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Original article

Molecular identification and antifungal susceptibility testing of *Candida* species isolated from dental plaques

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ABSTRACT

Objective. – The aim of the present study was to provide insight into the prevalence and susceptibility profiles of *Candida* species isolated from the dental plaque of Iranian immunocompetent patients. As a biofilm, *Candida* species are responsible for several disorders common to the oral cavity including gingivitis, dental caries, periodontitis, and the less common severe systemic infections specifically in immunosuppressed individuals.
Method. – PCR-RFLP was performed to identify yeasts isolated from the dental plaques of 40 immunocompetent patients. Moreover, antifungal susceptibility testing was performed in according to CLSI guidelines (M27-A3).
Results. – Among 40 yeasts isolated from the dental plaques of immunocompetent patients, *Candida albicans* was the most common species (92.5%), followed by *P. kudriavzevii* (7.5%). It is the first isolation of *P. kudriavzevii* from dental plaques and the first evaluation of antifungal effect of the new imidazole, luliconazole and echinocandins against these samples worldwide. Luliconazole, voriconazole, amphotericin B and anidulafungin showed the best activity with the lowest geometric mean (GM) 0.03, 0.06, 0.08 and 0.09 μg/ml, respectively, followed by miconazole (0.14 μg/mL), caspofungin (0.24 μg/mL) fluconazole (0.38 μg/mL) and itraconazole (0.5 μg/mL).
Conclusion. – The current study demonstrated luliconazole and echinocandins displayed excellent activity against all *Candida* isolates from dental plaques, presenting promising and potent alternative for all oral Candidiasis.

1. Introduction

Dental plaques are sticky biofilms of microorganisms such as fungi and bacteria that routinely found on the teeth [1]. They are commonly problematic because can participate in the formation of dental caries (tooth decay) and trigger systemic diseases in the presence of predisposing factors such as different types of malignancies, immunodeficiency, poor oral hygiene, use of dental devices, metabolic disorders like diabetes mellitus and frequent use of broad-spectrum antibiotics [2]. The most important causative fungi, which colonize the dental plaques and oral cavity, are yeasts consisting of different *Candida* spp. especially while the balance between the host and the microorganism is altered [3]. The *Candida* genus presents over 150 species of which 10 are responsible for infections in humans. Of these, *Candida albicans* mostly resides in the dental plaques and oral cavities of most healthy humans or medically compromised individuals [3,4]. Although, others like *Candida dubliniensis*, *Candida glabrata*, *Candida tropicalis* and *Pichia kudriavzevii* are also involved in the colonization and oral disorders [5]. In general, oral use of fluconazole, nystatin and itraconazole is the treatment of choice for oropha-

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ryngeal/esophageal Candidiasis (OPC/EC), however for severe or azole-resistant Candidiasis, treatment with amphotericin B may be necessary. Azole-based treatment currently is preferred, despite acquired or intrinsic resistance to azoles has been frequently documented in different *Candida* species [6,7]. Some species like *P. kudriavzevii* is intrinsically resistant to fluconazole and *C. glabrata* has low susceptibility to these agents [8,9]. Therefore, it seems important to differentiate the *Candida* species involved in dental plaques using a reliable and rapid procedure and determine the susceptibility patterns of *Candida* isolates of dental plaques to common antifungal agents. The purpose of this study was to identify 40 *Candida* strains isolated from dental plaques using PCR-RFLP test and evaluation of the *in vitro* susceptibility of these species to 8 antifungal drugs including fluconazole (FLC), itraconazole (ITC), miconazole (MIC), voriconazole (VOR), caspofungin, (CAS), anidulafungin (ANI), luliconazole (LULI) and Amphotericin B (AMP).

2. Material and Methods

2.1. Fungal isolates and Molecular Identification

During May 2015 to June 2016, among immunocompetent patients with dental plaques referred to the Dentistry Clinic of Mazandaran University of Medical Sciences, 40 samples were collected using a sterilized wooden toothpick explained with de Carvalho FG et al. [10]. Specimens were examined initially in 10% KOH, followed by inoculation on malt extract agar (MEA, Difco) supplemented with chloramphenicol and CHROMagar Candida medium (CHROMagar Company, Paris, France) to ensure purity, and incubated at 37 °C for 24 h. Subsequently, genomic DNA was extracted from all test isolates and all strains were identified by the previously described PCR-RFLP method [11]. Participation in our survey was voluntary and all the patients gave their informed consents.

2.2. Antifungal susceptibility testing

MICs (minimum inhibitory concentration) of isolates were determined according to instructions stated in the Clinical and Laboratory Standards Institute (CLSI) M27-A3 and M27- S4 for all of *Candida* isolates to eight antifungal drugs through the broth microdilution method [12]. Briefly, susceptibility testing was performed in 96-well microdilution trays by using RPMI 1640 (Sigma), 34.53 g of morpholinepropanesulfonic acid (MOPS; Sigma) and distilled water to 1000 ml, pH 6.9–7] was prepared and sterilized by filtration. The final concentration of the fungal inocula ranged from $0.5–2.5 \times 10^3$ cells/ml. The plates were incubated at 35 °C for 24 h to determine visually the MICs for caspofungin and anidulafungin, and 48 h for the other tested antifungals. For each isolate, drug - free (growth control well) and yeast - free were included and all isolates were tested in duplicate. For amphotericin B, the MIC was the lowest concentration at which inhibits visual growth completely and for azole and echinocandin derivatives, the MIC were defined as the lowest drug concentration inhibiting $\geq$ 50% of growth when compared to the growth control well [13,14]. Amphotericin B (AMB; Sigma, St. Louis, MO, USA), fluconazole (FLU; Pfizer, Groton, CT, USA), miconazole (MIC; Sigma-Aldrich Steinheim, Germany), itraconazole (ITR; Janssen Research Foundation, Beerse, Belgium), luliconazole (LUL; Nihon Nohyaku Co, Osaka, Japan), voriconazole (VOR; Pfizer), caspofungin (CAS; Merck Sharp and Dohme BV, Haarlem, The Netherlands) and anidulafungin (AFG; Pfizer) were dissolved in 1% dimethyl sulfoxide (DMSO, Sigma) and were prepared at a final concentration of 0.016–16 μg/ml for amphotericin B and azoles; 0.063–64 μg/ml for fluconazole, and 0.008–8 μg/ml for caspofungin and anidulafungin. The resistance breakpoints for the antifungals were as follows: fluconazole $\geq$ 8, itraconazole $\geq$ 1, voriconazole$\geq$1, amphotericin B > 1 anidulafungin $\geq$ 1 and caspofungin $\geq$1 μg/ml. The resistant breakpoint for luliconazole and miconazole has yet to be established, as per CLSI document M27-A3 and M27-S4 [15,16]. The CLSI recommended strains *Candida parapsilosis* (ATCC 22019) and *P. kudriavzevii* (ATCC6258) were included as quality controls to be used with every new series of MICs plates.

2.3. Ethical considerations

The ethics committee of the Mazandaran University of Medical Sciences, Sari, Iran, reviewed and approved the study (No. 1298) and written informed consent was obtained from all patients who participated in the current study.

3. Results

Candida isolates were identified using CHROM agar and PCR-RFLP test as 37 *C. albicans* (92.5%) and 3 *P. kudriavzevii* (7.5%). It is the first isolation of *P. kudriavzevii* from dental plaques worldwide. The *in vitro* antifungal susceptibility profile of 8 antifungal agents against *Candida* species is summarized in Table 1. Although, considering the limited number of *P. kudriavzevii* isolates, MIC_{50}, MIC_{90}, and GM MIC were not measured for these species. According to the MIC values obtained for fluconazole, itraconazole, miconazole, voriconazole, caspofungin, anidulafungin, luliconazole and amphotericin B, the isolates were classified to susceptible and resistant according to the breakpoints proposed by CLSI that summarizes in Table 1. Based on GM MIC (0.03 μg/ml), the *in vitro* activity of luliconazole against all the isolates was more potent than voriconazole (0.06 μg/ml), amphotericin B (0.08 μg/ml),

Table 1
In vitro susceptibilities of 40 clinical isolates to eight antifungal agents. MIC range, geometric mean, MIC50, and MIC90 values are expressed in μg/ml.

MIC (μg/ml) (No. of strains)	Antifungal agents	MIC range	MIC50	MIC90	GM	Resistant (Number)
All clinical strains (n=40)	Amphotericin B	0.016–4	0.016	2	0.08	9 (22.5%)
	Fluconazole	0.063–64	0.125	8	0.38	9 (22.5%)
	Itraconazole	0.125–4	0.5	2	0.5	14 (35%)
	Voriconazole	0.016–4	0.031	1	0.06	7 (17.5%)
	Luliconazole	0.008–32	0.031	0.031	0.03	–
	Miconazole	0.016–4	0.063	2	0.14	–
	Caspofungin	0.016–4	0.25	1	0.24	8 (20%)
	Anidulafungin	0.016–1	0.063	0.5	0.09	2 (5%)
Candida albicans (n=37)	Amphotericin B	0.016–4	0.016	2	0.07	9 (24.32)
	Fluconazole	0.063–64	0.125	8	0.29	6 (16.21%)
	Itraconazole	0.125–2	0.5	1	0.44	12 (32.43%)
	Voriconazole	0.016–2	0.031	0.5	0.05	5 (13.51)
	Luliconazole	0.008–16	0.016	0.25	0.03	–
	Miconazole	0.016–2	0.063	1	0.12	–
	Caspofungin	0.016–2	0.25	0.5	0.2	5 (13.51%)
	Anidulafungin	0.016–1	0.063	0.125	0.08	2 (5.4%)
Pichia kudriavzevii (n=3)	Amphotericin B	0.125–0.25	–	–	–	–
	Fluconazole	2–64	–	–	–	3 (100%)
	Itraconazole	0.5–4	–	–	–	2 (66%)
	Voriconazole	0.031–4	–	–	–	2 (66%)
	Luliconazole	0.063–1	–	–	–	–
	Miconazole	0.031–4	–	–	–	
	Caspofungin	1–4	–	–	–	3 (100%)
	Anidulafungin	0.031–4	–	–	–	–

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