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MALDI-TOF MS identification and antifungal susceptibility of *Candida* strains isolated from vulvovaginal candidiasis by the AST-YS08® Card with Vitek 2® in Dakar, Senegal

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The objective of this study is to determine the susceptibility profile of different *Candida* strains isolated from vulvovaginal candidiasis and identified by MALDI-TOF MS, using the AST-YS08 Card with Vitek 2. The AST-YS08 card with Vitek 2 was used to test the antifungal susceptibility of 38 clinical isolates of *Candida* recovered from vulvovaginal swabs throughout the period 2017 to 2018. The study included *Candida albicans* (n = 28), *Candida tropicalis* (n = 6), *Candida glabrata* (n = 3) and *Candida parapsilosis* (n = 1), previously identified by MALDI-TOF MS. As antifungals, polyenes (amphotericin B, AMB), azoles (fluconazole FLC and voriconazole VRC) and echinocandins (caspofungin CPF and micafungin MCF) were tested. All isolates of *C. tropicalis*, *C. glabrata* and *C. parapsilosis* were sensitive to FLC, VRC, CPF, MCF and AMB as well as for 100% of *C. albicans* isolates to FLC, CPF, MCF and AMB. However, 5% (n = 2) of *C. albicans* isolates showed resistance to VRC with MIC values four and eight times higher than for *C. albicans* breakpoint, respectively. Feasible and easy germ tube test detection of VVC strains showed a very high sensitivity (100%) but with specificity 80% judged by MALDI-TOF MS. Decreased susceptibility to VRC remains a little worrying since this molecule constitutes currently a recourse in case of resistance to FLC.

Key words: Antifungal susceptibility, Candida, vulvovaginal candidiasis, AST-YS08 card, Vitek 2, MALDI-TOF MS, Dakar.

INTRODUCTION

Vulvovaginal candidiasis (VVC), caused by *Candida* species, is a common and recurrent disorder in women (Belayneh et al., 2017; Lirio et al., 2019). Adequate treatment requires species determination confirmed by laboratory findings for effective treatment (Sobel, 2016). Majority of VVC cases is caused by *Candida albicans*

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infections in women with recurrent VVC infections resembling about 80 to 95% of examined cases (Fornari et al., 2016; Alfouzan et al., 2015). However, increasingly, episodes due to non-albicans species as *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, *Candida kefyr* and *Candida lusitaniae* appear in immunocompromised patients as well as in immunocompetent women (Fornari et al., 2016; Alfouzan et al., 2015; ElFeky et al., 2016).

In Senegal retrospectively, VVC prevalence was estimated at 24, 34.8 and 27.2% during 2006, 2008 and 2015, respectively (Diongue et al., 2018a). However, these studies were limited about the identification of the causative species due to the fact that only germ tub test (GTT) was performed to distinguish *C. albicans* from non-*albicans* species. More recently in 2018, a study was carried out using MALDI-TOF MS as identification tool showing high percentage (70%) of VVC cases caused by *C. albicans*. The other 30% were non-*C. albicans* species as 15% caused by *C. glabrata*, 8% by *C. tropicalis* and 7% by *C. krusei* (Kechrid, 2018). Since these non-*C. albicans* species do not have the same susceptibility to antifungals, it is of great importance to test them specifically using the techniques (automated and marketed) with a good adaptation to the routine such as the AST-YS® cards with Vitek 2® which is a reliable quick and feasible technique to determine antifungals susceptibility of *Candida* species (Pfaller et al., 2007; Posteraro et al., 2009; Cuenca-Estrella et al., 2010), approved from the United States Food and Drug Administration (FDA) (Pfaller et al., 2007). Therefore, the purpose of this study was to determine the susceptibility profile of different *Candida* strains isolated from VVC and identified by MALDI-TOF MS, using the AST-YS08 Card with Vitek 2.

MATERIALS AND METHODS

This is a cross-sectional descriptive study that was carried out on clinical vulvovaginal *Candida* isolates collected from women at the Parasitology and Mycology laboratory in Aristide Le Dantec University Hospital in Dakar, Senegal from 2017 to 2018.

Candida strains isolation and identification process

VVC samples were cultured on media containing Sabouraud-chloramphenicol dextrose agar incubated at 30 to 37°C for 24 to 48 h. Positive cultures for yeasts colonies were submitted to GTT according to a previous article (Sy et al., 2018) read once after 2 h of incubation, post negative urease test orienting to *Candida* spp. GTT led to distinction between *C. albicans* and non-*C. albicans* strains which were all maintained on brain-heart infusion broth supplemented with 15% glycerol and stored at -20 °C for downstream analyses. Then, they were randomly selected with a sampling interval of 3 until 40 samples were achieved (number of tests for two reagent kits 40). Selected isolates were identified at species level by MALDI-TOF MS in "IHU Méditerranée Infection'' in Marseille, France (Diongue et al., 2018b) in three steps as the manufacturer's instructions, post obtaining fresh colonies, as follows. The first step; fungal proteins were extracted using a mixture of 900 µL of anhydrous ethyl alcohol (Carlo Erba SDS, Val de Reuil, France) and 300 µL of sterile water (Water HPLC, Prolabo, BDH, Fontenay-sous-Bois, France) in a microtube. The second step under a chemical safety cabinet, consisted of suspending the pellet after centrifugation, in a volume-volume mix of formic acid (Sigma-Aldrich, Lyon, France) and acetonitrile (Prolabo BDH). While the third step after a last centrifugation, the supernatant containing the whole fungal proteins was deposited on the target plate (two spots of 1 µL of protein extract per isolate), covered with 1 µL of α-cyano-4-hydroxycinnamic acid matrix (Sigma-Aldrich, Lyon, France).

The MALDI-TOF MS analyses were conducted using a Microflex LT system (Bruker Daltonics GmbH, Bremen, Germany). The Biotyper software compared the protein profile of the microorganisms obtained from the reference spectra. For the clinical validation, identification of the spectra, with related logscore values, was performed with the Biotyper using either the in-house library (5945 spectra) or the commercially-available Bruker database dedicated to fungi (4111 spectra). Logscore values were therefore not strictly used as interpretative criteria for the reliability of the identification but were provided to indicate that according to the manufacturer. Scores <1.70 indicated unreliable identification, while scores of 1.70-1.99 and ≥2.0 indicated acceptable genus and species level identification.

Antifungal susceptibility testing

The Vitek 2 card AST-YS08 (BioMérieux, France) was used. The susceptibility tests were performed according to the manufacturer's recommendations. Briefly, a yeast suspension was adjusted to 2 McFarland (range: 1.8-2.2) using the DensiCheck (BioMérieux). Each suspension was diluted by transferring 280 µl to a tube containing 3 ml of saline solution. It was incubated for 10 to 26 h and read automatically, after inserting the card with the yeast suspension. The results were expressed as MICs. The Vitek 2 AST-YS08 card contains serial dilutions ranging from 1 to 32 µg/ml for amphotericin B, from 0.12 to 8 µg/ml for caspofungin, from 2 to 64 µg/ml for fluconazole, from 0.06 to 4 µg/ml for micafungin and from 0.5 to 8 µg/ml for voriconazole.

Susceptibility breakpoints

Clinical Laboratory Standard Institute (CLSI) *in vitro* susceptible and resistant breakpoints for *C. albicans*, *C. tropicalis, C. glabrata* and *C. parapsilosis* to fluconazole (FLC), voriconazole (VRC), caspofungin (CPF) and micafungin (MCF) were applied according to Pristov and Ghannoum (2019) completed by European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (v.9.0) for amphotericin B (AMB) for the same species (EUCAST, 2019).

Quality control

This was ensured by testing the *C. krusei* (ATCC 6258) strain.

Statistical method

The study data was entered and processed with the Microsoft® Excel 2016 software. Only percentage and frequency calculations were performed.

RESULTS

A total of 39 randomly selected isolates which were all

Table 1. Distribution of *Candida* strains identified by MALDI-TOF MS.

Clinical strains	Number	Percentage
Candida albicans	28	71.8
Candida tropicalis	6	15.4
Candida glabrata	3	7.7
Candida parapsilosis	1	2.55
*Saccharomyces cerevisiae**	1	2.55
Total	39	100

*The strain of *Saccharomyces cerevisiae* was finally tested but non-included and discarded away as it as non-*Candida* spp.

Table 2. Evaluation of the sensitivity and specificity of germ tub test versus MALDI-TOF MS.

Germ tub test	MALDI-TOF MS		
	C. albicans	**non-*C. albicans***	**Total**
C. albicans	28	2	30
non-albicans *Candida*	0	8	8
Total	28	10	38

Sensibility: 100%; Specificity: 80%; Positive predictive value: 93.3%, Negative predictive value: 100%.

clinical strains isolated from VVC were studied. Based on GTT, 77% (30/39) of them were identified as *C. albicans*/*dubliniensis* and 23% (9/39) as non-*C. albicans*. According to the MALDI-TOF MS identification of the same strains, *C. albicans* was predominant with 71.8% (28/39) of isolates, followed by *C. tropicalis* with 15.4% (6/39), *C. glabrata* with 7.7% (3/39) and *C. parapsilosis* with 2.55% (1/38) (Table 1). One strain (1/39 as 2.55%) was non-*Candida* spp. identified as *Saccharomyces cerevisiae* and finally non-included.

Three discrepancies were recorded between the GTT and the MALDI-TOF MS identification because two non-*Candida albicans* species (*C. tropicalis* and *C. parapsilosis*) were misidentified as *C. albicans* as well as the isolate of *S. cerevisiae* which was misidentified as a non-*C. albicans* strains with the GTT. Thus, the GTT presented a very good sensibility (100%) with a good specificity (80%) compared to MALDI-TOF in identifying the *Candida* spp. (Table 2).

All *C. tropicalis*, *C. glabrata* and *C. parapsilosis* isolates were sensitive to FLC, VRC, CPF, MCF and AMB as well as for 100% of *C. albicans* isolates to FLC, CPF, MCF and AMB. However, 5% (n = 2) of *C. albicans* isolates showed resistance to VRC with MIC values four and eight times higher than for *C. albicans* breakpoint (BP), respectively (Table 3).

DISCUSSION

Since VVC is so common, women disorders threaten recurrency (Belayneh et al., 2017; Lirio et al 2019), specific antifungal treatment is of great need, while, few studies concerned antifungal susceptibility of *Candida* isolates in Senegal are available (Sylla et al., 2019; Dieng, 2001) recommended inconvenient of Fungitest®. Thus, in this context, the objective of this present study was to determine the antifungal susceptibility profile of different strains of VVC *Candida* spp. as well as determination of MIC values of these antifungals with respect to the species and/or strains. As Vitek 2 instrument allows a reliable and fast detection of the weakest levels of resistance of *Candida* strains with a gain in time less than 24 h, its performance in antifungal susceptibility testing on *Candida* spp. has already been demonstrated (Alfouzan et al., 2015; Gonzalez-Lara et al., 2017). On this basis, it has been shown that the results provided by Vitek 2 system are identical with those of the conventional method (Gonzalez-Lara et al., 2017) and those of the E-test (Alfouzan et al., 2015) with a substantial gain in time.

In total, antifungals susceptibility testing was performed on 39 clinical isolates from VVC identified in 77% (30/39) as *C. albicans*/*dubliniensis* and 23% (10/39) as non-*C. albicans* strains based on GTT while MALDI-TOF MS identification concluded on 28 *C. albicans* and 10 non-*Candida albicans Candida* which included *C. tropicalis* (n = 6), *C. glabrata* (n = 3) and *C. parapsilosis* (n = 1) plus one strain of *S. cerevisiae* finally excluded.

C. krusei exhibits natural resistance to fluconazole while *C. glabrata* has variable or even dose-dependent azole susceptibility (Alfouzan et al., 2015), though,

Table 3. Antifungal susceptibility profile of randomly selected *Candida* isolates between 2017 and 2018 (n = 38).

Species (No. tested)	Fluconazole	Voriconazole	Caspofungin	Micafungin	Amphotericin B
C. albicans **(n = 28)**					
Range (mg/L)	≤2 - ≥8	≤0.12 - ≥1	≤0.25 - ≥1	≤0.25 - ≥1	≤1 - >1
S (%)	100	95	100	100	100
I (%)	0	0	0	0	0
R (%)	0	5	0	0	0
C. tropicalis **(n = 6)**					
Range (mg/L)	≤2 - ≥8	≤0.12 - ≥1	≤0.25 - ≥1	≤0.25 - ≥1	≤1 - >1
S (%)	100	100	100	100	100
I (%)	0	0	0	0	0
R (%)	0	0	0	0	0
C. glabrata **(n = 3)**					
Range (mg/L)	ND - ≥64	ND - ND	≤0.12 - ≥0.5	≤0.06 - ≥0.25	≤1 - >1
S (%)	100	100	100	100	100
I (%)	0	0	0	0	0
R (%)	0	0	0	0	0
C. parapsilosis **(n = 1)**					
Range (mg/L)	≤2 - ≥8	≤0.12 - ≥1	≤2 - ≥8	≤2 - ≥8	≤1 - >1
S (%)	100	100	100	100	100
I (%)	0	0	0	0	0
R (%)	0	0	0	0	0

ND: Not defined.

regarding the GTT identification, two non-*Candida albicans* strains; *C. tropicalis* and *C. parapsilosis* were misidentified as *C. albicans* as well as a strain of *S. cerevisiae* as a non-*Candida albicans* strains. ElFeky et al. (2016) also noted the first discordance with a GTT positive in 38 isolates whereas only 33 of them were identified as *C. albicans* by PCR-RFLP. Thus, they found the same specificity of GTT as we noticed with 86.8% of sensibility vs. 100 for us.

Therapeutic application against *C. parapsilosis* isolates have been found to be increasingly resistant to azoles with rates of fluconazole resistance found to be five times higher than those in *C. albicans.* Also, *C. tropicalis* shows resistance to azoles which is specifically high for fluconazole (Pristov and Ghannoum, 2019), demonstrating the usefulness of MALDI-TOF MS which has proved to be a rapid and reliable method for identification of *Candida* strains in the clinical laboratory (Yaman et al., 2012). Our findings showed that all our isolates of *C. albicans* tested were sensitive to FLC, VRC, CPF, MCF and AMB. However, 5% (n = 2) of *C. albicans* isolates showed resistance to VRC with MIC values four and eight times higher than for *C. albicans* breakpoint, respectively. Similar results with *C. albicans* were found in Kuwait in 2015 using E-test (AB Biodisk, Solna, Sweden) on vaginal isolates, but without resistance to FLC and VRC (Alfouzan et al., 2015). Contrarily, vaginal *C. albicans* resisted FLC and VRC using semi-solid medium microdilution technique, ATB1 Fungus 3 (BioMérieux, France) (Djohan et al., 2012). Resistance to fluconazole affects 2% of *C. albicans* strains, especially in patients at risk of fungal infections subjected to fluconazole prophylaxis (Dignani et al., 2009). The absence of such a prophylactic protocol in our context could explain the absence of fluconazole resistance of our strains. On the other hand, the two cases of *C. albicans* resisted VRC with very high MICs from four to eight times higher than the *C. albicans* BP (range: ≤0.12 and ≥1) seem to us somewhat surprising whereas they exhibit sensitivity to fluconazole with respective low MICs ≤0.5 and 1 µg/mL. As VRC is not available in Senegal and so, drug pressure was excluded. In fact, there are many ways in which *Candida* spp. may become resistant to azoles with the most common mechanism found on *C. albicans* isolates is the constitutive overexpression of ERG11 via gain-of-function mutations in the transcriptional activator Upc2. Subsequently, this overexpression resulted in efflux pumps, then cross-resistance between azoles is often seen in *C. albicans*, both *in vitro* and clinically (Pristov and Ghannoum, 2019; Revie et al., 2018). In the present study, concerning six strains of *C. tropicalis* tested, susceptibility was found for

all tested antifungals. Similar results were found in *Candida* VVC using disk diffusion method on Muller-Hinton agar supplemented (ElFeky et al., 2016). Sensitivity of *C. tropicalis* found in the present study was recommended as usual profile of susceptibility to antifungal (Pristov and Ghannoum, 2019), while vice versa through a recent study in Dakar (Sylla et al., 2019) and China where higher proportion of *C. tropicalis* FLC resistant was observed using a commercial agar diffusion test (A/S Rosco, Taastrup, Denmark) (Liu et al., 2014). These differences may be attributed to the fact that *C. tropicalis* exhibits resistance to azoles, more particularly to fluconazole, with an increase in the number of efflux pumps (Pristov and Ghannoum, 2019). The three strains of *C. glabrata* as well as for the *C. parapsilosis* strain tested in this study exhibited total susceptibility to all antifungals (AMB, FLC, VCR, CPF and MCF). Generally, *C. glabrata* is susceptible to echinocandins and has a low sensitivity to azoles due to overexpression of efflux pumps (Pristov and Ghannoum, 2019). While poor FLC activity on *C. glabrata* strains among *Candida* species isolates was achieved by E-test (Badiee and Alborzi, 2011) or intermediate sensitivity to FLC of all their *C. glabrata* strains (Scapaticci et al., 2018). Despite the fact that *C. glabrata* is also susceptible to AMB, but delayed *in vitro* killing kinetics (Pristov and Ghannoum, 2019). This is inconsistent to the present findings due to availability of these molecules in these countries contrary to our context in Senegal.

Conclusion

Feasible and easy GTT detection of VVC strains showed a very high sensitivity (100%) but with specificity, 80% judged by MALDI-TOF MS. Most of the present *Candida* isolates notably *C. albicans, C. tropicalis, C. glabrata* and *C. parapsilosis* were susceptible to all tested antifungals: azoles, echinocandins, and amphotericin B to be as promising remedy for this recurrent disease. In contrast, resistance to voriconazole was noted for two isolates of *C. albicans* with high MICs. This last observation seemed surprising especially when voriconazole, a triazole, is supposed to be a remedy for fluconazole-resistant isolates.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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