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Resistance of *Candida albicans* to antifungal drugs in Abidjan (Cote d'Ivoire)

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This study aimed to evaluate the resistance levels of strains of *Candida albicans* to the antifungals commonly used in Abidjan, Cote d'Ivoire. This is a prospective study that was carried out from July to October 2017 at the mycology laboratory of the Institut Pasteur of Cote d'Ivoire. 105 *Candida* isolates, obtained from swabs taken from patients receiving out-patient treatment, were seeded on chromogenic medium. Identification of *Candida* species was carried out by MALDI-TOF mass spectrometry (Vitek MS bioMérieux). The susceptibility of *C. albicans* strains to 5-fluorocytosine, amphotericin B, fluconazole, itraconazole and voriconazole was evaluated using the microdilution technique in a semi-solid medium to determine the minimum inhibitory concentration with the ATB1 Fungus 3 kit. Out of 105 *Candida* strains, 68 (64.8%), including *C. albicans*, were identified on the chromogenic medium and confirmed by MALDI-TOF spectrometry. These *C. albicans* strains exhibited varying levels of resistance to the antifungals tested: 1.5% for 5-fluorocytosine, 26.3% for fluconazole, 39.7% for itraconazole, 27.9% for voriconazole. No resistance to amphotericin B was observed. *C. albicans* strains taken from ear pus swabs exhibited greater resistance (*P* = 0.0113). *C. albicans* is developing increasing resistance to common antifungals, hence the need for regular surveillance in resource-poor countries.

Key words: Candidiasis, *Candida albicans*, resistance, mycosis, antifungual drug.

INTRODUCTION

Candida albicans is a yeast that forms part of the commensal flora of healthy individuals. However, when the host-parasite equilibrium is disrupted, the yeast becomes opportunistic and colonises the skin and mucous surfaces in humans and many animal species. In humans, this yeast poses a serious health threat, especially in patients with immune deficiency or undergoing immunosuppressive therapies. It is implicated in more than 80% of yeast infections (Gonsu et al., 2014). Its varied clinical spectrum ranges from superficial infections, in particular of the respiratory, digestive and genital mucosa, to deep (pulmonary mycosis) and

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disseminated (septicaemic mycosis) infections (Badillet et al., 1987).Traditional identification requires yeasts to be grown in biochemical test galleries or on chromogenic media and necessitates an incubation period of between 24 and 72 h (Bernal et al., 1996).

Unlike these so-called classical methods, matrix-assisted laser desorption ionization- time of flight (MALDI-TOF) mass spectrometry directly analyses the various bacterial macromolecules, especially proteins, and therefore yields results more quickly (Lindsay et al., 2010). It meets the need for precise, rapid diagnosis to deal more effectively with candidiasis.

Indeed, it is admitted that antifungal drugs are classified in five groups: (i) Antifungals that affect ergosterol. (ii) Antifungals acting on the fungal cell wall, (iii) Nucleic acid inhibitors, (iv) Mitosis inhibitors and (v) Protein synthase inhibitors. Four types of antifungals are currently used to treat fungal infections (5-fluorocytosine, polyenes, azoles and echinocandins). In limited resource countries, echinocandins, a recent class, is not yet available (Bounouman-Ira et al., 2011). Management of *Candida* infections often runs into a number of issues, including the small number of effective antifungal drugs, the toxicity of available antifungals, *Candida* resistance to common antifungals, recurrence of *Candida* infections, as well as the high cost of antifungal drugs (Khan et al., 2003, Klepser 2001). In addition this fact, in Cote d'Ivoire, antifungals are most often prescribed before susceptibility of the pathogenic fungi to the antifungals has been determined. The aim of this study was to determine the resistance profile of *C. albicans* strains isolated in Abidjan.

MATERIALS and METHODS

Patients

This is a prospective study carried out in the mycology laboratory in Institut Pasteur of Cote d'Ivoire (Cocody and Adopodoumé sites) from July to October 2017, on *Candida* isolates obtained from swabs taken from patients receiving out-patient treatment. *Candida* isolates came mainly from vaginal exudates, oropharyngeal and sperm swabs. The other swabs were taken from ear pus, sputum and stools.

Culture on chromogenic media

The isolates were cultured on chromogenic media (*Candida* Chromogenic agar, Condo S.A.Madrid, Spain), which allowed rapid identification of *Candida* species using the quadrant technique. After seeding, incubation was at 37°C for 24 to 48 h. Colonies were identified on the basis of their colour: *C. albicans* produces pale green colonies, *C. tropicalis* are blue-green, *C. krusei* are pink, and other species are white-pink.

After identification of the *Candida* species, confirmation of the results was sought with MALDI-TOF mass spectrometry (Vitek MS BioMerieux, France) following manufacturer's instructions. A colony of the calibration strain, Escherichia coli ATCC 8739, was spotted onto a MALDI-TOF plate with 1 µl of matrix (α-cyano-4-hydroxycinnamic acid, MS CHCA ref 411071). Using a sterile loop, samples of each colony were then deposited in target wells for testing in duplicate. 0.5 µl of formic acid (Vitek MS-FA, ref 411071) was added to each well. After air drying (approximately 5 min) 1 µl of matrix was added on each spot and these were again dried. Once this was done, the slide was inserted into the Vitek MS, and analysis was instigated after transferring the data from the Prep Station to the Vitek MS. Sample preparation was performed using the Prep Station, a module consisting of a computer and a barcode reader, which are used to enter the various sample data and their sites onto the slide. Measurements were performed with the MALDI BioTyper MYLA® software and the spectra obtained were compared with those from the database for validation. The results were measured by two parameters, namely the degree of confidence or percentage score, and the confidence level of the different colours. Green colour and a score between 99.9 and 60% indicates good identification, orange colour and a score of < 60% indicates a low probability of identification, and when the colour is red with zero percentage, then no identification has been made.

Resistance of *C. albicans* to antifungal drugs

Anti-fungal susceptibility testing (Zhang et al., 2014) was done for 68 isolates of *C. albicans* by using ATB Fungus 3® of Biomérieux. This method enables to determine the susceptibility of the *C. albicans* isolates to the antifungal agents in a semi-solid medium following the conditions recommended by the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) (National Committee for Clinical Laboratory Standards, 1997). ATB Fungus 3® was performed following manufacturer's instructions. Briefly, ATB Fungus 3®of Biomérieux strip consists of 16 pairs of cupules including two growth control wells and five antifungal drugs at different concentrations: 5-Flucytosine (4, 16 µg/ml), Amphotericin B (0.5 to 16 µg/ml), Fluconazole (1 to 128 µg/ml), Itraconazole (0.125 to 4 µg/ml) and Voriconazole (0.06 to 8 µg/ml). The inoculated strips were used in duplicate (c and C) and were read visually after incubation at 37°C for 24 h. For each antifungal agent, the reading of the strips was started with the lowest concentration. The growth score was recorded for each of the wells and compared with the control wells as follows: No reduction in growth (4), slight reduction in growth (3), distinct reduction in growth (2), very weak growth (1) and no growth (0). For Amphotericin B, the minimum inhibitory concentration (MIC) of the *Candida* species corresponded to its lowest concentration, thus enabling complete growth inhibition. For Fluconazole, Itraconazole and Voriconazole, as the possibility of a trailing growth existed, the MIC corresponded to the lowest concentration of the anti-fungal agent, with which a score of 2, 1 or 0 was obtained. For Flucytosine, a growth was looked for and was quantified in both the wells and tested for two concentrations. The results obtained gave an MIC that helps to classify the strain insensitive, intermediate or resistant. The anti-fungal breakpoints used followed the CLSI guidelines (National Committee for Clinical Laboratory Standards, 1997).

Statistical analysis

The data were statistically analysed using the Graphpad instat 3 software using the Chi-square test (x2) and the Pearson's correlation test at an α risk of 5%. The p value < 0.05 was considered statistically significant.

RESULTS

The 105 *Candida* isolates came mainly from vaginal

Table 1. *Candida* species identification rates by using MALDI-TOF MS (N=105).

Species	Total number of samples Identification		Score
	N=105	Rates (%)	
C. albicans	68	64.8	99.9
C. tropicalis	15	14.3	99.9
C. glabrata	9	8.6	99.9
C. parapsilosis	7	6.6	99.9
C. krusei	4	3.8	99.9
C. guillermondii	2	1.9	99.9
Total	105	100	99.9

N: Total number of identification; C: *Candida*.

Table 2. *C. albicans* in resistance pattern reported to antifungal drugs.

Pattern	Antifungal drugs [No. (%)]				
	5 FC	AMB	FCA	ITR	VRC
S	67 (98.5)	68 (100)	41 (60.3)	34 (50)	48 (69.2)
I	0	0	9 (13.2)	7 (10.3)	2 (2.9)
R	1 (1.5)	0	18 (26.3)	27 (39.7)	18 (27.9)

Table 3. *C. albicans* in resistance pattern reported to antifungal drugs by localization.

Antifungal drugs (%)	Localisation of samples								
	Vaginal			Oropharyngeal			Ear pus		
	N=47			N=15			N=2		
	n (%)								
	S	I	R	S	I	R	S	I	R
5FC	46 (97.9)	0	1(2.1)	15(100)	0	0	2(100)	0	0
AMB	47(100)	0	0	15(100)	0	0	2(100)	0	0
FCA	29(61.7)	7(14.9)	11(23.4)	7(46.7)	2(13.3)	6(40)	1(50)	0	1(50)
ITR	25(53.2)	4(8.5)	18(38.3)	6(40)	2(13.3)	7(46.7)	0	0	2(100)
VRC	32(68.1)	2(4.3)	13(27.7)	11(73.3)	0	4(26.7)	1(50)	0	1(50)

N: Total number of identification; n represents the number front of the percentage.

exudates (70.5%), oropharyngeal (19.1%) and sperm (4.4%) swabs. The other swabs were taken from ear pus (2.9%), sputum (1.5%) and stools (1.5%).

Culture on chromogenic media and MALDI-TOF MS identification results

A total of 68 strains of *C. albicans* (64.8%), 15 strains of *C. tropicalis* (14.3%) and 4 strains of *C. krusei* (3.8%) were identified by culture on chromogenic media. There were a further 18 strains (17.1%) of other species of *Candida* sp. All results were confirmed and *Candida* spp correctly identified by mass spectrometry with a score of 99.9%. *C. albicans* which was the most prevalent (64.8%) species (Table 1).

Resistance of *C. albicans* to antifungal drugs results

A total of 68 strains of *C. albicans* were subjected to *in vitro* antifungal susceptibility testing. No resistance to amphotericin B was observed with a minimum inhibitory concentration of 0.5 µg/ml, while 1.5% of strains exhibited resistance to 5-fluorocytosine. Regarding the azoles tested, resistance to itraconazole was particularly high at 39.7%, followed by voriconazole (27.9%) and fluconazole 26.3% (Table 2). Concerning the type of sample, resistance was higher in *C. albicans* strains taken from ear pus ($p = 0.0113$) (Table 3).

DISCUSSION

Candidosic infections are most frequently caused by *C. albicans*, as evidenced by epidemiological studies carried out in the United States of America (Cleveland et al., 2015), Europe (Klingspor et al., 2015), the Middle East (Sharifzadeh et al., 2013) and Africa (Kechia et al., 2015). *C. albicans* was the most prevalent strain (64.8%) in our series, as in several other studies (Bailly et al., 1995; Djohan et al., 2011; Kechia et al., 2015; Lacroix et al., 2014). The predominance of *C. albicans* could be explained by its considerable ability to adhere to host constituents, as well as by its ability to modify its behaviour according to the environment and the secretion of lytic enzymes (Calderone and Fonzi, 2001), which involves specific ligand/receptor interactions with mannoproteins of the yeast wall (Hoyer et al., 1998). In undergoing dimorphic transition from the blastospore to filamentous state, *C. albicans* increases its adhesion properties, its intercellular penetration capacity and its secretion of proteases. The blastospores appear to initiate the infection, while hyphae are involved in it spreading. Hyphae are less easily phagocytosed because of their morphology, and their large size may cause the death of the macrophages. They are also able to penetrate easily into the epithelial and endothelial layers (Karkowska-kuleta et al., 2009; Roman et al., 2007). Moreover, the secretion of hydrolytic enzymes during infection promotes virulence by degrading the surface of the host's mucous membranes and immune defences. These enzymes are aspartyl proteinases (Saps), phospholipases and lipases (Arslan, 2016; Schaller et al., 2005). Although *C. albicans* is the species most commonly responsible for this infection, there are increasing reports of a rise in candidiasis due to other *Candida* species (Amouri et al., 2010; Bonouman-Ira et al., 2011; Panizo et al., 2009).

C. albicans was isolated on *Candida* chromatic chromogenic medium along with *C. tropicalis* (14.3%) and *C. krusei* (3.8%), whereas in the Bernal et al. (1996) study, four *Candida* species: *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* were identified using CHROMagar *Candida* with a very high percentage of reliability. With respect to *C. albicans* identification, the susceptibility and specificity obtained in another study using the same CHROMagar *Candida* chromogenic medium were 100% similar for each of the above-mentioned parameters (Odds and Bernaerts, 1994).

All strains were identified by MALDI-TOF mass spectrometry with a score of 99.9 (Table 1). Six *Candida* species were identified, in contrast to Lacroix et al. (2014), who took surface and deep swabs from hospital patients in haematology, intensive care and undergoing kidney transplants, and obtained an overall identification rate of 98.2% with four species predominating: *C. albicans* (88%), *C. dubliniensis* (3.2%), *C. krusei* (4.6%) and *C. tropicalis* (4.1%) (Lacroix et al., 2014). Nocon (2013), on the other hand, identified 88.8% of bacteria at the species level: *C. albicans* (93.3%) and *C. glabrata* (66.6%).

Our evaluation of *C. albicans* resistance to antifungals by ATB Fungus 3, a method of microdilution in a semi-solid medium, revealed an increase in the level of *C. albicans* resistance to azole antifungals over the 9 years since Djohan's study on the susceptibility of *C. albicans* strains of vaginal origin from the Institut Pasteur of Côte d'Ivoire. The level of resistance to itraconazole increased from 22.2 to 39.7%, to voriconazole from 11.1 to 27.7% and to fluconazole from 2.2 to 26.3%. However, the data collected during the earlier study provide no indication of whether the patients received treatment before the examination, nor is it possible to clarify whether resistance is primary or secondary.

Furthermore, abusive use of these molecules has led to rising incidences of antifungal resistance (Vandeputte et al., 2012). According to a study carried out in Abidjan in 2008, *C. albicans* accounted for 72.6% of isolated strains of vaginal origin with varying rates of resistance to common antifungals: 2.2% for fluconazole, 11.1% for voriconazole and 22.2% for itraconazole (Djohan et al., 2011). In Cameroon, more than half of *Candida* yeasts were resistant to fluconazole in 2012 (Gonsu Kamga et al., 2014). Elsewhere in the world, high *C. albicans* resistance to azoles has been reported by several authors (Bagg et al., 2005; Chryssanthou, 2001; Nasrollahi et al., 2015; Sandra et al., 2005). These high rates of antifungal resistance provide good reason for regular monitoring of *C. albicans* susceptibility to these drugs to ensure effective treatment of candidosic lesions.

Azole antifungals are often the preferred treatments for many *Candida* infections because, on the one hand, they are inexpensive and, on the other, have low toxicity and can be administered orally (Berry et al., 1992; Whaley et al., 2017). Fluconazole is the most frequently prescribed antifungal for most *C. albicans* infections (Pfaller et al., 2002). Its resistance rate varies a lot, so that while a higher resistance rate (94%) was observed in Tehran in 2015 (Nasrollahi et al., 2015). In addition, Gonsu et al. (2014) found that over half of *Candida* yeasts were resistant to fluconazole. But, several authors have found low levels of fluconazole resistance (Jin-sol et al., 2007; Saporiti et al., 2001, Skrodeniene et al., 2006, Sobel et al., 2003), for example, St-Germain et al. (2001) found that only two out of 43 *C. albicans* isolates were fluconazole resistant, and these were isolates from one patient with AIDS and one with leukaemia, both of whom had already been treated with fluconazole. According to these authors, only patients who have already undergone long-term treatment with it are resistance to fluconazole. In contrast, El-Din et al. (2001), Sobel et al. (2004) and Khosravi et al. (2008), all reported no *C. albicans* resistance to fluconazole.

The high levels of resistance to voriconazole and to itraconazole found in our study are not consistent with the results of some other studies. Indeed, several authors have reported no voriconazole resistance (Jin-Sol et al.,

2007; Kronvall and Karlsson, 2001; Panizo et al., 2009; Pfaller et al., 2002; Tortorano et al., 2003). As for Itraconazole, moderate resistance rates of 13, 16.2 and 18% were reported, respectively, by Chryssanthou (2001), Bagg and Sandra (2005), in contrast to Khosravi, who found no *C. albicans* resistance to this drug in 2008.

Resistance to azoles frequently occurs when the target (14-α-demethylase) is modified. This enzyme is involved in synthesis of ergosterol within the membrane and is encoded by the Cyp51 gene (also called ERG11). Spot modifications of Cyp51 reduce the azole's affinity for its target. Only mutations at specific positions lead to resistance to an azole or to all azole drugs. In yeasts such as *C. albicans*, resistance to azoles is also related to increased activity of efflux pumps, which leads to rapid elimination of the antifungals (Guillot and Dannaoui, 1995).

Our study confirmed the excellent *in vitro* activity of amphotericin B on *C. albicans* with an MIC ranging from 0.5 to 1 mg/L (Khosravi et al., 2008; Panizo et al., 2009; Sandra et al., 2005; Skrodeniense et al., 2006). Several authors have also observed low levels of resistance to fluorocytosine (Godoy et al., 2003; Sandra et al., 2005; St-Germain et al., 2001), although Khosravi found increased levels of resistance (83.2%) in Tehran in 2008. Fluorocytosine resistance develops rapidly when the molecule is used alone, which has to do with a combined deficiency of its penetration (alteration of a purine-cytosine permease) or its metabolism (alteration of cytosine deaminase or UMP pyrophosphorylase) in the fungal cells (Dannaoui et al., 2012).

Low levels of fluconazole resistance in *C. albicans* strains of oropharyngeal origin have been observed by Bailly et al. (1995). In their study, nine of the 108 strains (8.3%) exhibited microbiological resistance to fluconazole, a result consistent with previous studies that identified resistant *C. albicans* strains *in vitro* (Regli et al., 1992; Ruhnke et al., 1994). It was not possible to verify whether patients from whom five of the resistant *C. albicans* strains were isolated had taken fluconazole in the 30 days preceding specimen collection. The remaining 4 strains were isolated from patients receiving fluconazole chemoprophylaxis, a situation consistent with secondary resistance.

Previous studies have shown that *C. albicans* is usually sensitive to most azoles (Amouri et al., 2010; Jin-Sol et al., 2007). It would be interesting to realize the resistance of *C. albicans* to antifungals from other tests, based on the principle of MIC, developed according to the protocol of the CLSI or the EUCAST, by incorporating sensitive and resistant reference strains, to compare the data on the resistance of *C. albicans* to antifungals.

Conclusion

Our study shows that *C. albicans* which was the most prevalent (64.8%) species, is not resistant to Amphotericin B, medicine commonly used to cure candidosic affections in Côte d'Ivoire. However, the relatively high level of resistances observed with itraconazole, voriconazole and fluconazole constitute a real challenge and calls for national strategies to monitor the resistance patterns of the antifungals used and to determine the different underlying mechanisms, particularly in African countries, where the burden of HIV/AIDS is still a problematic issue.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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