International Journal of Antimicrobial Agents 41 (2013) 65–69

Contents lists available at SciVerse ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Chlorhexidine is a highly effective topical broad-spectrum agent against *Candida* spp.

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ARTICLE INFO

Article history:
Received 13 March 2012
Accepted 24 August 2012

Keywords:
Fluconazole
Chlorhexidine
Candida
Susceptibility
Minimum inhibitory concentration

ABSTRACT

The objective of this study was to compare the in vitro antifungal activities of chlorhexidine (CHX) and fluconazole (FLZ) against *Candida* isolates comprising eight different species associated with oral candidosis. A broth microdilution method as described in Clinical and Laboratory Standards Institute (CLSI) protocol M27-A3 was used to determine susceptibility. A total of 79 clinical isolates and reference strains belonging to eight different *Candida* spp. was tested. The minimum inhibitory concentration (MIC) was the lowest drug concentration that reduced growth by 50% for FLZ at 48 h and by 80% for CHX at 24 h and 48 h. The geometric mean MIC (and MIC range) at 48 h for CHX was 3.03 mg/L (0.78–6.25 mg/L) and for FLZ was 19.12 mg/L (≤0.125–256 mg/L). Of the 79 isolates, 14 (18%) were resistant to FLZ (MIC ≥ 64 mg/L). All isolates were effectively inhibited by ≤6.25 mg/L CHX, and *Candida* CHX MICs are below the CHX levels found in saliva following normal dosing. No cross-resistance between CHX and FLZ was detected (r_s = 0.039, P = 0.733). CLSI M27-A3 methodology proved to provide reproducible results with clear endpoints for CHX. In conclusion, the findings showed that CHX has excellent broad-spectrum antifungal activity in vitro. It was effective at concentrations detected in saliva when using standard dosing regimens. Moreover, no cross-resistance was detected between CHX and FLZ, even among *Candida* spp. highly resistant to FLZ.

1. Introduction

Being the disease of the diseased, oral candidosis predominantly affects immunosuppressed and medically compromised patients [1]. In these high-risk patients, the oral cavity may provide a source for *Candida* causing systemic infection [2]. Oral candidosis has become a significant challenge in patients with persisting risk factors and a recurrent need for antifungal treatment [2]. In particular, repeated courses of fluconazole (FLZ) have been shown to constitute a risk for persistent colonisation with microbiologically and clinically resistant *Candida* [2]. Oral candidosis is a mixed multispecies candidal–bacterial biofilm infection that provides multiple challenges for its management [3]. The biofilm lifestyle is commonly associated with poor drug penetration and antimicrobial recalcitrance as well as a risk of development of resistance [3,4].

A number of antifungal agents are available for the management of fungal infections [5]; however, the choice of antifungals suitable for the treatment of oral candidosis is limited [2]. FLZ is a widely used systemic antifungal agent that is well tolerated, with low toxicity and mild side effects [5], although in elderly patients with reduced saliva production there is a risk of low drug levels in the oral cavity and the emergence of resistance [6].

Moreover, non-*albicans Candida* spp. such as *Candida glabrata* and *Candida krusei* are intrinsically resistant to FLZ and are common causes of oral candidosis [1,7,8]. In addition, its penetration into candidal biofilm is poor, leading to low drug concentrations, which again has a potential risk for selection and development of resistant strains [9]. Nystatin is a highly effective topical antifungal with few drug interactions. However, its four times daily dosage is a significant challenge for patient compliance [10,11]. Echinocandins are highly effective agents against *Candida* and *Candida* biofilms [12]. However, their availability only as an intravenous formulation and their high cost negate their use for the treatment of oral candidosis [2].

Chlorhexidine (CHX) has been used as an adjunctive therapeutic option for topical use owing to its broad-spectrum antimicrobial efficiency [13]. It is effective at low concentrations and has unique

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http://dx.doi.org/10.1016/j.ijantimicag.2012.08.014

substantivity extending its therapeutic effect in the oral cavity [14,15] owing to its high adsorption capacity such that it can be retained in the oral cavity for long periods (up to 12 h) [14]. Consequently, less frequent dosing can be used [14,16]. The mode of action of CHX on *Candida* is still unclear but it has been suggested that it inhibits cell wall synthesis by binding to negatively charged groups in the candidal cell wall, followed by intracellular material leakage and cell death (reviewed in [17]). It appears to inhibit candidal replication and the adhesion of *Candida* to epithelial cells and denture surfaces, all being crucial prerequisites for fungal infection [14]. CHX has been described to have significant activity against *C. albicans* in vitro, but less data exist for *Candida* spp. other than *C. albicans*, such as *C. glabrata*, *Candida tropicalis* and *C. krusei* [13,17]. It has also been shown to have superior efficacy against *Candida* biofilms compared with FLZ in vitro and in vivo [13,18–21]. Furthermore, it can be used to impregnate denture liners to act as a long-term self-release drug carrier [22].

There is a clear clinical and microbiological need for evaluation of the in vitro antifungal activity of CHX. The present study aimed to investigate the antifungal activity of CHX against a panel of isolates belonging to a number of different *Candida* spp. commonly isolated from patients with oral candidosis and to compare its activity with that of FLZ. The null hypotheses were: first, that CHX is effective against a broad spectrum of *Candida* spp.; and second, that it has a comparable activity to FLZ at levels seen in saliva.

2. Materials and methods

2.1. Organisms and media

A total of 79 *Candida* isolates belonging to eight different species, comprising 76 clinical isolates and 3 reference strains, were tested against CHX and FLZ. Clinical isolates were obtained from the culture collection of the Mycology Reference Centre Manchester (UK) and were predominantly obtained from mucocutaneous and haematogenous sources from patients, including those with immunodeficiency, candidaemia and tissue-invasive disease. American Type Culture Collection (ATCC) strains *C. albicans* ATCC 90028, *C. krusei* ATCC 6258 and *C. tropicalis* ATCC 750 were used as reference strains [23]. Isolates were identified by standard biochemical methods, including CHROMagar™ *Candida* medium (CHROMagar, Paris, France), API ID32C (bioMérieux, Lyon, France) assimilation tests, and *Candida dubliniensis* agglutination test (Bichro-Dubli Fumouze®; Fumouze Diagnostics, Levallois-Perret, France). Isolates were stored at −80 °C and each isolate was plated twice on Sabouraud agar (Oxoid Ltd., Basingstoke, UK) and incubated at 37 °C for 48 h before use to check viability and purity. A total of 32 *C. albicans*, 13 *C. glabrata*, 10 *C. dubliniensis*, 6 *Candida parapsilosis*, 6 *Candida guilliermondii*, 6 *C. tropicalis*, 5 *C. krusei* and 1 *Candida kefyr* were tested. RPMI-1640 with 2% glucose, buffered with morpholinopropanesulfonic acid (MOPS) (Sigma-Aldrich, Dorset, UK) and adjusted to pH 7.0 was used as growth medium for FLZ and CHX.

The reproducibility of the method was evaluated by retesting 20% of randomly selected isolates (16/79) against each drug. The same batch of medium was used throughout the study, including reproducibility studies.

2.2. Susceptibility testing and antifungal agents

The broth microdilution method as described in Clinical and Laboratory Standards Institute (CLSI) document M27-A3 [24] was used to determine susceptibility. FLZ (Pfizer, Sandwich, UK) and CHX (Sigma-Aldrich) were obtained in pure powder form from their respective manufacturers. Briefly, a two-fold dilution series of FLZ (0.125–2048 mg/L) and CHX (0.1–50 mg/L) was prepared in sterile distilled water, and an inoculum of 1×10^3 organisms/mL was used. Following incubation at 37 °C, growth in each well was determined by measuring the optical density at 490 nm (OD_{490}) with a spectrophotometer (BMG Labtech, Aylesbury, UK). For FLZ, the minimum inhibitory concentration (MIC) was the lowest drug concentration that reduced the OD_{490} by 50% at 48 h compared with the drug-free control. CLSI standard breakpoints for FLZ were used for susceptibility interpretation [23,25]. Isolates were designated susceptible, susceptible dose-dependent or resistant based on their MICs and according to CLSI standards [23,25]. For CHX, the MIC was the lowest drug concentration that reduced the OD_{490} by 80% at 24 h and 48 h compared with the drug-free control [23]. Sabouraud agar and blood agar plates were inoculated with 10 μL of each organism suspension to check the viable count and culture purity. Geometric means (GMs) and ranges were calculated.

2.3. Statistical analysis

SPSS statistical package v.18.0 (SPSS Inc., Chicago, IL) was used to analyse all data. Kruskal–Wallis test was used to verify differences in susceptibility between species against CHX at $P < 0.05$ with post hoc Mann–Whitney *U*-tests. A Wilcoxon test was performed to compare MICs at 24 h and 48 h for each species against CHX. The correlation between the antifungal activity of CHX and FLZ against *Candida* spp. was evaluated using Spearman's rho (r_s) coefficient. The ranking was used to establish whether this correlation coefficient is significantly different from zero. The significance level was determined at $P \leq 0.05$.

3. Results

The GM MIC for CHX for all *Candida* isolates was 2.22 mg/L at 24 h and 3.03 mg/L at 48 h (Table 1) and the MIC_{90} (MIC for 90% of the organisms) was 6.25 mg/L (range 0.78–6.25 mg/L) at 48 h. The MIC at 48 h was significantly higher than at 24 h for five species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. guilliermondii* and *C. krusei*) ($P \leq 0.05$). For three species (*C. dubliniensis*, *C. tropicalis* and *C. kefyr*), the incubation time did not have an impact on the MIC ($P > 0.05$), although only a small number of isolates were tested for some species. The GM MIC for *C. albicans* and *C. glabrata* was significantly higher than that detected for all other species at 48 h ($P < 0.05$).

The cumulative percentage of isolates for each species of *Candida* inhibited at each concentration of CHX and FLZ throughout the broth microdilution series is presented in Table 2A and B, respectively. CHX demonstrated antifungal activity against all tested isolates, with low MICs ranging from 0.78 mg/L to 6.25 mg/L; trailing endpoints with CHX were usually not encountered and 100%

Table 1
Chlorhexidine geometric mean minimum inhibitory concentration (MIC) results for 79 *Candida* spp. isolates belonging to eight different species at 24 h and 48 h incubation.

Candida spp. (No. of isolates)	MIC (mg/L)		*P*-value[a]
	24 h	48 h	
C. albicans (32)	4.05	5.03	0.001
C. glabrata (13)	3.13	4.78	0.005
C. dubliniensis (10)	3.13	3.13	1.0
C. parapsilosis (6)	1.56	3.13	0.014
C. guilliermondii (6)	0.78	1.10	0.046
C. tropicalis (6)	2.78	3.13	0.317
C. krusei (5)	1.56	3.13	0.025
C. kefyr (1)	0.78	0.78	
All isolates (79)	2.22	3.03	

[a] Differences between MICs at 24 h and 48 h were tested by Wilcoxon test.