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In-vitro Susceptibility of *Candida* Pathogens Isolated From Clinical Specimens to Available Antifungal Agents in Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author DOO conceived and designed the study protocol, drafted the article and was involved in revising it critically for important acceptability. Author OATA was part of the design from the onset, played a major role in critical review for important intellectual content and did the statistical analysis. Authors OSA and ASO were involved in acquisition of data including collection of assembly of isolates. All Authors were involved in interpretation of data and final approval of manuscript for submission.

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Case Study

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ABSTRACT

Aim: To characterize clinical isolates of *Candida* species from a tertiary hospital in South West Nigeria and also to determine their susceptibility to antifungal agents in order to guide in the course of empirical treatment of patients visiting hospitals in Nigeria.
Study Design: This was a cross sectional study.
Place and Duration of Study: Medical Microbiology and Parasitology Laboratory, LAUTECH Teaching Hospital, Osogbo, Nigeria. Study duration was Five (5) months.
Methods: One hundred and twenty-four *Candida* species obtained from various body sites were identified and speciated using conventional and analytical profile index (API) for *Candida* and the susceptibility to 5 antifungal agents was determined using disc and macrodilution methods.

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Results: Highest number of *Candida* was obtained from urine, 44 (35.5%); followed by High Vaginal Swabs (HVS), 32 (25.8%) and blood, 20 (16.1%). Highest frequencies were obtained from *C. Krusei* and *C. tropicalis*, 32 (25.8%) each, followed by *C. Albicans* and *C. pseudotropicalis*, 24 (19.4%) each, and *C. gulliermondii*,12 (9.7%). Susceptibility to amphotericin B was the highest (74%), followed by itraconazole (52%) while least susceptibility was found in ketoconazole (19.0%). Strains of *C. krusei* and *C. guilliermondii* demonstrated 100% resistance to fluconazole and clotrimazole, respectively. MIC_{50} in most cases were greater than clinical break points and MIC_{90} values ranged between 16 and >64 µg/ml for all antifungal agents except amphotericin B, 0.5 to 1 µg/ml.

Conclusion: Majority of *Candida* isolates are resistant to azole drugs. Amphotericin B is a reasonable alternative drug for empirical treatment of candidiasis since routine drug susceptibility testing *Candida* does not exist yet in any hospital in Nigeria.

Keywords: Candida; infection; antifungal agents; susceptibility; Nigeria.

1. INTRODUCTION

Yeasts of the genus *Candida* are another group of opportunistic pathogens which cause oral and vaginal infections in humans, known as candidiasis. *Candida* is commonly found as commensal yeast in the mucus membrane of humans and other warm-blooded animals. Sometimes, these same strains can become pathogenic [1]. Candidiasis is the most common fungal infection in humans and is predominantly caused by *Candida albicans*, although other species of *Candida* are increasingly recognized. Rates of systemic infections due to *Candida* species have steadily increased over the past four decades, and such infections represent an important cause of morbidity for severely ill hospitalized patients [2,3]. Any organ in the body can be invaded by *Candida*, but vaginal infection and oral thrush are the most common forms. This later is seen in the very young, in the elderly, following antibiotic therapy and in those who are immunocompromised. Cutaneous candidiasis typically occurs in intertriginous areas but vaginal infections are the most common [4]. Dissemination of candidiasis may lead to haematogenous spread, with meningitis, pulmonary involvement and endocarditis or osteomyelitis. *Candida* species produce a wide spectrum of diseases ranging from superficial mucocutaneous disease to invasive illness such as hepatosplenic candidiasis, *Candida* peritonitis and systemic candidiasis.

There has been increase in the incidence and severity of yeast infections due to *Candida* species, and many of the yeasts associated with human infections have been found to be innately resistant or develop resistance to the most common antifungal agents. The introduction of appropriate therapy for these invasive infections depends largely on the rapid and accurate identification of the aetiological agents [5] and the need for antifungal susceptibility testing has become increasingly important since marked differences exist in species distributions and antifungal drug susceptibilities between different countries. *Candida* infections are still largely managed empirically without recourse to antifungal susceptibility testing in Medical Microbiology diagnostic laboratories in Nigeria. This may due largely to inability of the routine clinical laboratories to carry out adequate identification and susceptibility testing because of cost or cumbersomeness of the techniques involved. It was in view of this, that this study was instituted with the following objectives (i) To characterize clinical isolates of *Candida* species and (ii) To determine the susceptibility pattern of the *Candida* species to antifungal agents with the hope the results of this study will guide the empirical treatment of patients infected with these organisms.

2. MATERIALS AND METHODS

2.1 Strains and Identification

One hundred and twenty-four *Candida* species were collected from diagnostic Medical Microbiology laboratory of a tertiary hospital in south western Nigeria from various clinical specimens received. For routine examination. Species identification of *Candida* was done using standard morphological and physiological methods, including fermentation and growth on carbon sources, growth on nitrogen sources and growth at various temperatures [6]. Analytical profile index (API) *Candida* was used for confirmation of various species following manufacturer's instruction (Biomerieux, France). They were stored in slopes of Sabouraud dextrose agar at 4°C prior to use.

2.2 Preparation of Inoculum

The inocula were prepared from 18- to 24- h cultures of *Candida* species grown on Sabouraud dextrose agar. The inocula were emulsified in 5 ml of normal saline and this was vortexed for 20 seconds to achieve homogenous suspension. The suspension was adjusted to a 0.5 McFarland standard (1×10^6 to 5×10^6 CFU/ml) using spectrophotometer to monitor the turbidity. The suspensions were further diluted to a ratio of 1:100 in 5 ml sterile saline and then diluted to a ratio of 1:20 in RPMI 1640. Each dilution was further vortexed for 30 seconds before proceeding.

2.3 Susceptibility Testing

2.3.1 Disc Diffusion

The medium used was prepared thus; 2% dextrose Mueller-Hinton agar and 5 µg of methylene blue dye/ml. The agar plates were inoculated by dipping a sterile cotton swab into the inoculum suspension, rotated several times, and pressed firmly against the inside wall of the tube above the fluid level to remove excess fluid. The entire dried agar surface was evenly streaked in two different directions. Varying concentrations of antifungal discs; clotrimazole (10 µg), ketoconazole (10 µg), itraconazole (5 µg), fluconazole (10 µg), amphotericin B (5 µg) made from powder supplied by Sigma Aldrich, UKwere dispensed onto the inoculated agar surface. They were incubated at 35°C for 20 to 24 h. Inhibition zone diameters around the discs were measured to the nearest whole millimetre at the point at which there was prominent reduction in growth. The susceptible inhibition zone diameter break point used throughout the study for each antifungal to the *Candida* species was based on CLSI recommendation [7].

2.3.2 Broth macrodilution

The broth medium used was RPMI 1640 broth buffered with MOPS buffer (0.165 M) and 0.2% dextrose adjusted to pH 7.0 at 25°C. Antifungal agents were diluted at 10× concentration ranges with RPMI 1640 containing L-glutamine without bicarbonate, buffered to pH 7.0 with 0.165 MOPS buffer following CLSI protocol [8]. 0.9 ml of test inoculum was added to 0.1 ml of 10× drug concentration to give drug dilution ranges of 0.03 – 64 µg/ml. The MIC tubes were incubated at 35ºC without agitation for 48 h in ambient air. Results were read at 24 h and rechecked at 48 h. The growth (turbidity) was visually graded. For amphotericin B, the MIC was read as the lowest concentration that would prevent any discernible growth in form of turbidity and for azoles, the MIC was defined as the lowest drug concentration that would reduce growth by 80% relative to that of the growth control. Some azoles, particularly fluconazole, exhibit a phenomenon known as trailing, trailing occurs when the turbidity continually decreases as the drug concentration increases but the suspension fails to become optimally clear (partial inhibition of growth over an extended range of antifungal concentrations).

2.3.3 Quality control and test specimens

The reference strains - *C. Krusei* ATCC 6258 and *C. Parapsilosis* ATCC 20019 were tested in the same manner as the other test strains in disc and macrotube methods. The growth control macrotube was inoculated with a 0.9 ml volume of the inoculum suspension and a 0.1 ml volume of drug-free medium. In addition, 1 ml of uninoculated drug-free medium was included as a sterility control.

2.3.4 Statistical analysis

Collation of data was carried out using Epi-info software from Centre for Disease control and prevention, USA. Data were analysed using statistical package within the Epi-info software. Chi square was used to determine the difference in the rate of isolation of *Candida* species from different clinical samples and the degree of susceptibility to different antifungal agents. The p value less than 0.05 was considered to be significant.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Identification of *Candida* species

One hundred and twenty-four *Candida* species collected from a tertiary hospital in south west Nigeria were obtained from various body sites. Highest number of *Candida* was obtained from urine, 44 (35.5%); followed by HVS, 32 (25.8%) and blood, 20 (16.1%). Highest frequencies were obtained from *C. Krusei* and *C. tropicalis*, 32 each, followed by *C. Albicans* and *C. pseudotropicalis*, 24 each, *C. gulliermondi*, 12 (Table1). There was no significant difference in the rate of isolation of *Candida* species from different clinical samples (Chi Square = 5.52; p = 0.063; p > 0.05).

3.1.2 Disc susceptibility testing

The *Candida* species were resistant to one or more of the five antifungal agents used. Susceptibility to amphotericin B was the highest (74%), this was followed by itraconazole (52%) while least susceptibility was found in ketoconazole (19.0%). The comparative disc sensitivities are shown in Table 2. Statistically, there was a significant difference in the degree of susceptibility to different antifungal agents (Chi square = 85.37, $p = 0.0001$; $p < 0.01$). Strains of Ckrusei and *C. guilliermondi* demonstrated 100% resistance to fluconazole and clotrimazole, respectively. *C. pseudotropicalis* also had least resistance to clotrimazole, itraconazole and amphotericin B; overall, *C. guilliermondii* appeared to be more resistant than the rest of *Candida* species.

3.1.3 Minimum inhibitory concentrations

The MIC results further confirmed level of resistance to available antifungal agents used (Table 3). MIC_{50}in most cases were greater than clinical break points while MIC_{90} values (MIC for 90% of the organisms) were between 16 and >64 µg/ml for all antifungal agents except amphotericin B, 0.5 to 1 µg/ml.

4. DISCUSSION

With the increasing frequency of fungal infections, as well as increase in resistance to antifungal agents, it is imperative that clinical applicable antifungal susceptibility testing should be made available. This study provides information on the species distribution and *in-vitro* drug susceptibility of *Candida* species obtained from different clinical samples in a tertiary hospital from Nigeria. *C. krusei* and *C. tropicalis* were the predominant pathogens and were largely isolated from urine. *C. krusei* accounted for most cases of vaginal candidiasis while in other studies *C. albicans* has been incriminated in cases of vaginal candidiasis [9,10]. There is a decline in the prevalence of *C. albicans* isolated from blood in the last decade in United States with corresponding tilt in increase of non-*C. albicans* specifically *C. glabrata* from the same site [2,11]. *C. parapsilosis* was not isolated in any of the clinical specimens/sites including blood culture. Several reports have shown that *C. parapsilosis* are the most incriminated *Candida* from blood stream infections [12,13]. This difference might be attributable to differences in population studied, geographical locations, and variations in healthcare practices could also be a significant factor.

Table 1. Sources of isolation of *Candida* species

Source	*C. albicans*	*C. guilliermondii*	*C. krusei*	*C. pseudotropicalis*	*C. tropicalis*	Total
HVS	4	8	12	0	8	32(25.8)
Wound Swab	0	0	4	0	0	4(3.2)
Urethral Swab	0	0	4	0	0	4(3.2)
Ear Swab	4	0	0	0	0	4(3.2)
Mouth Swab	0	0	0	4	0	4(3.2)
Blood	4	0	4	0	12	20(16.1)
Sputum	4	0	0	0	0	4(3.2)
CSF	0	0	0	4	0	4(3.2)
Catheter tip	4	0	0	0	0	4(3.2)
Urine	4	4	8	16	12	44(35.5)
Total	24(19.4)	12(9.7)	32(25.8)	24(19.4)	32(25.8)	124

( ): number in parentheses are percentages

Table 2. Comparative disc susceptibility pattern of *Candida* species

Strains (n)	Clotrimazole		Ketoconazole		Itraconazole		Fluconazole		Amphotericin B	
	S	R	S	R	S	R	S	R	S	R
C. albicans (24)	4(17)	20(83)	16(67)	8(33)	16(67)	8(33)	4(17)	20(83)	18(75)	6(25)
C. guilliermondii (12)	0(0)	12(100)	4(33)	8(67)	4(33)	8(67)	4(33)	8(67)	6(50)	6(50)
C. krusei (32)	12(37)	20(63)	20(62)	12(38)	18(56)	14(44)	0(0)	32(100)	30(94)	2(6)
C. tropicalis (32)	20(62)	12(38)	16(50)	16(50)	16(50)	16(50)	4(12)	28(88)	20(62)	12(38)
C. pseudotropicalis (24)	20(83)	4(17)	12(50)	12(50)	20(83)	4(17)	4(17)	20(83)	22(92)	2(8)

( ): number in parentheses are percentages

Table 3. MICs of a cumulative percentage of Strains with Inocula of 10^4 CFU (MIC Range: 0.0125 – 64 µg/ml)

Strains	N	Agents	MIC_{50}	MIC_{90}
C. albicans	24	Clotrimazole	32	>64
		Itraconazole	16	64
		Fluconazole	16	64
		Ketoconazole	32	>64
		Amphotericin B	0.125	0.5
C. guilliermondii	32	Clotrimazole	4	16
		Itraconazole	4	32
		Fluconazole	16	64
		Ketoconazole	2	16
		Amphotericin B	0.125	0.5
C. krusei	32	Clotrimazole	8	32
		Itraconazole	32	>64
		Fluconazole	64	>64
		Ketoconazole	16	64
		Amphotericin B	0.5	1
C. tropicalis	32	Clotrimazole	16	64
		Itraconazole	8	64
		Fluconazole	16	64
		Ketoconazole	8	32
		Amphotericin B	0.125	0.5
C. pseudotropicalis	24	Clotrimazole	4	32
		Itraconazole	16	64
		Fluconazole	8	16
		Ketoconazole	16	64
		Amphotericin B	0.125	0.5

The MICs of *C. krusei* and *C. albicans* to fluconazole were very high and *C. krusei* was less susceptible than *C. albicans*. This is a similar scenario to the relative growth data obtained by Odds [14]. There is a rapidly growing literature describing failures of fluconazole treatment in patients infected with these species and showing emergence of *C. krusei* in groups of at-risk patients who receive fluconazole prophylactically [15,16]. The fluconazole susceptibility testing results obtained for *C. krusei* is consistent with the intrinsic resistance nature of *C. krusei* to fluconazole. However amphotericin B was more active against *C. krusei,* which is inconsistent with those other reports for this organism [17,18]. It is noteworthy the isolation of *C. guilliermondii* from HVS and urine, which is an uncommon species of *Candida* until recently. They have been more reported in blood stream from Latin America than any other continent [19]. A localized infection could become systemic if not treated adequately or in a case of treatment failure. Elsewhere, *C. guilliermondii* was found to be associated with onychomycosis [20] and was rarely seen as a cause of invasive fungal infection [21,22]. *C. guilliermondii* appeared to be more resistant than the rest of *Candida* species with susceptibility of 50% to amphotericin B and 67% to fluconazole. In 1985, a patient died of disseminated candidias is due to *C. guilliermondii* despite amphotericin B therapy. The organism was later shown to be resistant to amphotericin B by *in vitro* testing [23]. There are other reported drug failures of these organisms with fatal human and economic losses [24]. In another related study where there was increased frequency of candidaemia due to *C. guilliermondii,* the isolates were generally susceptible to amphotericin B (100%), fluconazole (91%), and voriconazole (95%) [19]. These varying susceptibility patterns of *C. guilliermondii* in different countries, regions or clinical sites underscores the importance of local or specific susceptibility testing in the management of candidiasis.

As earlier stated *C. tropicalis* is one of the most predominant isolate including *C. krusei* and were predominantly found in candidaemic and candiduric patients. *C. Pseudotropicalis* share similar susceptibility pattern to *C. tropicalis.* These isolates were highly resistant to azole drugs with MIC_{90} ranged between 32 and 64 µg/ml. The only drug with good susceptibility was amphotericin B. In India, *C. tropicalis* is the most common cause of nosocomial candidaemia; 67-90% of nosocomial candidaemia cases are due to *Candida that are not C. albicans* of which *C. tropicalis* is the most dominant [25]. Resistance to fluconazole in clinical isolates of *C. tropicalis* had also increased in this study. There are several reports on azole resistance in *C. tropicalis,* which showed moderate level of fluconazole resistance to *C. tropicalis* [26,27].

Like in bacteria, there are several factors probably responsible for the development of drug resistance in various clinical conditions. Apparently, the epidemiology of drug resistance in *Candida* species appears complex and varies among different clinical sites, patients, care units, hospitals or geographical locations.

5. CONCLUSION

The majority of *Candida* isolates in this study have been found to be resistant to azole drugs. Our study suggests use of amphotericin B as a reasonable alternative drug for empirical treatment of candidias is since routine drug susceptibility testing of *Candida* does not exist yet in any hospital in Nigeria. However, we advocate a regional or national surveillance study in order to comprehensively elucidate the

epidemiology and susceptibility of *Candida* to antifungal agents.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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