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Fluconazole and voriconazole susceptibility in oral colonization isolates of *Candida* spp. in HIV patients.

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Key words: candidiasis; HIV; antifungal susceptibility; non-*albicans* species.

Abstract. The identification of *Candida* species and their antifungal susceptibility is important for the treatment of infected patients. The aim was to determine the susceptibility to fluconazole and voriconazole in isolates from oral colonization of *Candida* spp. in HIV patients. From the 135 patients studied, 33.3% were females and 66.7% males, with a mean age of 36.6 years and 83.7% of them were under treatment. The identification of the specie was performed by the API20CAUX® test and the antifungal susceptibility was determined by the disc diffusion test. Strains of *C. parapsilosis* and *C. krusei* were used as quality controls. The *Candida* species identificated were: *C. parapsilosis complex* 52%, *C. albicans complex* 36% and *C. famata* 12%. 60% of the isolates were susceptible to fluconazole and 40% were dose-dependent. All were susceptible to voriconazole. In this study, none of the patients had oropharyngeal candidiasis however, 18.5% had *Candida* spp. colonization, this percentage being below other studies in HIV carriers. We found a high proportion of non-*albicans* species but no difference in the CD4+ counts between patients.

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Susceptibilidad a fluconazol y voriconazol en aislados orales de *Candida* spp. en pacientes con VIH.

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Palabras clave: candidiasis; VIH; susceptibilidad antifúngica; especies no-*albicans*.

Resumen. Identificar las especies de *Candida* y la susceptibilidad a los antifúngicos es importante para el tratamiento adecuado de pacientes infectados. El objetivo fue determinar la susceptibilidad a fluconazol y voriconazol de *Candida* spp. en aislados de colonizaciones orales en pacientes con VIH. La identificación se realizó mediante la prueba API20CAUX® y la prueba de difusión de disco para la sensibilidad a los antifúngicos. Se utilizaron cepas de *C. parapsilosis* y *C. krusei* como control de calidad. De los 135 pacientes, el 33,3% eran mujeres y el 66,7% hombres con una edad media de 36,6 años y el 83,7% recibían tratamiento. Las especies de *Candida* identificadas fueron: complejo *C. parapsilosis* 52%, complejo *C. albicans* 36% y *C. famata* 12%. La susceptibilidad al fluconazol 60% eran susceptibles y 40% dependía de la dosis. Todas eran susceptibles al voriconazol. En este estudio, ninguno tenía candidiasis orofaríngea, sin embargo, el 18.5% tenía colonización por *Candida* spp., aunque el porcentaje está por debajo de otros estudios en portadores de VIH. Encontramos una alta proporción de especies no-*albicans*. No hubo diferencias en el recuento de $CD4^+$ entre los pacientes.

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INTRODUCTION

In HIV patients under antiretroviral therapy (ART) a reduction of oral candidiasis by *C. albicans* have been observed; however, the emergence of non-*albicans* species with resistance to antifungal agents has also been observed and the presence of oral *Candida* spp. in these patients can predict the development of oral candidiasis (1-3). To identify the *Candida* species involved and the study of antifungal susceptibility is important for the epidemiological mapping of the species and to improve the treatment of these patients(4). Fluconazole is used in the treatment of HIV/AIDS associated opportunistic yeast infections and prolonged exposure is associated with the increase of resistance (1,4,5). The aim of this work was to determine the susceptibility to fluconazole (FCZ) and voriconazole (VCZ) in the isolates of the oral colonization of *Candid*a spp. in HIV/AIDS patients.

METHODS

A *cross-sectional* study was made in 135 seropositive HIV patients previously diagnosed by ELISA and confirmed by Western blot at the University Hospital (SAHUM) HIV/AIDS program, Maracaibo - Venezuela. None had clinical evidence of oral candidiasis. Patient's age, $CD4^+$ T-lymphocyte count, World Health Organization (WHO) immunological classification for established HIV infection (5) and ART were obtained from their medical records.

Ethical clearance was obtained by the Medical board in accordance with the ethics committee of SAHUM and participants signed informed consent. Identification and susceptibility were reported to each subject and the attending physician.

Samples: A swab was collected from the oral mucosa, floor of the mouth and dorsal tongue. Samples were cultured in Sabouraud-dextrose agar (SDA - BD Difco™ MD, USA) with ampicillin (500mg/L) for 24h at 35ºC. Blastoconidies were observed.

Identification: Pure colonies were plated in CHROMAgar® Candida (Biomedics®) for 48h at 35°C following manufacturer's instructions. Morphology and color on CHROMAgar® Candida colonies were noted. Germ tube test was achieved inoculating 0.5 ml of sheep sterile serum with a scoop (5 μl) of yeast, incubated for 3h at 37°C. Isolates with germ tube-positive were presumed to be *C. albicans complex*. Chlamydospores production was assessed by culturing on cornmeal agar supplemented with 1% Tween-80 (SIGMA-ALDRICH®) at 25°C for 72h followed by microscopic observation. The isolates were identified by API20CAUX® test (bioMérieux®SA, Marcy-l'Etoile, France) in accordance with manufacturer's instructions.

Antifungal susceptibility of identified isolates as outlined in NCCLS M44-A2 document for disc diffusion susceptibility testing in yeasts using Mueller Hinton + 2% glucose + 0.5 g/mL methylene blue dye (GMB) agar and FCZ (25μg) and VCZ discs (1μg) (Bioanalyse®). After 24h at 37°C of incubation, the zones of inhibition were measured. FCZ breakpoint for *C. albicans, C. parapsilosis* and *C. tropicalis* was: sensible (S) ≥17; dose-dependent sensibility (DDS) 14-16 and resistance (R) ≤13. For *C. glabrata* (S) ≥17; SDD ≥15 y R ≤14. VCZ breakpoint was S ≥17; DDS 15- 16 and R ≤14 to *C. albicans, C. parapsilosis, C. tropicalis* and S ≥15; DDS 13-14; R ≤12 to *C. krusei* (6,7). Minimal inhibitory concentration (CMI) results were interpreted according to CLSI interpretive criteria (6,7). *As quality control*, ATCC strains of *C. parapsilosis* 22019 and *C. krusei* 6258 were used. For *statistical analysis*, the chi-square test and the Fisher's exact test using the Graph Pad Prism 5.0 program were used.

RESULTS

A total of 135 patients with a confirmed diagnosis of HIV were enrolled on this study, 33.3% (n=45) females and 66.7% (n=90) males with a mean age of 36.6 years (SD±11.28) ranging from 21 to 60 years ($p=0.21$). According to WHO (5) immunological classification for established HIV infection ($mm^3/CD4^+$): 27.4% (n=37) of the patients had not significant, immunosuppression; 22.2% (n=30) mild; 16.3% (n=22) advanced and 17.8% (n=24) severe with less than 200cel/mm^3. 16.3% (n=22) had no information on the medical chart because they are new incomings patients to the HIV program at the day that sample was collected ($p=0.48$). 83.71% (n=113) patients were on ART and 16.9% (n=22) were not because they were the new incoming patients ($p=0.96$). The most frequent treatment regimen was the combination with nucleoside reverse transcriptase inhibitor (NRTI) plus a protease inhibitor (PI) followed by a non-nucleoside transcriptase inhibitor (NNRTI) and a nucleotide reverse transcriptase inhibitor (NtRTI) or PI. Only one patient used a fusion inhibitor (FI) and none used integrase inhibitor (II) ($p=0.96$). Of all 135 patients 67.4% (n=91) were not under antimicrobial therapy, but 11.9% (n=16) reported use of any antifungal (fluconazole, ketoconazole or amphotericin B), 20% (n=27) antibacterial (cotrimoxazole, amoxicillin, azithromycin or clarithromycin) and less than 1% (n=1) antiparasitic (metronidazole) ($p=0.60$). Non-statistical significance was observed between age, gender, CD4+ and use of the ART or antimicrobial ($p>0.05$) (Table I).

TABLE I
GENDER, CD4+ CEL/MM^3, TREATMENT AND *Candida* spp. CULTURE

Characteristics	Positive *Candida* spp. (n=25)		Negative *Candida* spp. (n=110)		Total	
	Fx	%	Fx	%	Fx	%
Age, mean ± SD (years)	39.92±10.85		35.83±11.34		36.6±11.28	
Gender						*p=0.21*
Female	11	8.14	34	25.18	45	33.33
Male	14	10.37	76	56.29	90	66.66
CD4+ cel/mm^3 (WHO Classification)						*p=0.48*
>500 Not significant	8	5.92	29	21.48	37	27.40
350-499 Mild	8	5.92	22	16.29	30	22.22
200-349 Advanced	2	1.48	20	14.81	22	16.29
<200 Severe	3	2.22	21	15.55	24	17.77
Not Information*	4	2.96	18	13.33	22	16.29
Antiretroviral therapy (ART)						*p=0.96*
1-4 Drugs	21	15.55	92	68.14	113	83.71
New patient No ART	4	2.96	18	13.33	22	16.29
Antimicrobial Therapy						*p=0.60*
Antifungal	2	1.48	14	10.37	16	11.85
Antibacterial	6	4.44	21	15.55	27	20.00
Antiparasitic	0	0.00	1	0.74	1	0.74
No Antimicrobial therapy	17	12.59	74	54.81	91	67.4

* New patients without information in the medical chart at the sample collection day.

All 135 patients had no clinical evidence of oral candidiasis (angular cheilitis, leukoplakia or oral ulcerations), however, 18.5% (n=25) were colonized by *Candida* spp. Of these, 52% (n=13) were identified as *C. parapsilosis complex* (*C. parapsilosis sensu stricto, C. orthopsilosis* and *C. metapsilosis*); 36% (n=9) *C. albicans complex* (*C. dubliniensis, C. stellatoidea* and *C. africana*) and 12% (n=3) *C. famata*. The fluconazole MIC at 48 hours showed that 60% (n=15/25) were susceptible, of these, 24% (n=6/25) *C. parapsilosis complex*, 24% (n=6/25) *C. albicans complex* and 12% (n=3/25) *C. famata* and 40% (n=10/25) had doses dependent susceptibility, 28% (n=7/25) *C. parapsilosis complex* and 12% (n=3/25) *C. albicans complex*. None were resistant (*p=0.75*). All species of *Candida* identificated were susceptible to voriconazole MIC (*p=0.43*). Four patients with *Candida spp*. colonization were new incomings patients to the HIV/AIDS program without ART. One of these was admitted to the hospital with complicate tuberculosis plus oral *C. parapsilosis* and other with a neurocryptococosis plus oral *C. famata*. Also, two of the seven *C. parapsilosis* with doses dependent susceptibility was using FCZ daily for more than a month (*p=0.47*). Non-statistical significance was observed (*p>0.05*) (Table II).

TABLE II
CANDIDA SPECIES IDENTIFICATION BY GENDER, ANTIFUNGAL SUSCEPTIBILITY, CD4+ CEL/MM3 AND TREATMENT.

Characteristic	*C. parapsilosis* (n=13)		*C. albicans* (n=9)		*C. famata* (n=3)		Total	
	Fx	%	Fx	%	Fx	%	Fx	%
Gender								*p=0.61*
Female	6	24.00	4	16.00	1	4.00	11	44.00
Male	7	28.00	5	20.00	2	8.00	14	10.37
MIC Fluconazole (mg/L)								*p=0.75*
Susceptible	6	24.00	6	24.00	3	12.00	15	60.00
Doses Dependent susceptibility	7	28.00	3	12.00	0	0.00	10	40.00
Resistance	0	0.00	0	0.00	0	0.00	0	0.00
MIC Voriconazole (mg/L)								*p=0.43*
Susceptible	13	52.00	9	36.00	3	12.00	25	100.00
Doses Dependent susceptibility	0	0.00	0	0.00	0	0.00	0	0.00
Resistance	0	0.00	0	0.00	0	0.00	0	0.00
CD4+ cel/mm3 (WHO Classification)								*p=0.06*
>500 Not significant	4	16.00	3	12.00	1	4.00	8	32.00
350-499 Mild	3	12.00	4	16.00	1	4.00	8	32.00
200-349 Advanced	1	4.00	1	4.00	0	0.00	2	8.00
<200 Severe	3	12.00	0	0.00	0	0.00	3	12.00
Not Information*	2”	8.00	1	4.00	1º	4.00	4	16.00
Antiretroviral therapy (ART)								*p=0.94*
1-4 Drugs	11	44.00	8	32.00	2	8.00	21	84.00
New patient No ART	2”	8.00	1	4.00	1º	4.00	4	16.00
Antimicrobial Therapy								*p=0.47*
Antifungal	2^	8.00	0	0.00	0	0.00	2	8.00
Antibacterial	3	12.00	2	8.00	1	4.00	6	24.00
Antiparasitic	0	0.00	0	0.00	0	0.00	0	0.00
No Antimicrobial therapy	8	32.00	7	28.00	2	8.00	17	68.00

* New patients without information in the medical record at the sample collection day.

“ New patient with tuberculosis

^ Both with doses dependent susceptibility to fluconazole

º New patient with neurocryptococosis

DISCUSSION

The mean age of the patients was 36 years showing that HIV still is an infection among the young population as reported in other countries (5, 8-10). Within the 135 patients involved in this study, 90 were men and 45 were women, resulting in the ratio of two cases in men for every one case in women. Against what happens in some African countries with a female prevalence of 4.6% *vs.* 2.7% male prevalence and could be explained by the cultural, social, and economic vulnerability of women (8,9,11). The last HIV database from Venezuela show that more than 100.000 men were seropositive and approx. 38.000 women, so even with the non-statistical significance observed in our study, data shows that the incidence of HIV in men remains high, with a slow increase in women and both are worrying (12).

Candida spp. oral colonization among HIV patients may predict the development of oropharyngeal candidiasis (OPC) as the most common opportunistic infection in these patients and the progression is faster and more severe as a progressive depletion of $CD4^+$ T cells and/or failure to ART (3,5,8). No patient from this study had oropharyngeal candidiasis, however, 18.5% had *Candida* spp. colonization, being lower than the studies in HIV carriers in Argentina (72%), Brazil (62%), Cote d´Ívore (79.4%), India (68%) and Thailand (61%) (9,13-17). We found no difference in the $CD4^+$ counts between patients colonized by *Candida* spp. as other studies had found (11). Suggesting, as other authors also observed, a protective effect of ART regimens against OPC (1,5,18).

The patients without ART therapy one with complicated TBC and other with neurocryptococosis, both were considerate as severe immunodepression, using the WHO clinical staging as a guide for decision-making regardless of age or $CD4^+$ testing, particularly when the $CD4^+$ count is not available (5,18).

The susceptibility to an antifungal can be different between species and populations and the emergence of antifungal resistance made the identification of *Candida* spp. an essential to guide the therapeutic choice and clinical treatment (8,19-22). We have found no resistance against FCZ or VCZ, but 40% (n=10/25) of the species had DDS to FCZ: 12% (n=3/25) *C. albicans complex* and 28% (n=7/25) *C. parapsilosis complex*. Two of that seven were taking fluconazole, suggesting the exposure during ART provided a positive selection pressure for yeasts being less sensitive (3,4,20,22), or could be because *C. parapsilosis sensu extricto* show more resistance to FCZ (23-26).

This study addresses the importance on identification and antifungal susceptibility as guide to proper treatment in any mycosis. We find a high proportion of non-*albicans* species, against most of the studies in HIV carriers where *C. albicans* complex remains as the most common species (3,11,17). However, our data support Venezuelan´s studies, where *C. parapsilosis* is the most frequent species isolated (21,25,27-29). The epidemiology of *Candida* infections is changing: new species, better adapted virulence factors, further antifungal resistance and worse clinical presentations.

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