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Antifungal Susceptibility *In Vitro* Determined by the Etest® for *Candida* Obtained from the Oral Cavity of Irradiated and Elderly Individuals

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This study aimed to evaluate the *in vitro* antifungal susceptibility of Candida species of head-and-neck-irradiated patients (Group 1), non-institutionalized (Group 2) and institutionalized elders (Group 3) using Etest® methodology. Candida was isolated from saliva and presumptively identified by CHROMagar Candida®, confirmed by morphological criteria, carbohydrate assimilation (API 20C AUX®) and genetic typing (OPE 18). The collection was made from 29, 34 and 29 individuals (Groups 1, 2 and 3, respectively) with 67 isolates. Etest® strips (ketoconazole, itraconazole, fluconazole, amphotericin B and flucytosine) on RPMI (Roswell Park Memorial Institute) agar, on duplicate, were used to evaluate susceptibility. ATTC (American Type Culture Collection) 10231 (*Candida albicans*) was used as quality control. Among the 67 isolates of Candida species, most were susceptible to azoles, flucytosine and amphotericin B. None of the isolates showed resistance and dose-dependent susceptibility to amphotericin B. There were nine strains resistant to itraconazole, six to fluconazole and two to ketoconazole and ten dose-dependent, mainly to flucytocine. The highest MIC (minimum inhibitory concentration) to *C. albicans*, *C. tropicalis*, *C. parapsilosis* was 2.671 $\mu g.mL^{-1}$, 8.104 $\mu g.mL^{-1}$, 4.429 $\mu g.mL^{-1}$, all for flucytosine. *C. krusei* and *C. glabrata* were associated with higher MIC for azoles and *C. glabrata* with higher MIC to flucytosine. In summary, susceptibility to all tested antifungal agents was evident. The isolates were more resistant to itraconazole and dose-dependent to flucytosine. A comparison of *C. albicans* in the three groups showed no outliers. Higher MIC was associated with *C. krusei* and *C. glabrata*.

Key Words: *Candida*, susceptibility, Etest®, radiotherapy.

Introduction

Emergent resistant populations of *Candida* spp. treated with antifungals have been frequently reported, demonstrating the need to determine the levels of susceptibility of clinical isolates (1,2). Invasive fungal infections are important infections and their prevalence is increasing in immunocompromised patients, such as oncologic patients (3). Candidiasis presentation, in the course of radiotherapy for head and neck, is variable, including erythematous and pseudomembranous forms. The occurrence of oral candidiasis in patients undergoing radiation treatment is mainly related to the subsequent qualitative and quantitative changes in the salivary gland after radiation (4). During the course of radiotherapy, the diversification of *Candida* species as well as the growing incidence of colonization by species such as *C. albicans* and *C. tropicalis* are increased (4-8). There are few studies investigating thoroughly the effectiveness/resistance of antifungal agents in irradiated head and neck cancer patients and resistance to certain antifungals (6,8).

Etest® is a reliable method to verify the sensitivity/resistance of *Candida* species to antifungal agents. The assays using this method involve investigating *in vitro* responses using antimicrobial strips on RPMI (Roswell Park Memorial Institute) culture media (9).

Using the Etest methodology, this study aimed to measure the susceptibility/resistance profiles of isolates of *Candida* sampled from the oral cavity of patients irradiated on the head and neck, and institutionalized and non-institutionalized elderly individuals.

Material and Methods

Origin of Biological Samples

Isolates of fungi were collected from in the oral cavity of individuals categorized into three groups: Group 1: 29 individuals undergoing radiation therapy on head and neck and undergoing cervicofacial field radiotherapy (10^{th} and 23^{rd} sessions), with daily doses from 180 to 200 cGy (centiGray). Among them, 24 (82.8%) were males and 5 (17.2%) were females with a mean age of 61.4 years (median: 61 years). Users of antifungal or other broad spectrum antimicrobial substances or concomitant chemotherapy were excluded. Group 2 was composed by 34 individuals without malignant neoplasms and without

conditions predisposing to candidiasis (broad spectrum antimicrobials, xerostomia, immunosuppression), grouped by age (over 60 years old). Of them, 20 (58.8%) were males and 14 (41.2%) were females with a mean age of 66.35 years (median: 64.5 years). Group 3 was the reference group and consisted of 29 elderly residents of a nursing home, without the candidiasis predictive conditions of Group 2. Of them, 7 (24.1%) were males and 22 (75.9%) were females with a mean age of 73.69 years (median: 74 years). All groups presented mostly partial or totally edentulous individuals without prosthetic rehabilitation.

Isolation, Cultivation, Identification and Conservation of Yeast

The isolation of yeasts was performed using saliva samples collected from the oral mucosa and tongue with a sterile swab. After collection, each swab was placed in a sterile tube containing 1.5 mL of saline (NaCl, 0.85%), placed in a cooler (≈20 °C) and delivered to the laboratory. The content of organic material retained on the swab was detached into the saline solution, and 100 mL aliquots were distributed on 90 mm duplicate plates containing CHROMagar *Candida*® and incubated at 37 °C, allowing for the selective growth and morphological differentiation of *Candida* species (10).

The colonies grown on the selective medium were presumptively identified and isolated in Sabouraud agar supplemented with 1% chloramphenicol. Each colony of *Candida* spp. isolate was catalogued and then stored at -2 °C in YPD (Yeast Peptone Dextrose) broth (1% yeast extract, peptone 2% and 2% dextrose) with glycerol (40% v/v). Sixty-seven different strains were obtained. Among these, 36 isolates were from Group 1, 20 isolates from Group 2, and 11 isolates were from Group 3.

The taxonomic confirmation of isolates was performed using morphological criteria, referencing structures such as blastoconidia, chlamydosphore and pseudohyphae, as well as carbohydrate assimilation using the API 20 C AUX® identification test (BioMérieux Company, Marcy-l'Étoile, France) and genotyping by RAPD-PCR using the OPE 18 primer (11). When it was necessary to differentiate *C. albicans* and *C. dubliniensis* from other species, growth was assessed at different temperatures (12,13). Some isolates were not consistent with the taxonomy using the applied methods; these cases were treated as *Candida* spp.

Evaluation of Antifungal Susceptibility

The MIC (minimum inhibitory concentration) was measured using Etest strips (Probac Brazil, São Paulo, SP, Brazil) (14-17). The antifungal agents used for susceptibility testing in this study were azoles (fluconazole (FL), ketoconazole (KE) and itraconazole (IT)), amphotericin B (APB; polyenic) and flucytosine (FC; pyrimidine).

Preparation of Inoculates

Colonies identified with 24 h of culture in Sabouraud agar supplemented with chloramphenicol (1% v/v) were suspended in saline (NaCl 0.85%), which was calibrated by turbidimetry, using as reference 0.5 on the McFarland scale. A 0.1 mL aliquot of the inoculum calibrated suspension was spread on Petri dishes (150 mm) using a sterile swab in RPMI-1620 agar, alternated with glucose (0.2%) and MOPS (0.165 M, pH 7.0) (Probac Brazil) (18). The procedure was performed as recommended by the manufacturer (AB Biodisk, Solna, Sweden). The reading of MIC values was done between 24 and 48 h after incubation at 37 °C. The reference strain ATCC 10231 - *C. albicans* was used for quality control.

MIC Readings

After incubation, when growth was visible (24 and 48 h), the value of MIC was read at the point of intersection between the halo and the Etest strip. For APB, the MIC was read at the point of complete inhibition (100%) for FC and almost complete (95%) inhibition of growth. The MIC for azoles was read at the first point of significant inhibition (80% of visible growth). The MIC values ($\mu g.mL^{-1}$) of the Etest tape were interpreted as sensitive and resistant using the NCCLS reference (18).

Statistical Analysis

The statistical analysis was performed with SPSS 17.0 (SPSS, Chicago, IL, USA) using descriptive parameters, position and dispersion of values within each group of sampled individuals. For the most frequently encountered *Candida* species, the confidence interval (CI) was calculated with a significance level α=0.05.

Ethical Aspects

The work was conducted in accordance with the precepts established by resolution 466/12 of the Brazilian National Health Ministry of Health (Brazil), and by resolution 179/93 of the Code of Professional Dental Ethics.

Results

In Group 1 (irradiated patients), *C. tropicalis* (25.0%) was the most prevalent species, followed by *C. parapsilosis* and *C. albicans* (16.7% for both). In Group 2 (non-institutionalized elderly people), *C. albicans* was present in 45% of cases, followed by *C. parapsilosis* (20.0%) and *C. guilliermondii* (15.0%). In Group 3 (institutionalized elderly people), *C. albicans* was the only identified species.

Table 1 shows the percentage for testing susceptibility to FL, IT, KE, FC and APB for *C. albicans* of the three

study groups regarding susceptibility (S), dose-dependent susceptibility (S-DD) and resistant (R). There was one S-DD species for IT and one for FC in Group 1 and one S-DD strain for FC in Group 3. All the other *C. albicans* strains were S for all antifungals. The MIC for susceptibility testing of the quality control strain (*C. albicans* ATCC 10231) for azoles (average of duplicate plates) was 0.625 $\mu g.mL^{-1}$ for FL, 0.007 $\mu g.mL^{-1}$ for KE and 0.079 $\mu g.mL^{-1}$ for IT. For FC, the mean was 0.158 $\mu g.mL^{-1}$ and for APB it was 0.023 $\mu g.mL^{-1}$.

Based on the susceptibility test results with the Etest for 67 isolates of *Candida* species, most of them were susceptible to azoles, FC and APB. None of the isolates showed resistance or dose-dependent susceptibility to APB. Table 2 describes the overall conditions found for all species according to the Etest. This study found nine R to IT, six to FL and two to KE, and ten S-DD mainly to FC. Table 3 shows the MIC for antifungals, ranges, and lower and upper bounds, referring to species. For FL, the highest MIC was for *C. krusei*. For IT and KE, the highest MIC was for *C. glabrata* and *C. krusei*, respectively. The highest MIC for FC and APB were for *C. krusei* and *C. spp.,* respectively.

Discussion

The reproducibility and reliability of the Etest is evident. In agreement with this, the present study tested strains of *C. albicans* isolated from the oral cavity of patients with fixed orthodontic appliances and their susceptibility to antifungal agents (FL, KE and APB), based on the Clinical and Laboratory Standards Institute (M44-A protocol) and by Etest, showing overall 100% agreement between the disk diffusion method and the Etest for FC and APB (19) .

A study conducted by Belazi et al. (17) with patients irradiated on the head and neck using the Etest showed FL resistance for some *C. albicans* strains and for isolates of *C. krusei* (MIC>32 $\mu g.mL^{-1}$). The same strains were S-DD for IT. In this study, in Group 1 (irradiated patients) six strains (100%) of *C. albicans* were S for FL and one was S-DD for FC and IT. Only one strain (9.1%) of *C. albicans* (Group 3) was S-DD for FC and all *C. albicans* (Groups 2 and 3) were S for all the tested antifungals. These findings probably indicate different behaviors of this species in non-similar conditions. Considering all species, strains of *C. krusei* presented higher MIC values for FC, KE and FL, corroborating

Table 1. Levels of susceptibility of isolates of *Candida albicans* from the oral cavity of individuals in the three groups proposed by Wingeter et al. (21). (Sensitive - S; Sensitive dose-dependent - SDD and resistant - R)

Antifungal agent[1]	*Candida albicans* (Group 1)			*Candida albicans* (Group 2)			*Candida albicans* (Group 3)		
	S	S-DD	R	S	S-DD	R	S	S-DD	R
	n – %	n – %	n – %	n – %	n – %	n – %	n – %	n – %	n – %
Fluconazole	6 – 100	0 – 0	0 – 0.0	9 –100	0 – 0.0	0 – 0,0	11 – 100	0 – 0.0	0 – 0.0
Itraconazole	5 – 83.3	1 –16.7	0 – 0.0	9 –100	0 – 0.0	0 – 0,0	11 – 100	0 – 0.0	0 – 0.0
Ketoconazole	6 –100	0 – 0	0 – 0.0	9 –100	0 – 0.0	0 – 0,0	11 – 100	0 – 0.0	0 – 0.0
Flucytosine	5 – 83.3	1 –16.7	0 – 0.0	9 –100	0 – 0.0	0 – 0,0	10 – 90.9	1 – 9.1	0 – 0.0
Amphotericin B	6 –100	0 – 0	0 – 0.0	9 –100	0 – 0.0	0 – 0,0	11 – 100	0 – 0.0	0 – 0.0

Table 2. Levels of susceptibility of isolates of *Candida* from the oral cavity of individuals in the three groups proposed by Wingeter et al. (21) (Sensitive - S; Sensitive doses dependent – S-DD and resistant - R)

Susceptibility	Antifungal agent[1]									
	Azoles						Pirimidyne		Polyenic	
	FL		IT		KE		FC		APB	
	n	%	n	%	n	%	n	%	n	%
S	60	89.6	52	77.6	59	88.1	57	85.1	67	100.0
SDD	1	1.5	6	9.0	6	9.0	10	14.9	-	-
R	6	9.0	9	13.4	2	3.0	-	-	-	-
Total	67	100.0	67	100.0	67	100.0	67	100.0	67	100.0

[1]FL-fluconazole, IT-itraconazole, KE-ketoconazole, FC-flucytosine, APB-amphotericin B.

Table 3. MIC average and range for antifungals according to *Candida* species

Drugs	Species	n	Mean	95% confidence interval for mean		Minimum	Maximum
				Lower bound	Upper bound		
FL	*C. albicans*	26	.09623	.08229	.1017	.035	.190
	C. dubliniensis	3	.10967	.07116	.14818	.094	.125
	C. tropicalis	10	.61950	.36604	.87296	.190	1.250
	C. krusei	2	182.00	-1317.332	1681.332	64.000	300.000
	C. glabrata	3	67.000	-123.59991	257.59991	9.000	154.000
	C. parapsilosis	10	30.584	-37.13381	98.30361	.064	300.000
	C.guilliermondii	4	75.266	-163.13484	313.667	.250	300.000
	C. lusitaniae	3	.24667	-.08877	.58210	.110	.380
	C. kefyr	3	.04100	-.00554	.08754	.020	.056
	C. famata	1	.09400	.	.	.094	.094
	C. spp	2	150.02	-1755.60861	2055.65561	.047	300.000
	Total	67	22.118	4.18995	40.04718	.020	300.000
IT	*C. albicans*	26	.0523	.0376	.0671	.02	.19
	C. dubliniensis	3	.0540	-.0333	.1413	.03	.09
	C. tropicalis	10	.2518	-.0720	.5756	.02	1.50
	C. krusei	2	5.2500	-29.6921	40.1921	2.50	8.00
	C. glabrata	3	7.8333	-14.1177	29.7844	2.00	18.00
	C. parapsilosis	10	.2070	-.0661	.4801	.02	1.25
	C. guilliermondii	4	3.6438	-7.3459	14.6334	.06	14.00
	C. lusitaniae	3	.0347	-.0123	.0816	.02	.06
	C. kefyr	3	.0057	-.0044	.0157	.00	.01
	C. famata	1	.0470	.	.	.05	.05
	C. spp	2	2.1875	-20.8425	25.2175	.38	4.00
	Total	67	.8840	.1642	1.6039	.00	18.00
KE	*C. albicans*	26	.00585	.00516	.00653	.001	.010
	C. dubliniensis	3	.00633	.00116	.01150	.004	.008
	C. tropicalis	10	.01600	.00916	.02284	.007	.040
	C. krusei	2	.56500	-1.78565	2.91565	.380	.750
	C. glabrata	3	.48567	-.19338	1.16471	.285	.797
	C. parapsilosis	10	.11620	-.10608	.33848	.006	1.000
	C. guilliermondii	4	.26100	-.52296	1.04496	.010	1.000
	C. lusitaniae	3	.01133	.00560	.01707	.010	.014
	C. kefyr	3	.00200	.00200	.00200	.002	.002
	C. famata	1	.00800	.	.	.008	.008
	C. spp	2	.43900	-5.10091	5.97891	.003	.875
	Total	67	.09030	.03183	.14877	.001	1.000
FC	*C. albicans*	26	2.67119	-.52326	5.86564	.072	40.000
	C. dubliniensis	3	13.3753	-43.90303	70.65369	.047	40.000
	C. tropicalis	10	8.10400	-3.92172	20.12972	.040	40.000
	C. krusei	2	40.000	40.00000	40.00000	40.000	40.000
	C. glabrata	3	.13600	-.32879	.60079	.024	.352
	C. parapsilosis	10	4.42950	-4.54942	13.40842	.047	40.000
	C. guilliermondii	4	10.2585	-21.29739	41.81439	.064	40.000
	C. lusitaniae	3	.05933	-.05068	.16935	.028	.110
	C. kefyr	3	.77100	.14343	1.39857	.500	1.000
	C. famata	1	.02300	.	.	.023	.023
	C. spp	2	20.0005	-234.11724	274.11824	.001	40.000
	Total	67	5.95327	2.63808	9.26845	.001	40.000
APB	*C. albicans*	26	.0503	.0267	.0738	.02	.32
	C. dubliniensis	3	.0207	.0106	.0307	.02	.02
	C. tropicalis	10	.0972	.0387	.1557	.02	.22
	C. krusei	2	.1605	-.9767	1.2977	.07	.25
	C. glabrata	3	.2193	.0874	.3513	.16	.25
	C. parapsilosis	10	.1538	-.0593	.3669	.02	1.00
	C. guilliermondii	4	.1558	-.0735	.3850	.03	.35
	C. lusitaniae	3	.0713	-.0588	.2015	.02	.13
	C. kefyr	3	.0870	.0170	.1570	.06	.11
	C. famata	1	.0640	.	.	.06	.06
	C. spp	2	.2435	-2.2533	2.7403	.05	.44
	Total	67	.0971	.0625	.1318	.02	1.00

previous findings (17).

Moreover, considering the overall MIC, APB showed higher values and absence of R or S-DD, corroborating a previous result (20). In fact, S for APB determined by *in vitro* analysis is a common finding, although R has been found in few cases in another study (21). On bloodstream candidiasis, overall S rates were 98.0% for APB and 98.7% for FL (22). In our study, referring to FL, the rate was 89.6% to S. This difference could be explained by the different microbiota found in these studies, including different rates of prevalence.

Referring to IT and FL activities, the present study showed higher R overall in the evaluated species compared with other antifungals. In fact, a previous study that evaluated 160 strains of yeast-like fungi cultured from samples from the lower respiratory tract, blood, peritoneal cavity and other areas showed more R to IT in *Candida* species using the Etest (23). Another study investigating 159 clinical isolates of *Candida* species from patients with invasive candidiasis in a Kuala Lumpur hospital revealed six isolates R to FL, comprising two isolates of *C.albicans*, two of *C. parapsilosis*, one *C. tropicalis* and one *C. glabrata*; all of these isolates showed cross-resistance to IT (24).

Evaluating S to FC, a study of blood-borne candidiasis showed R to FC in two (0.8%) *C. albicans* isolates, seven (9.3%) *C. tropicalis* strains, three (1.6%) *C. parapsilosis* isolates and all ten (100%) of the *C. krusei* investigated isolates (25). Adding, in the present study, *C. glabrata* presented the highest MIC for IT. As previously shown, *C. glabrata* presents 100% susceptibility to APB and caspofungin and is the least susceptible to IT, posaconazole and voriconazole (23).

In summary, a different pattern of S-DD was observed to IT in *C. albicans* collected from irradiated patients. Nine R to IT, six to FL and two to KE, and ten S-DD mainly to FC were found. For FL, the highest MIC was for *C. krusei*. For IT and KE, the highest MIC was for *C. glabrata* and *C. krusei*, respectively. The highest MIC for FC and APB were for *C. krusei* and *C. spp.*, respectively.

Resumo

Esse estudo objetivou avaliar a susceptibilidade antifúngica *in vitro* de espécies de Candida obtidas de pacientes irradiados em cabeça e pescoço (Grupo 1), idosos não institucionalizados (Grupo 2) e idosos institucionalizados (Grupo 3) usando a metodologia Etest®. Candida foi isolada da saliva e identificada presuntivamente pelo teste CHROMagar Candida®, confirmada pelo critério morfológico, assimilação de carboidratos API 20C AUX® e identificação genética (OPE 18). A coleta foi feita em 29, 34 e 29 indivíduos (Grupos 1, 2 and 3, respectivamente) com 67 isolados. As fitas de Etest® (cetoconazol, itraconazol, fluconazol, anfotericina B and flucitosina) em meio ágar RPMI (Roswell Park Memorial Institute), em duplicata, foram utilizados para avaliar a susceptibilidade. A ATTC (*American Type Culture Collection*) 10231 (*Candida albicans*) foi usada como controle de qualidade. Dos 67 isolados de espécies de Candida, a maioria foi susceptíveis aos azoles, flucitosina e anfotericina B. Nenhum dos isolados mostrou resistência ou susceptibilidade dose-dependente a anfotericina B. Houve nove espécies resistentes ao itraconazol, seis ao fluconazol e duas ao cetoconazol e dez dose-dependentes, principalmente a flucitosina. Os maiores valores de MIC (mínima concentração inibitória) para *C. albicans*, *C. tropicalis*, *C. parapsilosis* foram, respectivamente, 2,671 $\mu g.mL^{-1}$, 8,104 $\mu g.mL^{-1}$, 4, 429 $\mu g.mL^{-1}$, todos para a flucitosina. *C. krusei* e *C. glabrata* foram associadas a um maior MIC para azoles e *C. glabrata* com maior MIC para flucitosina. Em resumo, a susceptibilidade a todos os antifúngicos testados foi evidente. Os isolados foram mais resistentes ao itraconazol e dose dependentes para a flucitosina. A comparação para *C. albicans* nos três grupos não mostrou diferença. Os maiores valores de MIC estavam relacionados a *C. krusei* e *C. glabrata*.

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