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A COMPARATIVE STUDY OF THE *IN VITRO* ACTIVITIES OF TWO ANTIFUNGAL AGENTS ON *CANDIDA ALBICANS*

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ABSTRACT

Candida albicans is the principal cause of candidiasis in women. A comparative study of the *in vitro* activities of two antifungal agents was carried out using clotrimazole and nystatin. Two hundred and fifteen (215) *Candida albicans* isolated from high vaginal swabs of different females with symptomatic cases of vaginal candidiasis were confirmed using Germ tube test. The minimal inhibitory concentrations (MIC) of the drugs on yeast isolates were determined by broth dilution susceptibility test and absorbance reading using spectrophotometer after 48 hours incubation at 27^{o}C to obtain IC_{10} and IC_{50} of the drugs. The results showed that the treatment of *Candida albicans* with selected sub-lethal concentrations of nystatin (250 mg/l) and clotrimazole (125 mg/l) respectively can induce a reversion to yeast growth known as drug resistance. Clotrimazole produced more therapeutic effect for the inhibition of some isolates with IC_{50} between 20.04 mg/ml and 17.42 mg/ml while nystatin produced more therapeutic effect for the inhibition of other isolates with IC_{50} between 10.73 mg/ml and 16.37mg/ml. This shows that 25% drug is required to inhibit the isolates by the drug of choice. Nystatin and clotrimazole showed different therapeutic effects on the isolates due to fungal, drug or host factors. Therefore, adequate antifungal susceptibility testing should be performed on individual basis to ascertain the drug of choice before prescription.

KEYWORDS: Antifungal drugs, *Candida albicans*, susceptibility test.

INTRODUCTION

Vulvovaginal candidiasis also known as vaginal thrush is one of the most common gynaecological disorders among females of reproductive age caused by mainly *Candida albicans* whichis part of the commensal flora of more than half of the healthy population. However, this yeast might become pathogenic when the normal environment of the vagina changes as a result of several precipitating factors (Thevissen, 2005).

Different solutions exist for the treatment of vulvovaginal candidiasis which include the boosting of immune system with nutrient-rich diet and probiotics, the use of antifungal creams, vaginal pessaries and oral medication (Moosa *et al.*, 2004). In clinical setting, yeast infections are treated with antifungal drugs, the choice of which depends on the site of infection and the immune status of the patient. The problem with the use of antifungal agents, apart from safety and cost is the development of drug resistant strains during treatment (Talaro and Talaro, 1996). The development of antifungal resistance among yeasts has been linked to misuse and inappropriate prescription of antifungal agents (Talaro and Talaro, 1996). Appropriate antifungal therapy depends upon a good knowledge of the agents causing the infections and their susceptibility patterns. The susceptibility of yeasts to antifungal agents cannot always be predicted and therefore testing individual yeast pathogens against the appropriate antifungal agents is often necessary. Antifungal susceptibility testing *in vitro* ensures that the drug that will be chosen will be active against the infecting organism and therefore provide beneficial therapeutic effect to the patient under treatment. Antifungal susceptibility testing also aids in drug development studies and as a means of tracking the development of antifungal resistance in epidemiologic studies (Rex and Pfaller, 2002). It was not until recently that the National Committee for Clinical Laboratory Standards (NCCLS) published its guidelines for a standardized broth macro- and micro dilution assay for *in vitro* testing of antifungal susceptibilities. The recognition of yeasts as agents of opportunistic infections, the chronic nature of diseases they cause, their unpredictable susceptibility patterns with the resultant increase of treatment failure and related costs make close monitoring of antifungal agents prudent (Spellberg *et al.*, 2006).

This study reports on a comparative assay of the *in vitro* activities of two antifungal agents on *Candida albicans* from high vaginal swabs.

MATERIALS AND METHODS

Description of Sample Location

The vaginal swabs were collected aseptically from female patients confirmed with cases of vulvovaginitis at Saint David's hospital in Owerri, Imo State, Nigeria. Specimen from the swabs were labelled appropriately, transported in an ice chest to Anthony Van Leeuwenhoek Research Centre, Nekede, Imo State, Nigeria, and stored in a refrigerator maintained at 4^{o}C prior to assay.

Preparation of sample

The refrigerated swab samples were allowed to warm to room temperature. The inocula were spread on freshly prepared and surface dried Sabouraud's Dextrose Agar (SDA) and Malt Extract Agar (MEA) to ensure even distribution before incubating the plates. The inoculated plates were incubated at room temperature (28 ± 02^{O}C) for 48 hours for the growth of yeast cells. Pure cultures were stored on SDA agar slant and kept in the refrigerator prior to characterization, identification and antifungal susceptibility test.

Morphological Characterization

Isolates were characterized based on colonial features presented on the culture plates. Each isolate was further subjected to microscopic characterization and confirmatory test using the Germ tube method (Saravan *et al.,* 2010).

Gram Staining (Microscopy)

Smears of the isolates obtained from high vagina swabs were prepared on the microscope slide and allowed to air-dry quickly. The smears were heat-fixed and flooded with crystal violet solution for one minute and then rapidly washed off with clean water. The smear was flooded with Lugol's iodine for one minute and washed off. After washing, the smears were decolorized with 95% ethanol and washed off immediately with clean water after which they were counter-stained with aqueous basic Fuchsin for 2 mins. The slides were air-dried and subjected to observation under the microscope using 40x objective and oil immersion objective lens 100 x (Saravan *et al.,* 2010).

Germ Tube Test

Germ tube test also known as the Reynolds-Braude phenomenon is a confirmatory test which differentiates vagina *Candida albicans* from other *Candida* spp. 0.5mls of human serum was pipetted into a small test tube. Using a sterile wire loop, the serum was inoculated with a yeast colony from the culture plate and emulsified. The test tube was incubated at 37^{O}C for 3 hours. Using a Pasteur pipette, a drop of the serum yeast culture was transferred to a glass slide, and covered with a cover slip. The preparation was examined using 10x and 40x objectives for sprouting yeast cells presented as tube-like outgrowths or germ tubes (Saravan *et al.,* 2010).

Preparation of 0.5 McFarland Standard

One gram (1g) of barium chloride was dissolved in 99 ml of water to obtain 1% Barium Chloride. 1ml of H_2SO_4 was obtained from stock bottle (1% H_2SO_4). 0.5 McFarland standard was prepared by mixing 0.05 ml of 1% Barium Chloride dihydrate ($BaCl_2.2H_2O$), with 9.95 ml of 1% Sulfuric acid (Saravan *et al.,* 2010).

Preparation of Stock Solutions

The solutions of vaginal tablets (nystatin and clotrimazole) were prepared taking into account the potency of the powders. The amount of powder required to prepare the standard solutions was calculated as follows:

$$\text{Weight (g)} = \frac{\text{Volume (L)} \times \text{Concentration (mg/L)}}{\text{Potency (mg/g)}}$$

$$\text{Volume (L)} = \frac{\text{Weight (g)} \times \text{Potency (mg/g)}}{\text{Concentration (mg/L)}}$$

The nystatin and clotrimazole were aseptically crushed to powder and weighed on an analytical balance that has been calibrated to two decimal places. Two grams of each powder was weighed using an analytical balance and dissolved in 20 ml of distilled water each to obtain stock solutions with concentrations of 100mg/ml. The drug stock solutions were filtered using whatman filter paper (110mm) to remove particles and obtain clear filtrates used for the dilution susceptibility test vaginal tablets (Anhalt and Washington, 1991).

Broth Dilution Susceptibility Test

This test was used to determine the minimal inhibitory concentration values of the antifungal drugs (nystatin and clotrimazole). Peptone broth was prepared following the standard preparation procedure. 100mg/ml of the antifungal drugs (nystatin and clotrimazole) as stock solutions were prepared and diluted with distilled water of different volumes in line with the dilution law; $C_1V_I = C_2V_2$ to obtain graded concentration of 40mg/ml, 30mg/ml, 20mg/ml, 10mg/ml, 5mg/ml and 0mg/ml (negative control) respectively. The yeasts inocula were standardized using 0.5 MacFarland standard (Matar *et al*., 2003). A constant volume of standardized yeast inocula (0.1 ml) and constant volume of peptone broth (0.4 ml) were added to the different stock concentrations of nystatin and clotimazole and mixed thoroughly on an electronic shaker. The same protocol was followed to obtain different volumes of water and stock with constant volumes of inoculum and medium to obtain a final volume of 4 ml. The inoculated test tubes were incubated at room temperature (28 ± 02^{O}C) for 48 hours. The amount of growth in the tubes containing nystatin and clotrimazole was determined spectrophotometrically and compared to the amount of growth in the control tubes. The lowest concentration at which the yeast isolates were completely inhibited (absence of visible growth) was recorded as the minimal inhibitory concentration (MIC).

Protocol Table for the Serial Dilution of Nystatin and Clotrimazole

Concentration (mg/ml)	0	5	10	20	30	40
Volume of inoculum (ml)	0.1	0.1	0.1	0.1	0.1	**0.1**
Volume of medium (ml)	0.4	0.4	0.4	0.4	0.4	**0.4**
Volume of stock (ml)	0	0.2	0.4	0.8	1.2	**1.6**
Volume of water (ml)	3.5	3.3	3.1	2.7	2.3	**1.9**
Final volume (ml)	**4**	**4**	**4**	**4**	**4**	**4**

RESULTS

Table 1 shows the morphology of *Candida albicans* isolated on Sabourau d's Dextrose Agar. The isolates grew luxuriantly and presented an oval shaped, cream coloured, raised elevation, round margined with smooth edged colonies. Microscopically, they are Gram positive oval and ellipsoidal clusters and 4-6 µm in diameter. Figure 1 shows the cultural morphology of *Candida albicans* growing on SDA medium presenting a creamy butyrous colonies.

Table 1: Morphology of *Candida albicans* isolates cultured on Sabouraud's Dextrose Agar.

Size	**4-6 µm**
Shape	**Oval**
Colour	**Cream**
Margin	**Entire**
Edge	**Smooth**
Elevation	**Raised**
Appearance	**Yeast-like**
Colony arrangement	**Grape-like clusters**

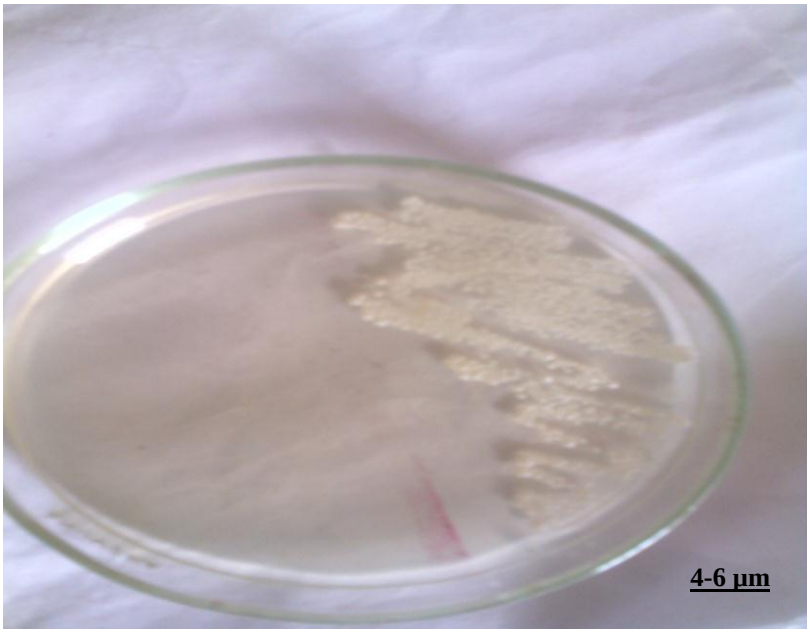

Figure 1: Morphology of *Candida albicans* on Sabouraud's Dextrose Agar.

Figure 2 show the germ tubes of *Candida albicans* under the microscope which was confirmed by the presence of slender, tube like extensions or non-septate germinating hyphae (germ tubes), arising laterally from a yeast cell, with no constriction at the point of origin. They are ½ the width and 3-4 times the length of the cell from which they arise.

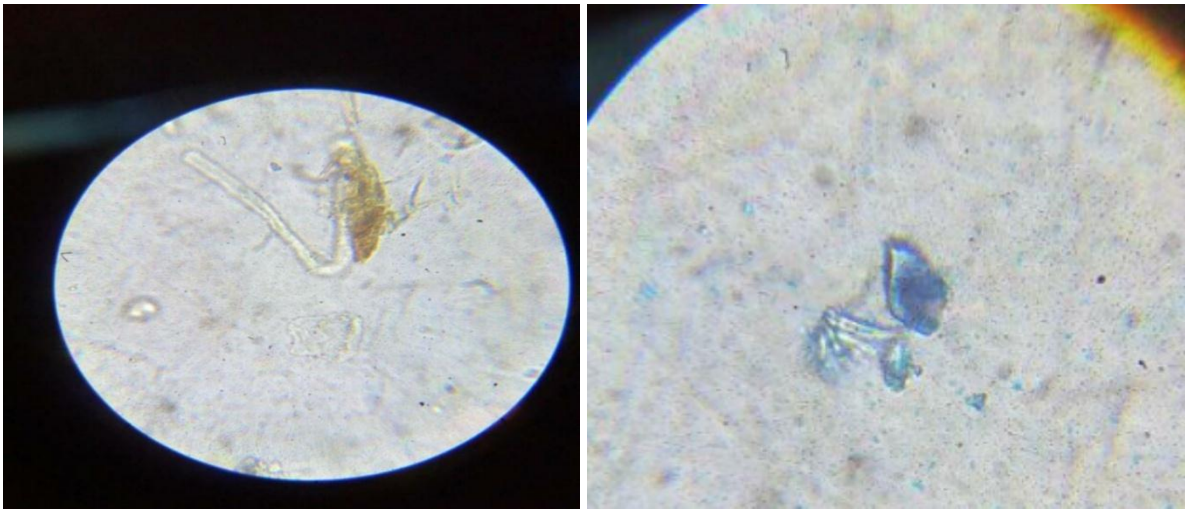

Figure 2: Germ Tubes of *Candida albicans* under the Microscope.

Table 2 shows linear equations obtained from linearized graphs of drug concentrations against percentage inhibitory concentrations (IC) for each sample using the formula Y= mx ± C. The IC_{10} (concentration that inhibited 10% of *Candida albicans*) and IC_{50} (concentration that inhibited 50% of *Candida albicans*) were calculated using the corresponding equations. From the table, a negative IC signifies stimulatory response (that is, growth of the isolate was stimulated at the concentration) while a positive IC signifies (that is, growth of the isolate was inhibited at the concentration).The drug of choice is determined from the table.The lower the IC_{50}, the more the therapeutic effective of the drug on each isolate. Clotrimazole produced more therapeutic effect for the inhibition of isolates in B and D category, with IC_{50} of 20.04mg/ml and 17.42mg/ml respectively while nystatin produced more therapeutic effect for the inhibition of isolates in category A, C and E with IC_{50} of 10.73mg/ml, 10.16mg/ml, and 16.37mg/ml respectively.

Table 2: IC_{10} and IC_{50} of Clotrimazole (C) and Nystatin(N) on *Candida albicans* isolates.

Sample Code	Linear Equation	R^2	IC_{10}	$1C_{50}$
EN	Y=2.1195X + 15.2977	0.9058	-2.4995	16.3728
EC	Y=1.6184X + 11.3437	0.9215	-0.8302	23.8855
DN	Y=1.8421X + 2.17	0.9338	4.2505	25.9649
DC	Y=2.1346X +12.805	0.9379	-1.3140	17.4248
BN	Y=1.8024X + 1.7178	0.9443	4.6012	26.7877
BC	Y=2.3658X + 2.5704	0.9422	3.1404	20.0480
CN	Y=3.3707X + 15.7282	0.9429	-1.6994	10.1675
CC	Y=2.4313X + 0.1371	0.9943	4.0566	20.5087
AN	Y=4.7267X – 0.7189	0.9879	2.2675	10.7302
AC	Y=1.9834X – 3.4424	0.9905	6.7774	26.9448

IC10= 10% Inhibitory concentration of test organism, IC50= 50% Inhibitory concentration of test organism, R^2 = Degree of fit

Y= mx ± C, Y = % inhibition, x = Concentration, m = Coefficient, C = Constant

Sample codes A, B, C, D, E = Isolates, C= Clotrimazole, N= Nystatin

DISCUSSION

With the rising frequency of candida vaginitis, as well as the increase in resistance to antifungal agents, it is imperative that clinical applicable antifungal susceptibility testing should be carried out on the isolates of *Candida albicans*. Over the years, clotrimazole and nystatin have been widely prescribed for the treatment of vaginal candidiasis (Pavel *et al*., 2005; Arikan *et al*., 2002). Nystatin is a polyene antifungal agent, discovered in the late 1950s and was isolated from the bacterium, *Streptomyces noursei*. Its mode of action involves binding to the main component of fungal cell membrane, the ergosterol and result in the formation of transmembrane channels that allow the leakage of cell contents along with K+ and Na+ ions leading to the damage and death of the fungal cells (Sanglard and Odds, 2002). Clotrimazole is a member of the azole family which disrupts the cell membrane by inhibiting the activity of the lanosterol 14- -demethylase enzyme involved in the biosynthesis of ergosterol (Hof, 2006). In my study, antifungal susceptibility testing was carried out by broth microdilution method and MIC using the spectrophotometer. Other studies showed the efficacy of nystatin and clotrimazole against *Candida* isolates using E-test, disk diffusion and well-diffusion methods (Matar *et al*., 2003).

Pietro *et al*. (2016), in their work determined the *in vitro* susceptibility testing of yeasts to nystatin and found low MIC values for nystatin against *Candida albicans*. The MIC of nystatin was in the range of 0.625 µg/ml after 24 hours spectrophotometric reading. Other yeast strains were also inhibited by nystatin *in vitro* at a comparable minimum inhibitory concentration ranging from 0.31µg/ml to 1.25µg/ml.

This result demonstrated that the antifungal drugs could develop inhibitory or stimulatory response to the isolates at IC_{10} and an inhibitory response at IC_{50} (half minimum inhibitory concentration). Negative values signify stimulatory response while positive values signify inhibitory response. This collaborates with the report of Calabrese and Blain (2005) which reported that hormesis response is a biphasic dose response characterized by low dose stimulation or beneficial effect and a high dose inhibitory effect.

The study also shows that nystatin and clotrimazole showed an inhibitory response at IC_{50} of 16.3725 mg/ml and 23.8855 mg/ml respectively for 'E' isolates. However, nystatin had a lower IC_{50} than clotrimazole and therefore is the drug of choice for inhibiting isolates in category 'E'.

Nystatin showed inhibitory response at IC_{10} (4.2502mg/ml) and IC_{50} (25.9649mg/ml for the isolates in category 'D'. However, with clotrimazole, the *Candida* isolates growth was stimulated at IC_{10} (-1.3140mg/ml) and inhibited at IC_{50} (17.4248mg/ml). For *Candida albicans* in the category, the drug of choice is clotrimazole which has lower MIC than nystatin for the isolate.

For isolates in category 'B', clotrimazole and nystatin showed inhibitory response at both IC_{10} (3.1404mg/ml, 4.6012mg/ml) and IC_{50} (20.0480mg/ml, 26.7877mg/ml) respectively. Clotrimazole has a lesser MIC value than nystatin and is therefore the drug of choice for inhibiting isolates in 'B' category.

Nystatin showed a stimulatory response at IC_{10} (-1.6994mg/ml) and inhibitory response at IC_{50} (10.1675mg/ml) while clotrimazole showed an inhibitory response at both IC_{10} (4.0566mg/ml) and IC_{50} (20.5087mg/ml) for *Candida* isolates in category "C". The drug of choice for the isolates is nystatin with a lower IC value.

For category isolates in 'A', nystatin and clotrimazole showed an inhibitory response for both IC_{10} (2.2675mg/ml, 6.7774mg/ml) and IC_{50} (10.7302mg/ml, 26.9448mg/ml) respectively. However, clotrimazole has a lower IC value and is therefore the drug of choice for inhibiting the isolates.

Generally, Clotrimazole produced more therapeutic effect for the inhibition of isolates in category B and D with IC_{50} of 20.04mg/ml and 17.42mg/ml respectively while nystatin produced more therapeutic effect for the inhibition of isolates in category A, C and E with IC_{50} of 10.73mg/ml, 10.16mg/ml, 16.37mg/ml respectively. This could be due to the fact that they have lower inhibitory concentrations than the other and requires lesser concentration of drug to inhibit the growth of the isolates. This shows that the drug of choice for each isolate differs with the physiology of the isolates.

The stimulatory response of clotrimazole and nystatin to some of the isolates signifies higher absorbanceleading to the cell growth. This is usually seen at low concentrations of IC_{10} while at higher concentrations of IC_{50}, nystatin and clotrimazole inhibited the growth of *Candida albicans*. Different isolates from different individuals respond in different ways to the presence of antifungal agents and factors surrounding them.

According to Theodore *et al*. (1998), factors that contribute to antifungal resistance include fungal factors (cell type, yeast or hyphae, switch phenotype, serotype, genomic stability of *Candida* strains, size of the population), drug factors (fungistatic nature of drugs, dosage, previous use of antibiotics, drug penetration and distribution) and host factors (immune status of host, site of infection, the severity of infection, presence of foreign materials like catheters, prosthetic valves and abscess formation).

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