specification. Briefly, 62 g of SDA powder was weighed and transferred into a conical flask and 1000 ml of distilled water added and allowed to soak for 5-10 min. The mixture was then swirled to mix and sterilized at 121 C, 15 Ib for 15 min. After sterilization, the media was allowed to cool to 45-47 C and then dispensed (18-20 ml) into Petridishes and allowed to solidify (18).

Isolation and identification of *Candida albicans*

For isolation of *C. albicans*, a loopful of urine sample was inoculated onto solidified and dried plates of SDA to make a pool, and then streaked in a zig-zag fashion towards the center of the plate. For HVS samples, the swab stick was used to make a pool on the SDA plate and a sterilized wire loop was used to make a streak as in the case of urine. The culture plates were then incubated at 37 C for 18-24 h. After incubation, soft, cream-coloured colonies with yeasty odour were picked and Gram stained (18).

Grams staining technique

This was carried out as described elsewhere (17). Briefly, 1-2 colonies of presumptive *Candida albicans* were smeared onto a slide with 1 drop of normal saline. The smear was air dried, heat fixed and allowed to cool. The smear was then covered with crystal violet stain and allowed for 1 minute. The stain was rapidly washed off, tipped off, and then covered again Lugol's iodine and allowed to stand for 1 minute. This was then rinsed with water and rapidly decolorized using acetone-alcohol for a few seconds and once again washed immediately with clean water. The smear was then covered with neutral red stain, allowed for 1 minute, washed off with water and allowed to dry. The dried smear was then examined under oil immersion at x100 magnification. Gram positive pseudohyphae or oval shaped large purple cells were identified as *Candida albicans.*

Germ tube test

This was carried out as earlier described (8). In this procedure, a small portion of , previously purified presumptive colony of *Candida albicans* on SDA earlier identified by Gram staining was inoculated into sterile test tubes containing 0.5 ml of blood serum using a sterile wire loop, and incubated in a 10% CO_2 jar for 1-2 h. At 10 min intervals during the incubation process, a

drop of the *Candida albicans*-serum mixture withdrawn using a sterile wire loop and placed on a clean microscope slide, covered with a cover slip and examined microscopically, using the x10 and x40 objective lenses. The appearance of small filaments projecting from the cell surface confirmed formation of germ tubes.

Preparation of antifungal discs

Filter paper discs were cut using paper puncher to give 4 mm disc sizes. The discs were soaked into solutions of three different antifungal agents (fluconazole, ketokunazole and nystatin that are commonly used in the treatment of candidiasis), in three different sets of test tubes to obtain drug concentrations of 2 mg/ml per disc. To obtain this concentration (2 mg/ml) of antifungal agent, 20 mg of the antifungal drug was soaked in 10 ml of distilled water in a test tube. 100 pieces of filter papers were then be added to the 10 ml of 2 mg/ml antifungal agent solution and allowed to soak for 15-30 min. The tubes with the discs were sterilized by autoclaving at 121 C, 15 Ib pressure for 15 min. After sterilization, the discs were transferred into sterile Petri dishes and dried in the oven at 45 C for 15-30 min. The dried discs were then stored in sterile bottles for further use (22).

Determination of antifungal susceptibility pattern of isolates

The disc diffusion method as described previuosly (18) was used for this purpose. Filter paper discs (4 mm diameter) impregnated with 2 mg/ml concentrations of the antifungal agents ketokunazole, flucunazole and nystatin were placed on SDA plates previously seeded with 2x 10^5 conidia/ml culture suspensions of *Candida albicans* isolates. Filter paper discs soaked in sterile distilled water without any antifungal agent was placed on the culture plate to serve as control. The culture plates were incubated at 37 C for 24 h. Antimicrobial activity was determined by measurement of zone of inhibition around the test organisms. Absence of zone of inhibition was considered as resistant by the test against the antifungal agent.

Results and Discussion

Results of the total number of isolates from various samples collected and yield of *Candida albicans* isolates after culture on SDA are shown in Table 1. A total of 82 urine samples were collected (25 from males, 57 from females) and 42 high vaginal swab (HVS) samples were collected. Of the total samples collected, all the urine samples from males did not yield any *C. albicans* isolates, while of all the samples collected from females, 8 yielded isolates of *C. albicans* and 6 of the HVS samples were positive for *Candida albicans.* All the isolates obtained showed a Gram positive pseudohyphae or oval shaped large purple cells after Gram staining and small filaments projecting from the cell surface after the respective germ tube tests (results not shown).

Table 1. Number of *Candida albicans* isolates obtained from the urine and HVS.

S/No.	Total Number of Samples		Male (Urine Only)		Female			
					Urine		HVS	
	Urine	HVS	No. Positive	No. Negative	No. Positive	No. Negative	No. Positive	No. Negative
	82	42	-	25	8	49	6	36

- = no *Candida albicans* isolated

Out of all the 25 urine samples examined from males, non was positive for *Candida albicans,* while 8 of the 49 (16.3%) urine samples and 6 (17%) of the 42 HVS samples from females yielded *Candida albicans.* It has earlier been reported that, though candidiasis infects males occasionally, the condition is more common in females (9, 12). It is therefore not surprising that the male samples did not yield any *Candida albicans* isolates. On the overall 14 isolates of *candida albicans* were obtained from a total of 114 samples collected representing 12.28% of the samples. By sample type, prevalence rate of 14.04 % of *Candida albicans* in urine samples and 14.29% in HVS samples was recorded, this prevalence rate is matter of concern. It is simply an indication that the infection might be very common and thus a wider survey of incidence of *Candida* infections is required.

Result of antifungal susceptibility of the isolates are shown in Table 2. From the results, only 3 (37.5%) out of the 8 isolates from female urine (FU) and none of the 6 (0%) isolates from HVS were susceptible to the antifungal agents tested. This simply means that 62.5 and 100% of the isolates from urine and HVS respectively were resistant to the antifungal agents tested. Isolate FU 1 was only susceptible to fluconazole (10 mm diameter zone of inhibition), while FU2 to fluconazole (7 mm), ketokunazole (15 mm) and nystatin (5 mm zone diameter of inhibition). Isolate FU22 on the other hand was only susceptible to ketokunazole and flucunazole (7 and 5 mm zone diameter of inhibitions respectively). None of the isolates from HVS samples showed susceptibility to any of the antifungal agents.

Table 2. Percentage of samples positive for *Candida albicans* and their antifungal susceptibility pattern

Sample	No. collected	No. positive (%)	No. (%) susceptible/Resistant		Zone diameter of inhibition (mm)		
					Fluconazole	Ketokunazole	Nystatin
Female Urine (FU)	57	8 (14.04)	3 (37.50)/62.50	FU1	10	0	0
				FU2	7	15	5
				FU22	0	7	5
HVS	42	6 (14.29)	0(0.00)/100.00	0	0	0	0
Male Urine	25	0(0.00)	0(0.00)	0	0	0	0

Key: FU = Female Urine; H = HVS (High Vaginal Swab)

These antifungal agents are the commonly prescribed in the treatment of candidiasis and the high rate of resistance to these agents is simply an indication that in the near futre, they cannot be used in treating candidiasis among the patients (23). Resistance to fluconazole by *Candida albicans* isolates from clinical samples (urine) has earlier been reported by other workers (21), as well as low susceptibility profile to other triazoles (14). This therefore stresses the need to further investigate more clinical samples for such incidences. All together 78.57% of the *Candida albicans* isolates (form the two samples) were resistant to flucunazole, ketokunazole and nystatin. This development is indeed worrisome indicating high level of antifungal resistance amongst the isolates. Infected individuals may encounter treatment failures thus further complicating chemotherapy thereby aggravating prevailing conditions (23).

Conclusions

This study showed a reasonable degree of antifungal resistance to some antifungals by some clinical isolates of *Candida albicans.* The number of serious invasive fungal infections has continued to increase due to the fact that more immunosuppressed patients are at risk for these infections. Clearly the importance of clinical resistance continues to increase so that the injudicious use of antifungals should be avoided (16). Adequate dosing and targeted use of azole therapy will optimize the utility of these agents in the therapy of invasive fungal infections and will keep antifungal resistance a manageable clinical problem. Chemotherapy of candidiasis and other invasive fungal Infections based on laboratory diagnosis will curb the incidences of antifungal resistance.

There is need for the populace to be enlightened on the need for strict compliance to laboratory tests before the use of any given antifungal agent. More investigations on a wider range of samples to determine the susceptibility profile of different *Candida* isolates to a variety of antifungal agents should be carried out.

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