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In vitro Sensitivity to Fluconazole through Vitek II Systems, of Strains of *Candida spp.* In Patients with Oropharyngeal Candidiasis and HIV/AIDS

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Abstract

Background: *Candida albicans* is a commensal fungus of the mucosa in humans that may become an opportunistic pathogen causing recurrent infections in immunocompromised hosts. In individuals with HIV/AIDS, Histatin-5 levels are significantly reduced, causing oropharyngeal candidiasis to become a pathological process. Clinical and *in vitro* resistance to azoles is common whether by selection or acquisition of *Candida*-resistant strains. In 2008, the Clinical and Laboratory Standards Institute (CLSI) defined the cutoff point for active agents against the isolates of *Candida* species. The objective of this study was to determine the frequency of fungal isolates of *C. albicans* through VITEK II system and their susceptibility pattern in patients with HIV/AIDS and oropharyngeal candidiasis treated at the Hospital for Infectious Diseases in Mexico City.

Methods: From June 2011-December 2012, there were 96 patients with HIV/AIDS and oropharyngeal candidiasis who were included in the study. Oral and esophageal specimens were directly examined to identify fungal structures. Cultures and sensitivity testing were done with the Vitek II method. Descriptive statistics and bivariate analysis were carried out.

Results: Of 96 patients, 87 had *C. albicans* oral isolates identified and 73 esophageal isolates. Non-albicans Candida (NAC) was identified in three and ten patients (oral and esophageal) respectively, and *Cryptococcus neoformans* was isolated in both sites. Sensitivity of *C. albicans* to fluconazole was demonstrated in 87/90 strains.

Conclusions: The fungal pathogen isolated was *C. albicans* followed by *C. glabrata* and *C. parapsilosis*. *C. albicans* was identified through VITEK II in 90% of cases susceptible to fluconazole.

Keywords: Fluconazole; *Candida spp.;* Vitek II; Oropharyngeal; AIDS

Introduction

The genus *Candida* comprises a large and diverse group of cultured yeasts that are part of the human microbiota and colonize the mucosa of the intestinal tract. *Candida* acts as an opportunistic pathogen that causes recurrent infection sunder immunodeficiency conditions. The oral cavity is the most common site of infection by *C. albicans*. It has been proposed that a potent salivary peptide with anti-Candida properties, Histatin-5, plays an important role in protecting against exposure of the oral mucosa to colonization by commensal strains of *C. albicans*. In individuals with HIV/AIDS, Histatin-5 levels are significantly decreased. Thus, oropharyngeal candidiasis is a pathological process directly related to ineffective immune response [1-4].

Variability in the pathogenic potential of this species of fungus is the result of its ability to adapt, evolve and invade the host immune defense through regulation of the factors determinant for virulence in a selective manner under predisposing conditions [5-8]. Among other pathogenic characteristics of *C. albicans* are the secretion of aspartic proteases and hydrolytic enzymes [2,3] as well as the phenotypic change, characterized by formation of hyphae or pseudo hyphae and with it the antigenic modulation that facilitates tissue invasion. These virulence factors may vary depending on type, stage and location of the infection and the nature of the immune response.

Other species of the *Candida* genus have been identified in esophageal an doral lesions including *C. tropical, C. krusei, C. glabrata, C. parapsilosis, C. guilliermondii,* and others [9-15]. Some species of this yeast are known to be intrinsically resistant to antifungal agents, i.e., *C. krusei* to fluconazole [5,16].

C. albicans readily develops resistance to fluconazole and may occur by the alteration in the sterol 14-alpha demethylase encoded by the ERG16 [17], drug inactivity and the presence of genes associated with the efflux pump such as the BENr, the Erg11 gene as well as the over expression of the genes Cdr1, Cdr2 and Mdr1 [18-20]. The adhesion to the cellular surface [21,22] forming a biofilm [23] confer different levels of resistance to fluconazole as well as cross-resistance.

Since 2008, the Clinical and Laboratory Standards Institute (CLSI) defined the epidemiologic cut off point (ECVs) for active agents against isolates of *Candida* sp. The minimum inhibitory concentration (MIC) <8 mg/ml established sensitivity (S),16-32 µg/ml established the sensitive dose-dependent sensitivity (D-DS) and >64 µg/ml was established as resistant [24-26], CLSI also established (ECVs) to evaluate the emergence of strains with reduced susceptibility to antifungal agents and served as a bridge for the establishment of species-specific susceptibility or clinical breakpoints (CBPs). In the case of CBPs for fluconazole, species-specific values were provided for *C. albicans*, *C. tropicalis* and *C. parapsilosis*. CLSI and EUCAST selected S ≤ 2 mg/mL, SDD4 µg/mL, and R ≥ 8 µg/mL [27].

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Recurrent infection promotes resistance to antifungal and drug interaction. Reduced serum azole promotes poor clinical response to established treatment and resistance. There are various antifungals for treatment; however, it is important to prioritize treatment based on local epidemiology, which the clinician must determine for an appropriate therapeutic selection and thereby reduce the problem of resistance to azoles in species identification and sensitivity. The objective of this study was through to VITEK II system to determine the frequency of fungal isolates of *C.albicans* and their susceptibility pattern in patients with HIV/AIDS and oropharyngeal candidias is treated at the Infectious Diseases Hospital in Mexico City.

Patients and Methods

We performed a prospective cross-sectional study. We included adult patients diagnosed with HIV/AIDS treated as outpatients or inpatients from June 2011–December 2012. Patients had clinical suspicion of oral candidiasis and/or oropharyngeal candidiasis due to symptoms compatible with esophagitis and had upper endoscopy performed. All patients provided signed informed consent.

The study did not include patients with severe respiratory insufficiency (pO_2<40 mmHg), obstructive nasal pathology such as tumors in the nasal vestibule, nasal stenosis after surgery, massive hematemesis, thrombocytopenia<50,000 platelets, and prothrombin time >5 sec with respect to control. We defined oral oresophageal candidiasis as the presence of erythematous white plaque lesions, inflammation or bleeding present in the oropharyngeal cavity. Fresh examination of these lesions with the presence of yeast and pseudomycelia and growth of colonies was suggestive of *Candida sp* in Sabourad and Chromogenic chromID Candida (bioMerieux).

Identification and susceptibility were performed using the Vytek II v.05.06 system with YST card (MIC update to May 28, 2011 by CLSI criteria with reference strains): *C. albicans* ATCC 10231, *C. parapsilosis* ATCC22019, *C. krusei* ATCC6258, and *C. glabrata* ATCCMYA2950.

Statistical analysis

For descriptive analysis, we used simple frequencies, proportions, and measures of central tendency and dispersion.

Bivariate analysis: We calculated prevalence odds ratios with 95% confidence intervals and χ^2 tests of association (p<0.05). After analysis of the population distribution, we applied the Mann-Whitney U test for non-normally distributed variables and student *t* test for normally distributed variables.

Ethical aspects

The study was submitted to the Local Committee on Health Research of the IMSS. All patients' signed informed consent for providing oropharyngeal specimens.

Results

We identified a male/female ratio of 6:1. The youngest patient was17 years and the oldest patient were 63 years. Average age was 35 years with a standard deviation (SD) of 9.2 years. CD4+ lymphocyte counts were in the range of 9-82 cells/mm^3 (mean 29 CD4 cells ± SD14.07).

Of the total patients, >90% had <1 months in diagnosis of HIV according to clinical condition and CD4+ and were in AIDS stage (Table 1). There was a higher frequency of patients with no apparent added symptoms. Six patients were co-infected with hepatitis B virus and hepatitis C virus (four and two cases, respectively). There was one case of histopathologically confirmed malignancy.

	Minimum	Maximum	Mean	SD
Age (years)	17	63	35.26	9.2
CD4+	9	82	29.26	14.07
Gender			**No.**	**%**
Female			9	9.4
Male			87	90.6
Time since HIV diagnosis				
<1 month			90	93.8
1-6 months			1	1.0
6 months-1 year			5	5.2
Antimicrobial use prior to taking specimens for diagnosis of candidiasis				
Yes			5	5.2
No			91	94.8
ARV treatment				
Without ARV			96	100
With ARV			0	0
HIV/AIDS and comorbidity				
Without apparent associated pathology			75	78.1
Viral hepatitis B-C			6	6.3
Latent syphilis			2	2.1
Pulmonary pneumocystis			4	4.2
Disseminated tuberculosis			3	3.1
Hodgkin's neoplasm			5	5.2
Encephalitis due to *Toxoplasma gondii*			1	1

Table 1: Characteristics of the patients with HIV/AIDS and oropharyngeal candidiasis.

Mouth (*n* = 96)	**Sensitive**		**Intermediate (D-RS)****		**Resistant**	
	No.	**%**	**No.**	**%**	**No.**	**%**
No development 5/96 (5.20%)	-	-	-	-	-	-
C. albicans 90/96 (77%)	87	96.60	3	3.30	0	0.00
C. glabrata 2/96 (2.1%)	0	0.00	2	100.00	0	0.00
C. parapsilosis 1/96 (1.07%)	0	0.00	1	100.00	0	0.00
Esophagus (*n* = 83)						
No development/no samples 14/96 (14.58%)	0	0.00	0	0.00	0	0.00
C. albicans 73/96 (87.95%)	69	94.50	4	5.47	0	0.00
C. glabrata 6/96 (7.22%)	2	33.33	4	66.66	0	0.00
C. parapsilosis 4/96 (4.81%)	1	25	3	75	0	0.00

Table 2: Susceptibility to fluconazole of the isolated strains from the mouth and esophagus.

Only 5/96 (5.2%) patients received some type of antimicrobial; 1/5 received fluconazole as primary prophylaxis, 1/5 ceftriaxone, 2/5 antituberculosis treatment and 1/5 clindamycin + pyrimethamine. None of the patients had begun antiretroviral therapy (Table 1).

With regard to the endoscopic findings, the presence of minimal lesions predominated. In 4/96 (4.2%) patients, stage IV of the Kodsi classification was identified, interpreted as abundant lesions and with light esophageal mucosa bleeding (Table 2). The most frequent oral clinical form was pseudo membranous candidiasis (93.7%). Only in

a few cases (6.3%) was the mixed form (atrophic or erythematous) identified.

Of the specimens taken on direct examination, we observed increased frequency of budding yeast and the presence of pseudomycelia. In other cases, we observed only yeast and, less frequently, blastoconidia. In the fungal isolates of oropharyngeal origin, *C. albicans* was more frequent. Of the genus non-albicans Candida (NAC), only two species were isolated: *C. glabrata* and *C. parapsilosis*. Identified yeast was *Cryptococcus neoformans* with oropharyngeal location. This patient underwent the study protocol and the organism was not identified elsewhere. The patient received antifungal treatment.

Susceptibility of *C. albicans* to fluconazole was ~90%. Isolates of NAC showed intermediate or dose-response sensitivity. No isolate was reported as resistant. Two different species were isolated from one patient: one from the mouth and the other from the esophagus. One case had intermediate sensitivity and the other one was sensitive.

There were 87/90 oral isolates of *C. albicans* with fluconazole sensitivity, and 3/90 had intermediate sensitivity. Of those patients, one had fluconazole prophylaxis and two patients had two *Candida* strains and different species: *C. albicans* + *C. glabrata and C. albicans* with *C. parapsilosis*. With regard to the NAC isolates, three were identified with intermediate resistance or dose-response susceptibility. In five cases, there was no development, probably related with the size of the inoculums and taking of the specimen (Table 2).

In the esophagus, 69/73 isolates of *C. albicans* were sensitive to fluconazole and <6% had intermediate sensitivity. It should be noted that 2/6 strains of *C. glabrata* showed sensitivity to fluconazole. It has been reported that the species has shown intrinsic *in vitro* resistance and acquired a high *in vivo* percentage. In the case of *C. parapsilosis*, 3/4 isolates expressed intermediate or dose-response sensitivity.

In patients with CD4+ lymphocytes between 51 and 100 cells/mm^3 there was a 6-fold increase for risk of developing this disease without identifying statistical power. If the time of diagnosis of HIV infection is >1 month and antiretroviral treatment has not been administered, the risk increased six times for developing oral candidiasis (95% CI, p>0.05, non-significant). In patients >50 years of age, this was a risk factor for the development of oral candidiasis, with 1.5 times more risk than other groups (95% CI, p=0.57 non-significant) (Table 3).

Regarding esophageal candidiasis, bivariate analysis showed that diagnosis of HIV infection for >1 month increased the risk of developing esophageal candidiasis 1.5 times (95% CI 0.26-9.02, p>0.05). Age range of 30-39 years was identified as an increased risk factor (1.6 times) for the development of esophageal candidiasis *vs.* other age groups in this sample.

Oral candidiasis				
Variable	POR	95% CI	χ^2	*p*
Gender				
Female	1	-	-	-
Male	0.3	0.05-1.76	1.92	0.19
Use of antimicrobials				
Yes	1	-	-	-
No	1.11	1.03-1.18	0.54	0.6
Susceptibility				
Intermediate	1	-	-	-
Sensitive	0.14	0.02-0.74	6.7	0.03
CD4 lymphocytes				
<50	1	-	-	-
51-100	5.9	0.91-38.25	4.32	0.09
Time since HIV diagnosis				
<1 month	1	-	-	-
>1 month	5.92	0.91-38.25	4.32	0.09
Age group (years)				
17-29	1	-	-	-
30-39	1.12	0.19-6.60	0.89	0.63
40-49	1.43	0.18-11.2	0.72	0.56
≥50	1.64	0.12-20.9	0.7	0.57
Esophageal candidiasis				
Gender				
Female	1	-	-	-
Male	1.18	0.22-6.1	0.04	0.6
Use of antimicrobials				
Yes	1	-	-	-
No	1.35	0.14-12.7	0.7	0.63
Susceptibility				
Intermediate	1	-	-	-
Sensitive	0.06	0.01-0.36	14.7	0.001
CD4 lymphocytes				
<50	1	-	-	-
51-100	0.73	0.64-0.83	2.13	0.16
Time since HIV diagnosis				
<1 month	1	-	-	-
>1 month	1.54	0.26-9.02	0.23	0.46
Age groups (years)				
17-29	1	-	-	-
30-39	1.62	0.50-5.25	0.41	0.3
40-49	1.14	0.25-5.02	0.85	0.57
≥ 50	1.33	0.20-8.70	0.76	0.55
POR, prevalence odds ratio.				

Table 3: Bivariate analysis of the risk factors for oral and esophageal candidiasis.

Discussion

In the group of HIV-immunocompromised patients, decreased cellular type immunity favors the presence of infections considered as opportunistic, among them oral infections due to *Candida* sp. In this study, we found that the MIC of fluconazole for *Candida* sp. and reported sensitivity was ≤1 µg/ml. For intermediate or dose-dependent sensitivity (D-DS), it was 44-32 µg/ml and ≥ 64 µg/ml as resistant according to the VYTEK II system and AST-YS01 cards with CLSI v.2011 criteria. Other species were not identified, probably due to the size of the sample.

We know that the constant and necessary use of antifungal agents in primary or secondary prophylaxis and treatment of oropharyngeal candidiasis in AIDS patients favors the selection of resistant strains and contributes to the change in the spectrum of *Candida non-albicans* species. Under these circumstances, this study determined the frequency of the identification of *Candida albicans* and in vitro susceptibility through Vitek II system, since it is an automated resource more accessible than micro dilution techniques.

With the standardization of antifungal susceptibility, procedures such as those established by the Clinical and Laboratory Standards

Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST), the need for automated commercial systems has been created, which has been considered as a way to optimize time and resources without neglecting the quality required. In the case of the Vitek system II, antifungal susceptibility is determined by spectrophotometry to identify the genus and species for determination of the MIC. This system was approved by U.S. FDA in 2006 for clinical use and to detect resistance to fluconazole [28,29].

The cutoff point for susceptibility was established by the CLSI as well as by EUCAST, both for fluconazole as well as for voriconazole vs. *Candida*. The CLSI suggested using values of ≤ 8 μg/mL as susceptible (S), 16-32 mg/mL as DDS, and ≥ 64 mg / mL as resistant (R) for all species.

We consider that when encountering epidemilogic cutoff point (ECVs), (24-26) allows us to assess the emergence of strains with established resistant. ECVs have also served as a bridge to establish specific species susceptibility and clinical breakpoints (CBPs), which in the case of fluconazole were determined for *C. albicans, C. tropicalis and C. parapsilosis*. CLSI and EUCAST selected S ≤ 2 mg / mL, SDD 4 μg/mL and R ≥8 μg/mL.

Fluconazole is the first-line antifungal drug in the treatment of oral and/or esophageal candidiasis [30,31]. The intervention of other species of non-albicans *Candida* in immune compromised hosts and the increased availability of new antifungal agents as well as frequent reports of resistance to *C. albicans* associated with therapeutic failures makes it imperative to have antifungal susceptibility testing in routine microbiology laboratories, a situation that guides clinicians.

The results of this study are from a single center performed with a VITEK II, incorporated into the proposal to continue the surveillance of antifungal susceptibility of *Candida albicans* and other species. In this paper, we describe fungal isolates of different species in the oral cavity and esophagus.

With regard to *Candida glabrata* isolates, we found that susceptibility was dose dependent (DDS) according to the findings by CLSI that chose to place the CBPs for fluconazole and *C. glabrata* ≤ 32 μg/mL for SDD and ≥ 64 μg/mL for R.

Factors that affect the clinical response are as follows: immunological status of the patient [32], treatment adherence and presence of a biofilm due to *Candida* sp. [33] as well as frequent drug interactions.

As for the resistance to antifungal agents used by the Vytek II system, none of the *Candida* sp. strains isolated was found to be resistant, although they were found with intermediate resistance. The explanation for this phenomenon is unclear, considering that the main risk factor is prolonged prior exposure to azoles, a circumstance not experienced by any of the patients.

Although candidiasis of the oropharyngeal mucosa is common in immunecompromised patients due to HIV/AIDS, chronic diseases with immunosuppression such as those of hematological, oncological, and metabolic origin along with the parallel increase of high-risk patients with neoplasms and transplants, low birth weight newborns, elderly patients or patients who have had extensive surgery performed and were hospitalized in the ICU, use of intravascular catheters and diagnostic therapeutic procedures or invasive support have coincided with a marked increase of invasive mycosis. Infections in these groups of patients also require identification of the genus and species of the fungus.

The choice of an antifungal agent should take into account several factors including previous exposure to antifungal agents and correct identification of the species.

Conclusions

There have been reports of resistance to fluconazole in *C. albicans*, which is the most frequent etiologic agent isolated in oropharyngeal candidiasis in patients immune compromised due to HIV/AIDS. We should consider that the occurrence of antimicrobial resistance is related to exposure to antimicrobial agents, dose, and target site and treatment adherence without neglecting drug interactions that may occur in each case. This study did not identify acquired resistance of *C. albicans* to fluconazole.

There is a need for automated commercial systems so as to optimize time and resources without neglecting the quality involved in detecting resistance to fluconazole. Vitek II was approved by the U.S. FDA. There are several publications that said that Vitek II system is highly reproducible with excellent categorical agreement with the CLSI microdilution reference procedure (>95%) for fluconazole, this system was able to determine the MIC endpoint after 9.1 to 27.1 h of incubation (mean 12 to14 h) the U. S. FDA approved in 2006 the clinical use of this system [28].

We found intermediate resistance to fluconazole in *C. glabrata*. All this confirms that we should always report susceptibility to antifungal agents. In the case of these types of species, we are aware of their intrinsic and acquired resilience and we should rule out the presence of non-albicans *Candida* in order to provide the best antifungal treatment to the patient. When added to the immune status, decreased drug interaction and treatment adherence would contribute to controlling the fungal infection process.

Regarding the p values greater than 0.05, they are correlated with confidence intervals, in this case they pass through the unit, these circumstances statistics are related to the size of the sample, which was small. Some data show increased risk, which would be clearer with increasing the sample size because the values of p, as the confidence intervals are directly influenced by the population studied [34].

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