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SPECIES DISTRIBUTION AND ANTIFUNGAL SUSCEPTIBILITY PATTERN OF CANDIDA ISOLATES FROM PREGNANT WOMEN IN A TERTIARY HOSPITAL IN NIGERIA

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ABSTRACT

Introduction: Information regarding the resistance pattern of *Candida* species in developing countries is limited. Most sensitivity studies were performed on few isolates and/or few antifungal agents using the disc diffusion method because of limited resources.

Methods and Material: We evaluated six antifungal agents against *Candida* isolates recovered from the vagina of apparently healthy pregnant women using the E-test method.

Results: One hundred and seventy *Candida* isolates recovered from 500 participants were identified and subjected to an antifungal susceptibility test. *Candida albicans*(53.5%) was the most common specie identified, followed by *Candida glabrata*(14.1%). *C. albicans* was mostly resistant to itraconazole (31.9%), with MIC 50 and 90 of 0.038 mg/L and 6 mg/L, respectively. Resistance to 5-fluorocytosine, fluconazole, and voriconazole was not observed for *C. albicans*. Caspofungin resistance was observed in 3 *C. albicans*and 1 *C. glabrata* isolates. Resistance to amphotericin B (50%) and itraconazole (100%) were the highest for *C. glabrata*. Flucytosine and voriconazole resistance was not observed in this study.

Conclusion: The observed species diversity and the presence of *C. albicans* resistance to 3 of the 6 antifungal agents tested justify the need for a regular surveillance of the sensitivity pattern to antifungal drugs in Nigeria.

LA REPARTITION DES ESPECES ET PROFIL DE SENSIBILITE ANTIFONGIQUE DES ISOLATS DE CANDIDA PROVENANT DE FEMMES ENCEINTES DANS UN HOPITAL TERTIAIRE AU NIGERIA.

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RESUME

Présentation : Informations concernant le profil de résistance des espèces de Candida dans le pays en développement est limité. La plupart des études sensibles ont été effectuées sur quelques isolats et/ ouquelques agents antifongiques en utilisant la méthode de diffusion sur disque en raison de ressources limitées.
Méthodes et Matériels : Nous avons évalué six agents antifongiques contre des isolats de Candida provenant du vagin des femmes enceintes en bonne santé apparente à l'aide de la méthode d'essai E.
Résultat : Cent soixante – dix isolats de Candida repris des 500 participantes ont été identifiés et soumis à un test de sensibilité aux antifongiques. Candida albicans (53,5%). Candida albicans est l'espèce la plus commune identifiée, suivi par le Candida glabrata (14,1%). C. albicans a été la plupart de temps résistant à l'itraconazole (31,9%) avec MIC 50 et 90 de 0,038 mg/L et 6 mg/L, respectivement. Résistance à la 5 – fluorocytosine, fluconazole et voriconazole n'a pas été observée pour C. albicans. Résistance à la casponfungine a été observée dans les isolats de 3C. albicans et dans les de 1 C. glabrata. La résistance à l'amphotericine B (50%) et itraconazole (100%)étaient le plus élevés pour C. glabrata. La résistance à la Flucytosine and voriconazole n'a pas été observée dans cette étude.
Conclusion: La diversité observée des espèces et la présence de la résistance de albicans aux 3 des 6 agents antifongiques testés justifient la cessité d'une surveillance régulière du profil de la sensibilité aux médicaments antifongiques au Nigeria.

INTRODUCTION

Candida species are components of the normal flora most commonly found in the gastrointestinal tract, mucous membranes of the mouth and vagina, and on the skin of many healthy individuals (1). A higher prevalence of vaginal colonization and symptomatic vaginitis is more often observed in pregnant women than in non-pregnant women(2).*Candida* species become pathogenic and invasive when the balance of the normal bacterial flora is disrupted because of antibiotic therapy(3)or if the immune system is compromised(4).

Candida infection is one of the most common opportunistic infections in health care settings. It is also the most common yeast infection in the community. There are more than 20 known *Candida* species associated with human infections, but only about four cause vast majorities of the clinical cases including *Candida albicans, Candida glabrata, Candida parapsilosis,* and *Candida tropicalis*(5,6).Most *Candida* isolates are now seen as a potential pathogen, unlike some decades ago when most of the non-*albicans Candida* species (NAC) were often dismissed as probable contaminant because they were viewed to be non-pathogenic. This change in perception is because many of the virulent factors that were once only attributable to *C. albicans* are now demonstrated in many NAC as well(7).Increase in the population of immunosuppressed patients that accompanied the era of HIV/AIDS as well as recent advances in patients care also contribute to the emerging transition of many *Candida* species to formidable multidrug resistance pathogens(8). While mucocutaneous candidiasis affects both immunocompetent as well as immunocompromised hosts, systemic infection is more prevalent in patients with reduced immunity such as AIDS patients, organ transplant patients and those with severe underlying heath conditions including diabetes mellitus(9).*C. albicans* is currently the commonest specie all over the world; infections by NAC are increasing in many countries(10). Epidemiology of the *Candida* species can also vary from one health care center to the other depending on the patient population as well as surveillance method employed. Even in a particular country, specie distribution of *Candida* infection is dynamic and should be constantly monitored. For example in recently published 16 year surveillance of the epidemiology of *Candida* species in Italy, pattern of infection was found to have changed over time. *C. albicans* was associated with less than 50% of all *Candida* infection over this period, while as much as 75% of the infections in 2013 alone were found to be due to NAC(11). A similar study that was carried out in the USA also showed that species distribution of *Candida* infection is unstable, and that discovery and utilization of new antifungal agents was partly responsible. For instance, the percentage of *C. albicans* infection was reduced following the era of fluconazole and echinocandins while the percentage of *C. glabrata* infection was increased over a period of 1983 to 1986 and 2004 to 2007(12). In another study carried out by the research group of Pfaller, increase in the incidence of NAC, as well as the emergence of uncommon *Candida* species were noted in most of the sentinel centers in North America. Previous antifungal treatment and debilitating patient conditions were cited as some of the factors that contributed to these changes. The study also showed that mortality rate was higher in patients infected with unknown *Candida* species as well as those infected with more than one isolate(13).

Parallel to the changing epidemiology of Candida infection from *albicans* towards NAC, the sensitivity pattern of many *Candida* isolates is also changing. This can be exemplified by the increase in fluconazole resistance of *C. glabrata* in United States from 9% between 1992 and 2001 to 14% between 2001 and 2007(14). Fluconazole resistant isolates of *C. glabrata* are expected to be resistant to other azoles because of the similar mechanism of action of this group of drug. Though echinocandins are the recommended agents for azole resistant *C. glabrata*, there are now concerns about emerging echinocandin resistant and multidrug resistant *C. glabrata* (10). Non *albicans Candida* isolates associated with increasing morbidity and mortality were also said to be more prevalent in neutropenic patients as well as those who have been previously treated with azole based antifungal agents in another review by a researcher in Germany(9). A study in Ghana that compared the susceptibility pattern of *Candida* isolates in 2003-2005 to those of 2010-2014 also found a reduction in the susceptibility of majority of the isolates to most of the antifungal drugs tested(15). Since, sometimes, invasive candidiasis is endogenously derived from species that already colonize the patient mucosal surface and skin, we decided to carry out this study to find out the prevalence of vaginal colonization, distribution of species as well as the antifungal susceptibility pattern of *Candida* isolates among a group at increased risk of colonization, pregnant women, in a Nigerian tertiary health institution. This will provide regional surveillance data regarding dynamic antifungal susceptibilities among *Candida* isolates, which are currently a global phenomenon.

SUBJECTS AND METHODS

Five hundred pregnant women attending the antenatal clinic at the Lagos University Teaching Hospital between September 2012 and March 2013 were recruited into the study following informed consent and ethical approval. The women presented no clinical feature of vulvovaginal candidiasis. Two sterile swabs were used to collect high vaginal swabs from each participant with the aid of a sterile speculum. The swabs were sent to the Department of Medical Microbiology, Lagos University Teaching Hospital, for preliminary identification of the isolates. One swab was used for Gram stain smear examination and the other was streaked on Sabouraud dextrose agar

(SDA) and incubated at 35°C aerobically. Yeast cell growth on SDA was confirmed based on colony morphology and Gram staining. In cases where multiple morphologies were observed per plate, the predominant morphology was selected so that each isolate represents a patient.

Species identification

Isolates from SDA cultures were transferred to the Institute of Medical Microbiology and Infectious Disease Epidemiology, University of Leipzig, Germany, for further identification and antifungal susceptibility testing. All fungal isolates were cultured on CHROMagar*Candida* (Becton Dickinson GmbH, Heidelberg, Germany) for preliminary identification and to rule out possible contaminants. Eventual identification to specie level was carried out by using Matrix- Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (BioMerieux Deutschland GmbH, Nurtingen, Germany).

Susceptibility testing and interpretation

Antifungal susceptibility testing (AST) was performed with Etest strips, (AB Biodisk, Solna, Sweden) in accordance with the standards described in the CLSI document M27-A3(16). The antifungal test strips used were: Amphotericin B, itraconazole, fluconazole, voriconazole, 5-fluorocytosine and caspofungin. The inoculum concentration of *Candida* isolates was prepared using sterile 0.85% saline. After mixing with a Vortex mixer, turbidity was adjusted to match 0.5 McFarland turbidity standards and inoculated onto RPMI 1640 agar plate supplemented with 2% glucose. Plates were read after incubation at 35°C for 24 h. The minimum inhibitory concentration (MIC) was considered as the lowest concentration at which the border of the elliptical zone of growth inhibition intersected with the scale on the test strip. The cutoff for the minimum inhibitory concentration (MIC) for azoles and caspofungin was set at 80% growth inhibition, while 90% to 100% growth inhibition was used as the cutoff for flucytosine and amphotericin B(17). Caspofungin was the brand of echinocandin tested in this study because alternative brands were not available throughout the duration of this investigation. Quality control strains were tested with the isolates, including *C. albicans* ATCC 90028, *C. krusei*ATCC 6258, and *C. parapsilosis* ATCC 22019. Results were interpreted according to the revised CLSI document M27-S4(18), except for the caspofungin results, which were interpreted according to the older CLSI version (M27-A2 document)(17).

Ethical issues

Ethical approval for this work was granted by the Research and Ethical Review Board of Lagos University Teaching Hospital, Nigeria. Written informed consent was obtained from each participant after the objectives of the study were explained to them in their respective local Nigerian languages.

RESULTS

One hundred and seventy *Candida* isolates were identified from the participants; representing a 34% carriage rate. Species distribution among the 170 *Candida* isolates (Table 1) included 91 (53.5%) *C. albicans*, 24 (14.1%) *C. glabrata*, 15 (8.8%) *C. parapsilosis*, 11 (6.5%) *C. tropicalis*, (2.4%) *C. krusei,* and 25 (14.3%) uncommon species, consisting of eight isolates of *Candida lipolytica*, four *Candida haemulonii*, three *Candida valida*, three *C. guilliermondii*, two *Candida membranaefaciens,* and one *Candida kefyr*, *Candida catenulate*, *Candida boidinii, Candida norvegensis*, and *C. dubliniensis*. The ranges of MICs and the MICs at which 50% (MIC50) and 90% (MIC90) of *Candida* species were inhibited after 24h of incubation are presented below. Resistance to amphotericin B was the highest for *C. krusei*(50%), followed by the uncommon *Candida* species (32%). Amphotericin resistance rate observed in *C. albicans* was 5.6%. Resistance to itraconazole was observed in all isolates and the highest in *C. krusei* (100%), followed by *C. glabrata* (83.3%), (Table 1). *C. glabrata* and *C. krusei* were the only common *Candida* species with MIC 50 above the ≤8 mg/L susceptible breakpoint for fluconazole and these are the only species with detectable resistance to this drug.

A large number of the uncommon candida species (44%) 1 as well as 50% of *C. glabrata* isolates fell within the susceptible dose dependence (S-DD) range of fluconazole, while the S-DD range of itraconazole was mostly occupied by *C. parapsilosis* (46.7%) and *C. tropicalis*(36.4%), (Table 2). One isolate of *C. tropicalis* (9.1%) fell within the intermediate susceptibility range of caspofungin. All *Candida* isolates presented MIC 50 and MIC 90 within susceptible breakpoint for caspofungin, but 3.3% of *C. albicans*and 4.2% *C. glabrata*isolates were resistant to this antifungal agent. We could not categorically identify the number of uncommon *Candida* species that were resistant to caspofungin because the resistance breakpoints for most of them were not yet established by CLSI. Voriconazole and flucytosine resistance was not observed in this study, (Table 2).

DISCUSSION

The predominance of *C. albicans* observed in our study is in general agreement with similar studies in Nigeria and some other countries(19-22). A similar study in another location in Nigeria by Akortha *et al* (19) indicated the highest rate of fluconazole resistance (100%) for *C. krusei,* but a 3.6% resistance rate for *C.albicans,* and none for *C. glabrata* and *C. tropicalis* isolates. This is partly in agreement with our results, showing the highest resistance rate for *C. krusei*and no resistance for *C. tropicalis*. However, in our study, the observed fluconazole resistance among the isolates was not in agreement with Pam *et al.* results in Lagos, who indicated a resistance rate of 7.7%, 10%, and 40% for *C. tropicalis, C. albicans,* and *C. krusei,* respectively (22). However, our results, regarding no fluconazole resistance for *C. tropicalis,* are similar to those of Pam *et al*. The observed differences may be due to differences in study populations, site, or the procedures employed. We used E test, while broth microdilution methods were used in

the other studies(19, 21). However, Etest has been found to be more sensitive when it comes to antifungal testing for *Candida* species (23).*C. krusei*is known to be intrinsically resistant to fluconazole (18).

TABLE 1: MINIMUM INHIBITORY CONCENTRATIONS (MICS) OF ANTIFUNGAL AGENTS AGAINST *CANDIDA* SPP. AND THE NUMBER (PERCENTAGES) OF RESISTANT STRAINS AS DETERMINED BY ETEST AFTER 24 H OF INCUBATION

	Susceptibilities to antifungal agents					
Species and MICs	AMB	5 FC	FLC	ITC	VRC	CAS
C. albicans(n =91)						
Range	0.025–2.5	<0.002–0.64	0.019–48	0.006–>32	<0.002–3	<0.002–0.94
MIC 50	0.5	0.008	1	0.038	0.032	0.016
MIC 90	1.0	0.065	12	6	0.38	0.25
N(%) Resistant	5(5.6)	0	0	29(31.9)	0	3(3.3)
C. glabrata (n=24)						
Range	0.25–3	<0,002–0.047	4–>256	0.025–>32	0.019–3	<0.002–0.75
MIC 50	0.75	0.006	32	>32	0.5	0.025
MIC 90	1.5	0.008	48	>32	3	0.125
N (%) Resistant	2(8.2)	0	3(12.5)	20(83.3)	0	1(4.2)
C. parapsilosis(n =15)						
Range	0.038–0.75	< 0.002–0.008	1.5-16	0.032–2	0.032–0.94	0.016–0.75
MIC 50	0.38	0.003	6	0.5	0.094	0.19
MIC 90	0.5	0.006	12	0.75	0.125	0.5
N (%)Resistant	0	0	0	2(13.3)	0	0
C. tropicalis(n=11)						
Range	0.038–1.5	< 0.002–0.023	0.125–16	0.125–>32	0.003–0.94	<0.002–0.38
MIC 50	0.5	0.002	0.5	1	0.47	0.008
MIC 90	1	0.004	1.5	32	0.25	0.19
N (%)Resistant	0	0	0	6(54.5)	0	0
C.krusei (n =4)						
Range	1–2	1.5–>2	192–>256	>32	1-1.5	0.125–0.25
MIC 50	1	1.5	192	32	1	0.125
MIC 90	2	2	>256		32	0.25
N (%) Resistant	2 (50%)	0	NA	4(100)	0	0
Others (n =25)						
Range	0.025–>32	0.0002–4	0.5–>256	0.025–>32	0.0125–2	<0.002–0.5
MIC 50	1	0.006	12	2	0.125	0.19
MIC 90	32	4	>256	32	0.75	0.5
N (%) Resistant	8(32)	0	5(20)	16(64)	0	-

MICs are reported in mg/L. AMB= Amphotericin B, 5FC=5- Flucytosine, FLC= Fluconazole, ITC= Itraconazole, VRC= Voriconazole, CAS= Caspofungin, MIC = Minimum inhibitory concentration. NA= Not applicable (Breakpoint not defined)

Hence, the results of studies with certain degrees of sensitivity to this drug must be interpreted with caution. Our findings regarding voriconazole and flucytosine sensitivity patterns were similar to those observed in

Ghana, another African country where no voriconazole resistance was found, and most of the isolates were also susceptible to flucytosine(24). This may be because these two antifungals are not widely available for use in developing countries where they may exert the selective pressure needed against the emergence of resistance. However, surprisingly, in India, another developing country, *C. albicans* was found to be resistant to fluconazole, itraconazole, and voriconazole at a rate of 38.9%, 19.3%, and 25.5%, respectively (25).

TABLE 2: ANTIFUNGAL SUSCEPTIBILITIES OF *CANDIDA* ISOLATES AS DETERMINED BY E TEST

Species and susceptibilities	Susceptibilities to antifungal agents					
	AMB, N(%)	5-FC, N(%)	FLC, N(%)	ITC, N(%)	VRC, N(%)	CAS, N(%)
C. albicans (n =91)						
Susceptible	53(58.2)	91(100)	79(86.8)	16(17.6)	87(95.6)	83(91.2)
S-DD	-	-	6(6.6)	6(6.6)	-	-
Intermediate	-	0	-	-	-	2(2.2)
Resistant	5(5.5)	0	0	29(31.9)	0	3(3.3)
C. glabrata (n =24)						
Susceptible	7(29.2)	24(100)	-	0	22(91.7)	22(91.7)
S-DD	-	-	12(50.0)	4(16.7)	-	-
Intermediate	-	0	-	-	-	1(4.2)
Resistant	2(8.3)	0	3(12.5)	20(83.3)	0	1(4.2)
C.parapsilosis(n =15)						
Susceptible	13(86.7)	15(100)	12(80)	3(20)	15(100)	15(100)
S-DD	-	-	3(20)	7(46.7)	-	-
Intermediate	-	0	-	-	-	0
Resistant	0	0	0	2(13.3)	0	0
C. tropicalis (n =11)						
Susceptible	8(72.7)	11(100)	11(100)	1(9.1)	11(100)	10(90.9)
S-DD	-	-	0	4(36.4)	-	-
Intermediate	-	0	-	-	-	1(9.1)
Resistant	0	0	0	6(54.5)	0	0
C. krusei (n =4)						
Susceptible	0	4(100)	NA	0	3(75)	4(100)
S-DD	-	-	NA	0	-	-
Intermediate	-	0	NA	-	-	0
Resistant	2(50)	0	NA	4(100)	0	0
Others (n =25)						
Susceptible	6(24)	25(100)	9(36)	4 (16)	24(96)	-
S-DD	-	-	10(44)	4(16)	-	-
Intermediate	-	0	-	-	-	-
Resistant	8(32)	0	5(20)	16(64)	0	-

AMB= Amphotericin B, 5-FC=5- Flucytosine, FLC= Fluconazole, ITC= Itraconazole, VRC= Voriconazole, CAS= Caspofungin, N= Number, S-DD= Susceptible dose dependence. NA= Not applicable (Breakpoint not defined)

Absence of flucytosine resistance in our study area also needs 1 to be interpreted with caution because resistance to this drug can rapidly emerge when the drug is used as a monotherapy (26). Hence, combination with other

antifungal agents is especially needed for serious infections. Similarities and differences were also observed while comparing our results to those obtained in developed countries. For instance, an Etest based *Candida* susceptibility testing in a German teaching hospital showed that similarly to our findings, *C. glabrata, C. krusei,* and uncommon *Candida* species presented fluconazole resistance(27). However, in contrast to our result, the same study found a flucytosine resistance rate of 3.6%, 3.7%, 58.3%, and 100% for *C. albicans, C. glabrata*, *C. tropicalis,* and *C. krusei,* respectively. Similar to our results, the results of an antifungal susceptibility study in Japan also showed that the species with the highest resistance to itraconazole was *C. glabrata* and that this species also exhibited voriconazole resistance (14.3%), but the same study did not evaluate amphotericin B resistance among the isolates(28). As observed in our study, amphotericin B resistance has been demonstrated among many *Candida* species in the USA, including *C. glabrata*(5.9%) and *C. krusei*(1.2%)(29). Another USA study indicated a *C. albicans* resistance rate of 3%, 2%, 4%, and 2% to flucytosine, fluconazole, itraconazole, and voriconazole, respectively, and a high (66%) itraconazole resistance for *C. glabrata* (17).However, in Israel, another developed county, resistance to itraconazole was 3.2%, 3.4%, and 93% for *C. albicans, C. parapsilosis,* and *C. glabrata,* respectively. Fluconazole resistance rate among the same species was 1.6%, 3.4%, and 21.4%, respectively (30). The observed regional differences in the susceptibility pattern to the various antifungal agents may be a reflection of the differences in the pattern of the drug used. However, because of the increase in human mobility, it is very impossible for resistant isolates to spread from one region to another. Hence, there is a need for continuous surveillance of the various *Candida* species resistance pattern. Only few studies tested the six antifungal agents used for fungal chemotherapy against a large number of *Candida* isolates in a single study with Etest in Nigeria as described herein. Most studies tested a limited number of *Candida* isolates and/or drugs at any points in time, mainly fluconazole and itraconazole (21-22). One of the limitations of this study is the fact that available epidemiological information of participants is not sufficient enough for it to be used for discussion of results. Also, broth microdilution is the gold standard for fungal sensitivity test, but it is labor intensive and has been replaced by Etest in most clinical laboratories. So our results provide a more practical and real life situation is most laboratory settings.

This study showed that, apart from *C. albicans* with a MIC 50 of 0.0388mg/L, all *Candida* isolates present MIC 50 and MIC 90 above the sensitive breaking point for itraconazole. Our result also showed that, besides the three isolates of *C. glabrata*, all the common *Candida* spp. were sensitive to fluconazole. This reinforce the need to encourage routine identification of *Candida* isolates at the species level as well as their antifungal susceptibility in developing countries to ensure that patients receive the optimum benefit when it comes to antifungal chemotherapy

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