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Major Article

Antifungal activity of synthetic antiseptics and natural compounds against *Candida dubliniensis* before and after *in vitro* fluconazole exposure

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Abstract

Introduction: This study evaluated the susceptibilities of oral candidiasis-derived *Candida albicans,* fluconazole-resistant (FR) *Candida dubliniensis,* and fluconazole-susceptible (FS) *C. dubliniensis* to synthetic antiseptics [chlorhexidine gluconate (CHX), cetylpyridinium chloride (CPC), and triclosan (TRC)] and natural compounds (carvacrol, eugenol and thymol). **Methods:** Susceptibility tests were performed based on the M27-A3 reference method. The fluconazole-resistant *C. dubliniensis* strains were obtained after prolonged *in vitro* exposure to increasing fluconazole concentrations. The geometric mean values for minimum inhibitory concentrations and minimum fungicidal concentrations were compared among the groups. **Results:** FS *C. dubliniensis* was more sensitive to CPC and TRC than FR *C. dubliniensis* and *C. albicans*. However, eugenol and thymol were more active against FR *C. dubliniensis.* The fungicidal activities of CHX and TRC were similar for the three groups, and FR *C. dubliniensis* and *C. albicans* had similar sensitivities to CPC. **Conclusions:** The resistance of *C. dubliniensis* to fluconazole affects its sensitivity to the synthetic antiseptics and natural compounds that were tested.

Keywords: Antiseptics. Susceptibility. *Candida dubliniensis.*

INTRODUCTION

Candidiasis is the most common fungal infection among immunocompromised patients. These infections frequently involve the oral cavity, as *Candida* spp. are commensal organisms, and may contaminate other lesions. *Candida albicans* is the most frequently occurring species, although other *Candida* species (e.g., *Candida dubliniensis*) are becoming more common. *C. dubliniensis* was recognized as a new species in 1995, when it was isolated from the oral cavity of patients with human immunodeficiency virus (HIV) infections and acquired immunodeficiency syndrome (AIDS)[1]. Although *C. dubliniensis* shares many phenotypic characteristics with *C. albicans*, *C. dubliniensis* has a notable ability to acquire resistance to fluconazole[2].

In odontology, as well as during treatment of cancer using antineoplastic and/or radiotherapy, mouthwash use has become an established adjunct to antimicrobial treatment. These mouthwashes have also been formulated to contain various antiseptics, such as chlorhexidine gluconate (CHX), cetylpyridinium chloride (CPC), triclosan (TRC), thymol, and eugenol[3-6]. These compounds have well-known antibacterial activities, although the susceptibility of fungi, especially *Candida* spp., remains unclear.

Among *Candida* spp., the development of antifungal resistance is an emergent phenomenon that can be confirmed using standardized susceptibility tests[7]. However, it remains unclear whether oral antiseptics can inhibit fluconazole-resistant (FR) *Candida* spp. Therefore, we compared the susceptibilities of *C. albicans*, fluconazole-susceptible (FS) *C. dubliniensis*, and FR *C. dubliniensis* to well-known antiseptics and several natural compounds (eugenol, carvacrol, and thymol).

METHODS

Microorganisms

The present study evaluated 20 *Candida dubliniensis* strains and 20 *Candida albicans* strains that were isolated from oropharyngeal candidiasis cases. Because the strains'

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susceptibility to fluconazole was already known, these isolates were classified as the FS *C. dubliniensis* group and the *C. albicans* group. Based on the methods of Fekete-Forgács et al.[8], a third group was created from the FS *C. dubliniensis* group by exposing the strains to increasing concentrations of fluconazole, and this group was named the FR *C. dubliniensis* group (n = 20). The three groups' minimum inhibitory concentration (MIC) ranges were found to be 0.25-4µg/mL (FS *C. dubliniensis*), 0.25-64µg/mL (FR *C. dubliniensis*), and 0.25-16µg/mL (*C. albicans*).

Antimicrobial agents

The studied synthetic compounds were CHX, CPC, and TRC. The studied natural compounds were carvacrol, eugenol, and thymol. All compounds were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Antifungal susceptibility tests

The antimicrobial agents were diluted to create stock solutions and testing concentrations: CHX (10mg/mL; 0.4-250µg/mL), CPC (10mg/mL; 0.04-25µg/mL), TRC (10mg/mL; 0.04-25µg/mL), carvacrol (20mg/mL; 1.22-625µg/mL), eugenol (50mg/mL; 2.44-1,250µg/mL), and thymol (10mg/mL; 1.22-625µg/mL). CHX, CPC, and TRC were diluted in distilled water, and carvacrol, eugenol, and thymol were diluted in methanol. One hundred-microliter aliquots of the two-fold diluted compounds were dispensed into 96-well microtiter plates, and the compounds' MICs were determined using the M27-A3 reference protocol (Clinical & Laboratory Standards Institute, 2008). The medium was Roswell Park Memorial Institute (RPMI) 1640 broth containing 2% dextrose buffered using 3-(N-morpholino) propanesulfonic acid. According to the M27-A3 protocol, the inocula were standardized by suspending the yeast (five colonies grown on Sabouraud dextrose agar) in saline solution (0.85%) and adjusting the turbidity. All tests were performed in triplicate, and each series included a positive control (diluted inoculum working solution) and a negative control (RPMI 1640 alone). The cell suspensions were diluted 1:50 using distilled water and 1:20 using RPMI 1640 medium. After adding the 100-µL cell suspension aliquots, the microdilution plate was incubated at 35°C for 48h. Yeast growth was monitored visually, and the MIC for each compound was defined as the lowest concentration required to arrest visible fungal growth at the end of the 48-h incubation.

The minimal fungicidal concentrations (MFCs) were determined by subculturing 0.01mL from each well without visible growth during the MIC assay onto Sabouraud dextrose agar plates. The lowest concentration of the antimicrobial agents to prevent growth was defined as the MFC value.

Statistical analysis

The groups' susceptibilities (MICs and MFCs) to each antiseptic compound were compared using the paired Wilcoxon test (FS *C. dubliniensis* vs. FR *C. dubliniensis*) and the unpaired Mann-Whitney test (*C. albicans* vs. FS *C. dubliniensis* and *C. albicans* vs. FR *C. dubliniensis*). P-values of <0.05 were considered statistically significant[9].

RESULTS

The MICs and susceptibility profiles of *C. albicans*, FS *C. dubliniensis*, and FR *C. dubliniensis* are shown in **Table 1.** The CPC tests revealed that FS *C. dubliniensis* group was significantly more susceptible, compared to *C. albicans* ($p < 0.001$) or FR

TABLE 1

Comparing the MICs of *Candida albicans* and fluconazole-susceptible and -resistant forms of *Candida dubliniensis* for synthetic and natural antiseptic compounds.

Antiseptics	Groups* (n = 20)	MIC range (µg/mL)	GM (µg/mL)	Comparisons	P-values
Cetylpyridinium chloride	A	0.78–6.25	1.56	A × B	<0.05
	B	1.56–6.25	3.44	A × C	<0.001
	C	3.12–6.25	4.26	B × C	ns
Chlorhexidine gluconate	A	0.97–7.8	3.63	A × B	ns
	B	1.95–15.6	3.51	A × C	<0.05
	C	1.95–7.8	5.14	B × C	<0.05
Triclosan	A	0.78–25	10.5	A × B	<0.05
	B	6.25–25	16.8	A × C	<0.05
	C	6.25–25	17.1	B × C	ns
Carvacrol	A	78.1–625	206.4	A × B	<0.01
	B	78.1–312.5	148.7	A × C	ns
	C	78.2–312.5	213.6	B × C	<0.05
Eugenol	A	312.5–625	603.7	A × B	<0.05
	B	156.5–625	371.6	A × C	ns
	C	156.2–625	583.1	B × C	<0.001
Thymol	A	39.06–625	272	A × B	ns
	B	156.2–625	286	A × C	ns
	C	78.2–625	301.9	B × C	ns

MICs: minimum inhibitory concentration; **GM:** geometric mean; **ns:** not significant. ***A:** fluconazole-susceptible *Candida dubliniensis*; **B:** fluconazole-resistant *Candida dubliniensis;* **C:** *Candida albicans.*

C. dubliniensis ($p < 0.05$). A similar susceptibility pattern was observed for TRC (FS *C. dubliniensis* vs. *C. albicans,* $p < 0.05$; FS *C. dubliniensis* vs. FR *C. dubliniensis*, $p < 0.05$). *C. albicans* was significantly less susceptible to CHX, compared to the FS *C. dubliniensis* and FR *C. dubliniensis* groups ($p < 0.05$). The susceptibility tests for TRC revealed that *C. albicans* was significantly less susceptible, compared to FS *C. dubliniensis* ($p < 0.05$). However, no differences were detected between the FR *C. dubliniensis* and *C. albicans* groups.

The FR *C. dubliniensis* group was more susceptible to carvacrol than the FS *C. dubliniensis* group ($p < 0.01$) and the *C. albicans* group ($p < 0.05$). The FR *C. dubliniensis* group was less susceptible to eugenol than the FS *C. dubliniensis* group ($p < 0.05$) and the *C. albicans* group ($p < 0.001$). The FS *C. dubliniensis* and *C. albicans* groups had similar susceptibilities to eugenol. No significant differences were observed among the three groups' susceptibilities to thymol.

The MFC values are shown in **Table 2**. Significantly differences in susceptibility were observed for CPC (FS *C. dubliniensis* was more susceptible than *C. albicans*; $p < 0.001$), carvacrol (FR *C. dubliniensis* was more susceptible than FS *C. dubliniensis*; $p < 0.05$), and eugenol (FR *C. dubliniensis* was more susceptible than FS *C. dubliniensis*; $p < 0.01$). All other tests did not reveal any significant differences among the groups.

DISCUSSION

The present study evaluated *Candida albicans* because it is the most studied yeast-like fungi that can be responsible for oral candidiasis in immunocompromised patients. We also considered *C. dubliniensis* because it can acquire resistance to fluconazole and its susceptibility to antiseptics remains largely unknown.

Fluconazole exposure may alter resistance to other antifungal agents. Thus, we created a group of FR *C. dubliniensis* using prolonged *in vitro* exposure to this triazole, in order to evaluate the effect of this resistance on susceptibility to other oral antiseptics. However, it is important to recognize that this form of induced resistance may be difficult from naturally occurring resistance in patients with oral candidiasis.

In general, the FS *C. dubliniensis* group was significantly more sensitive to the studied compounds' fungistatic activity, compared to the other groups. Furthermore, the MICs in the FR *C. dubliniensis* group were similar to those in the *C. albicans* group. However, measurement of the compounds' fungicidal activities did not reveal any significant differences in the groups' susceptibilities to CHX and TRC. Furthermore, the FS *C. dubliniensis* group was more sensitive to CPC, compared to the other groups, and the FR *C. dubliniensis* group was less sensitive to CHX. Although this reduced susceptibility was not evident in the MFC tests, we believe that it may be a sign of emerging resistance.

Chlorhexidine gluconate is a biguanide compound that is commonly found in toothpastes, hand soaps, and mouthwashes. In addition, it can be used as adjunct antifungal therapy for candidiasis, as it induces coagulation of nucleoproteins, inhibits budding, and causes changes in the cell wall that lead to cytoplasmic component escape and yeast death[5]. Similar to our findings, Shresta et al.[5] found that *C. tropicalis* was less

TABLE 2

Comparing the MFCs of *Candida albicans* and fluconazole-susceptible and -resistant forms of *Candida dubliniensis* for synthetic and natural antiseptic compounds.

Antiseptics	Groups* (n = 20)	MFC range (µg/mL)	GM (µg/mL)	Comparisons	P-values
Cetylpyridinium chloride	A	0.78–50	2.43	A × B	ns
	B	1.56–25	5.38	A × C	< 0.001
	C	3.12–25	7.69	B × C	ns
Chlorhexidine gluconate	A	0.97–500	7.3	A × B	ns
	B	1.95–250	7.06	A × C	ns
	C	3.9–31.25	6.56	B × C	ns
Triclosan	A	1.56–50	17.7	A × B	ns
	B	6.25–50	18.5	A × C	ns
	C	6.25–50	18.9	B × C	ns
Carvacrol	A	156–625	301.8	A × B	< 0.05
	B	156–625	256.6	A × C	ns
	C	156–312	262.8	B × C	ns
Eugenol	A	625–2,500	915.1	A × B	< 0.01
	B	312–2,500	625	A × C	ns
	C	625–1,250	769.5	B × C	ns
Thymol	A	312–625	420.6	A × B	ns
	B	312–625	388.1	A × C	ns
	C	78.12–625	359	B × C	ns

MFC: minimum fungicidal concentration; **GM:** geometric mean; **ns:** not significant. ***A:** fluconazole-susceptible *Candida dubliniensis.* **B:** fluconazole-resistant *Candida dubliniensis* . **C:** *Candida albicans.*

susceptible, compared to *C. albicans*, albeit using different methods. Fathilah et al.[10] have also reported elevated MICs for *Candida tropicalis* (75µg/mL) and *Candida krusei* (150µg/mL), which were much higher than the GM MICs from the present study (3.63-5.14µg/mL). These findings highlight the differences in the susceptibilities of *Candida* spp. to CHX. Thurnmond et al.[11] have also reported variations in the MICs of *C. albicans* after daily CHX exposure, with an increase in the MIC range from 5-10 during week 1 to 2.5-20µg/mL during week 8. These findings are consistent with reports regarding varying degrees of stomatitis that are related to reducing the numbers and occurrences of oral *Candida* spp., oral candidiasis, and *Candida*-related morbidity and mortality[12].

Cetylpyridinium chloride is a cationic quaternary ammonium compound that is widely used in mouthwashes to prevent or treat candidiasis and bacterial infections[13]. CPC alters the surface tension of the cell wall structure, which may lead to cell wall leakage. Based on the GM MIC values, we found that CPC provided greater activity (1.56-4.26µg/mL), compared to the results of Fathilah et al.[10], who found MICs of 66 µg/mL for *C. tropicalis* and 33µg/mL for *C. krusei*. Edling et al.[14] have reported that two strains of FR *C. albicans* have reduced CPC susceptibility, which suggests that mouthwashes with CPC might select for resistant strains. Our results did not confirm this possibility, because the FR *C. dubliniensis* and *C. albicans* groups had similar susceptibilities, although we did not test *C. tropicalis* and *C. krusei*.

Our results also revealed that the FR *C. dubliniensis* and *C. albicans* groups had similar sensitivities to TRC, although the FS *C. dubliniensis* group was more sensitive than the *C. albicans* and FR *C. dubliniensis* groups. In contrast, Jones et al.[15] reviewed the activity of TRC against fungi and reported MICs that ranged from 1.63µg/mL for *Epidermophyton floccosum* to 5,000µg/mL for *Blastomyces dermatitidis*; *C. tropicalis* ATCC 750 was inhibited by 2,500µg/mL of TRC. Furthermore, Yu et al.[16] studied the combination of TRC and fluconazole against FR *C. albicans,* and reported MICs of 32-64µg/mL. When fluconazole and TRC were combined, the MICs of TRC decreased to 4-8µg/mL[16], which suggested that fluconazole resistance affected the susceptibility to TRC. However, we did not detect this phenomenon in the present study.

Carvacrol, eugenol, and thymol are natural compounds that are contained in the main fractions of essential oils from *Origanum vulgare, Syzygium aromaticum,* and *Thymus vulgaris,* respectively. All three compounds are terpenoids and have antimicrobial activities against a wide range of pathogens, including *Candida* spp.[17] In contrast to our findings with the synthetic compounds, we found that the FR *C. dubliniensis* group was significantly more susceptible to carvacrol than the FS *C. dubliniensis* and *C. albicans* groups. The susceptibility of *C. dubliniensis* to carvacrol is poorly understood, and only a small number of isolates have been reported[4]. In the present study, the carvacrol MICs for *C. albicans* (78.2-312.5µg/mL) were higher than the 0.16µg/mL values for *C. albicans* and *C. dubliniensis* that were reported by Vale-Silva et al.[18]. Those authors also reported that the MFC for carvacrol was similar to the MIC[18], while we found that the MFCs were generally higher than the MICs.

Similar to the results for carvacrol, the MIC and MFC values for eugenol were higher in the FS *C. dubliniensis* group than in the FR *C. dubliniensis* group. Conflicting results have been reported by Ahmad et al.[19], who noted that FR strains had higher sensitivity to eugenol than the standard or clinical strains did. In addition to its use as an antiseptic agent, eugenol is applied topically to dental cavities, used as a component of dental protectives, and combined with zinc oxide to form zinc oxide eugenol, which has restorative and prosthodontic applications in dentistry[20].

The fungistatic and fungicidal activities of thymol were similar in the three groups. Guo et al.[21] have also studied the activity of thymol against FS and FR *C. albicans*, although our results (based on the MIC ranges) were higher than their results. Thymol causes protein denaturation and damage to cellular membranes, which results in the leakage of intracellular components[5]. As suggested by Ahmad et al.[19], the antifungal activities of carvacrol, eugenol, and thymol against FR and FS *C. dubliniensis* highlight the possibility that these compounds could expand the existing class of useful antifungal agents. Thus, these compounds might be used in pharmaceutical products, such as the antiseptic ingredients for mouthwashes.

In conclusion, our results indicate that FS *C. dubliniensis* were more sensitive to antiseptics than FR *C. dubliniensis* and *C. albicans*, which highlights the possibility that acquired resistance to fluconazole may alter antiseptic susceptibility. Interestingly, we did not observe this cross-resistance for the natural compounds (carvacrol, eugenol, and thymol). As stated by Fraise[22], changes in the cell wall may also contribute to cross-resistance between biocides and antibiotics, which most likely involves reduced permeability. Thus, researchers must be alert for changes in the susceptibility of yeasts to antiseptics, given the increasing number of antimycotics that may target the cell wall.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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