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Original Article

In vitro antifungal effect of mouth rinses containing chlorhexidine and thymol

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Abstract *Background/purpose:* In this *in vitro* study, we assessed the antifungal effect of mouth rinses containing chlorhexidine and thymol.
Materials and methods: The fungistatic activities of chlorhexidine- and thymol-containing mouth rinses were assessed by means of the minimum inhibitory concentration (MIC) and the fungicidal activity was determined by a time-kill assay.
Results: The chlorhexidine-containing mouthwash was able to kill all strains of *Candida albicans* and *Candida tropicalis* in shorter times compared to the thymol-containing mouthwash. Hexidine showed an MIC of 1:32 for both *Candida* species, whereas Listerine respectively showed MICs of 1:8 and 1:16 for *C. albicans* and *C. tropicalis*.
Conclusions: Antimicrobial agents used in the study had good *in vitro* activity against the two *Candida* species. Mouth rinses containing chlorhexidine showed superior antifungal and fungicidal activities compared to the thymol-containing mouth rinse. Both antimicrobial agents may be suggested for use as topical antifungal agents.

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Introduction

Candidiasis is the term used to denote infections caused by species of *Candida*. The spectrum of disease caused by *Candida* species includes infections of nails, skin, mucous membranes, and internal organs. Although over 100 species of *Candida* are recognized, not all are pathogenic to humans. Important pathogenic species are *Candida albicans, Candida tropicalis, Candida parapsillosis, Candida guilliermondi, Candida krusei*, and *Candida stellatoidea*. *C. albicans* is the most common pathogen of all *Candida* species. *Candida* species are harmless commensals of the human body and are a part of the normal flora of the pharynx, intestine, vagina, perianal skin folds, and mouth.[1] The dorsum of the tongue represents the primary oral reservoir for these organisms, but mucosal surfaces and dental plaque can also harbor them.[2]

Local or systemic factors must be involved for the organism to become infectious because it was proven difficult to initiate experimentally produced *Candida* infections in intact oral mucosa. Among precipitating factors are age, depressed host defenses, endocrine disorders, heredity, ill-fitting dentures, and protracted use of antibiotics, corticosteroids, or cytostatic drugs.[3] When changes occur in host defense mechanisms and the oral environment, yeasts may induce mucosal and periodontal opportunistic infections. The incidence of these infections has increased in parallel with the increase in numbers of immunocompromised patients, including HIV-infected individuals, transplant recipients receiving immunosuppressive agents, and patients undergoing cytotoxic chemotherapy and radiotherapy for cancer and bone marrow transplantation.[2]

A number of antimycotic drugs are available for treating oral candidiasis. Polyene antibiotics such as nystatin and amphotericin B are among the recommended drugs.[3] Mouth rinses containing antimicrobial agents exert beneficial clinical effects when used as adjuncts in treating periodontal disease. Antibacterial activities and the effectiveness in reducing or retarding plaque formation were comprehensively studied.[3] Furthermore, chlorhexidine gluconate is also considered an appropriate adjunct or alternative to specific antimycotic drugs, with administration of 0.2% chlorhexidine gluconate as a mouthwash being widely recommended.[2,4–6] Thymol is an essential oil which is also used as a mouth rinse and was tested for its effect in inhibiting the development of plaque and gingivitis.[7] Studies were carried out to evaluate the antifungal effectiveness of chlorhexidine gluconate and other antimicrobial agents, such as sanguinarine, triclosan,[2] aliphatic amines,[8] and tetracycline hydrochloride.[9] Chlorhexidine showed superior antifungal effects except in studies involving cetylpyridinium chloride[6] and Listerine.[7] The objective of this study was to investigate the *in vitro* antifungal effect of mouth rinses containing chlorhexidine and thymol.

Materials and methods

Specimen collection

Patients with signs of oral candidiasis were identified. As a part of the diagnosis procedure in the Department of Oral Medicine, Manipal College of Dental Sciences (MCODS), Mangalore, sterile cotton swabs (2 each) were rolled and pressed on the lesion to collect a specimen. Samples obtained were immediately transported to the diagnostic microbiological laboratory of the Department of Microbiology, Kasturba Medical College, Mangalore. Samples were subjected to further processing at the laboratory.

Microscopic examination

One of the swabs was used to prepare a smear on a clean glass slide. The smear was air-dried, heat-fixed, and stained by Gram's method.[10] The presence of many gram-positive yeast cells with pseudo-hyphae was considered significant.

Isolation and identification of *Candida* species

Candida species were isolated and identified using standard methods. The second swab was used to inoculate Saboroud's dextrose agar (SDA). The inoculated medium was incubated at 37°C for up to 14 days. The colony morphology of the fungal growth was examined. *Candida* species were identified by the colony morphology, Gram's stain, a germ tube test, chlamydospore test, and sugar fermentation layout.[11]

Study of the inhibitory effects of mouthwashes against *Candida*

The present study is a portion of a larger study investigating the *in vitro* and *in vivo* antifungal effects of various prescribed antiseptic mouth rinses. Samples obtained from oral candidiasis patients examined over a period of 6 months showed the presence of *C. albicans* and *C. tropicalis* in a ratio of 4:1. Hence, 4 isolates of *C. albicans* and 1 isolate of *C. tropicalis* were used in this study. The patients from whom these isolates were sampled were free of systemic disorders. Two male patients, 58 and 56 years old, suffered from pseudomembranous candidiasis of the dorsum of the tongue and 1 female patient, 52 years old, suffered from pseudomembranous candidiasis of the soft palate (Fig. 1). A 70-year-old female, wearing removable complete dentures, had denture stomatitis along with angular chelitis (Fig. 2). The fifth sample was taken from a 45-year-old male who had median rhomboid glossitis with an opposing palatal erythematous area.

Two commonly prescribed mouthwash products of Hexidine (ICPA Health Products Ltd., Mumbai, Maharastra, India) containing chlorhexidine and Listerine (Johnson & Johnson Limited, Mumbai, Maharastra, India) containing thymol, were procured from a local pharmacy. The antifungal effects of the mouthwash proportions were studied by a time-kill assay and broth dilution method.

Time-kill assay (suspension test)[11]

The test microorganisms in the mid-logarithmic growth phase were inoculated into several tubes of broth containing varying concentrations of mouthwash solutions and a growth control tube without the drug. These tubes were

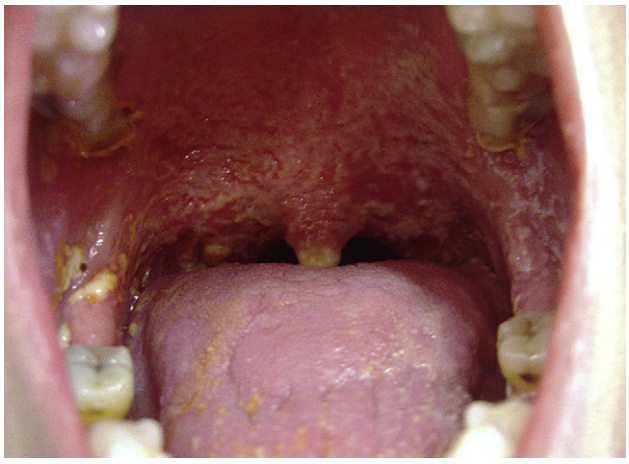

Figure 1 Clinical picture of oral candidiasis.

incubated at 35°C. Then small aliquots were removed at specific time intervals (of 0, 30, 60, 90, and 120 min), diluted to obtain countable numbers of colonies and plated onto agar for the colony count determinations. The number of organisms remaining in each sample was plotted over time to determine the rate of antimicrobial agent killing.

Time-kill was defined as the shortest interval that yielded no growth or 1 discrete colony.

Broth dilution method[11]

Two-fold serial dilutions each containing 1 mL of the antimicrobial agent were prepared. Saboroud's dextrose broth (SDB) is the medium recommended for *Candida* species. A standardized suspension of test bacteria was added to each dilution to obtain a final bacterial concentration of 5×10^5 colony-forming units (cfu)/mL. A growth-control tube (broth plus inoculum) and an uninoculated control tube (broth only) were used with each test. After overnight incubation at 35°C, the minimum inhibitory concentration (MIC) was determined visually as the lowest concentration that inhibited growth as demonstrated by the absence of turbidity.

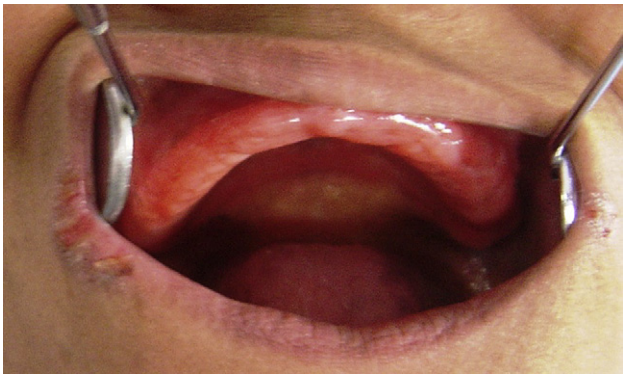

Figure 2 Clinical picture of denture stomatitis.

Results

Intraoral swabs taken from patients exhibiting symptoms of oral candidiasis were subjected to microscopic examination, isolation, and identification of *Candida* species. Most of the specimen revealed *C. albicans* and the rest *C. tropicalis*. *C. albicans* demonstrated true germ-tubes in that there was continuation of the germ tubes from yeast cells with no constriction at the junction between the yeast cell and germ tube. *C. tropicalis* showed pseudo germ-tubes, in that a constriction was present at the junction of the yeast cell and germ tube.

In total, 5 strains of *Candida* were studied: 4 *C. albicans* and 1 *C. tropicalis*. The time-kill assay and MIC procedures to determine the antifungal effects of the 2 different types of mouth rinses were carried out separately. The isolates considered in this study were limited and not representative of the entire cohort of patients. Hence, statistical analyses for significant results were not carried out.

Effect of mouthwashes

Table 1 shows the time-kill assay results for the chlorhexidine mouthwash. No colony of organisms of any of the 4 strains of *C. albicans* lasted beyond 30 min. *C. tropicalis* showed a colony count for up to 60 min. The MIC of the chlorhexidine-containing mouthwash was 1:32 (Table 2). As for Listerine, all 4 strains of *C. albicans* were killed within 60 min. *C. tropicalis* showed a colony count for up to 90 min. The MIC was 1:8 for the *C. albicans* strains and 1:16 for the *C. tropicalis* strain.

Discussion

This *in vitro* study to assess the antifungal activities of chlorhexidine- and thymol-containing mouth rinses was carried out by means of MIC and time-kill assays. The results compared favorably with investigations by Giuliana et al.[2,12] However, the concentration of chlorhexidine

Table 1 Time-kill assay (suspension test) results of Hexidine and Listerine mouth rinses.

Mouthwash	*Candida* species	Time (min)				
		0	30	60	90	120
		Colony count (cfu/mL)				
Hexidine	*C. albicans* (CA 1)	10^2	10^1	0	0	0
	C. albicans (CA 2)	10^2	0	0	0	0
	C. albicans (CA 3)	10^2	0	0	0	0
	C. albicans (CA 4)	0	0	0	0	0
	C. tropicalis (CT 1)	10^3	10^2	10^1	0	0
Listerine	*C. albicans* (CA 1)	10^4	10^3	10^2	0	0
	C. albicans (CA 2)	10^3	10^2	10^1	0	0
	C. albicans (CA 3)	10^3	10^2	10^1	0	0
	C. albicans (CA 4)	10^3	10^2	10^1	0	0
	C. tropicalis (CT 1)	10^4	10^3	10^2	10^1	0

cfu, colony-forming units.

Table 2 Minimum inhibitory concentrations (MICs) of Hexidine and Listerine mouth rinses.

Mouthwash	*Candida* species	MIC (dilution of mouthwash)
Hexidine	*C. albicans* (CA 1)	1:32
	C. albicans (CA 2)	1:32
	C. albicans (CA 3)	1:32
	C. albicans (CA 4)	1:32
	C. tropicalis (CT 1)	1:32
Listerine	*C. albicans* (CA 1)	1:8
	C. albicans (CA 2)	1:8
	C. albicans (CA 3)	1:8
	C. albicans (CA 4)	1:8
	C. tropicalis (CT 1)	1:16

required for growth inhibition of *C. albicans* was less than that required for *C. tropicalis*.

The 0.2% chlorhexidine-containing mouthwash was able to kill all strains of *C. albicans* in a shorter time compared to the thymol-containing mouthwash. Similar results were seen for *C. tropicalis*. However, both mouthwashes took longer to kill the strain of *C. tropicalis* compared to *C. albicans*. Studies conducted by Giuliana et al. showed that the kill-time of mouth rinses containing chlorhexidine were $\leq$180 seconds at half the concentration of commercial formulations.[2,8] No kill-times were achieved with the sanguinarine-containing mouth rinse, and mouth rinses containing either triclosan or hexetidine did not show a lethal effect on *C. albicans*.[2]

As the current study was a pilot study, only standard procedures of a time-kill assay were followed. However, from a clinical standpoint, patients retain a mouth rinse within the mouth for 30—60 seconds. A larger study, involving more species of *Candida* and more varieties of mouth rinses, should involve more-extensive testing and more replicates of all isolates against all mouth rinses with exposure times of 30, 45, and 60 seconds.

Time-kill studies to determine the period of exposure needed to kill *C. albicans* were also performed with antifungal drugs like amphotericin B and nystatin and compared to chlorhexidine. A comparison showed that of the 3 agents, chlorhexidine had the most rapid killing effect on this organism.[6]

The mode of action of chlorhexidine is not entirely understood. However, some clues have emerged from laboratory studies. The chlorhexidine molecule is a highly cationic chlorophenyl bisbiguanide and avidly binds to negatively charged surfaces including epithelial cells. In addition, it was shown to adsorb onto enamel and salivary proteins. It is therefore speculated that the crucial feature of chlorhexidine is its substantivity in the oral cavity. Indeed, about 30% of the total chlorhexidine dose may be retained in the mouth for 24 hours after a 1-minute rinse, although most of the agent is removed from the oral cavity within the first hour. The slow and sustained release from pellicle-covered oral surfaces appears to be an important pharmacodynamic feature of chlorhexidine.[13]

Ultrastructural studies of *Candida* exposed to chlorhexidine showed coagulation of nucleoproteins with inhibition of budding and cell wall changes with a possible escape of cytoplasmic components through the plasmalemma.[4] These morphological events lead to death of some cells while cells with previously protruding buds survived revealing a fungicidal as well as a fungistatic effect of the antiseptic.[13]

The Listerine antiseptic acts as an essential oil-containing antimicrobial mouth rinse. Its mode of action against bacterial cells involves protein denaturation and damage to the cell membrane, which results in leakage of the intracellular components.[4] Hence, thymol even when used at high concentrations, *in vitro*, allows the uninhibited growth of *Candida*, whereas chlorhexidine inhibits cell growth and replication.

Conclusions

A study was carried out to assess the antifungal effects of mouth rinses containing chlorhexidine and thymol. The results indicated that the antimicrobial agents used in the study have good *in vitro* activity against 2 *Candida* species. Mouth rinses containing chlorhexidine showed superior antifungal and fungicidal activities compared to the thymol-containing mouth rinse. Both antimicrobial agents can be suggested for use as topical antifungal agents. The current study is an *in vitro* study involving only 2 species of *Candida* and 2 different varieties of mouth rinses. A larger study involving more species of *Candida* and more varieties of mouth rinses is recommended. Continuity also needs to be given to *in vivo* studies to justify the antifungal properties of the mouth rinses and their value in management of oral candidiasis.

References

1. Prabhu SR, Wilson DF, Daftary DK, Johnson NW, eds. *Oral Diseases in the Tropics*, 1st ed. Oxford: Oxford University Press, 1992.
2. Giuliana G, Pizzo G, Milici ME, Musotto GC, Giangreco R. *In vitro* antifungal properties of mouthrinses containing antimicrobial agents. *J Periodontol* 1997;68(8):729—33.
3. Barkvoll P, Attramadal A. Effect of nystatin and chlorhexidine digluconate on *Candida albicans*. *Oral Surg Oral Med Oral Pathol* 1989;67(3):279—81.
4. Meiller TF, Kelley JI, Jabra-Rizk MA, DePaola LG. *In vitro* studies of the efficacy of antimicrobials against fungi. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2001;91:663—70.
5. MacNeill S, Rindler E, Walker A, Brown AR, Cobb CM. Effects of tetracycline hydrochloride and chlorhexidine gluconate on *Candida albicans*: an *in vitro* study. *J Clin Periodontol* 1997;24: 753—60.
6. Hamers AD, Shay K, Hahn BL, Sohnle PG. Use of a microtiter plate assay to detect the rate of killing of adherent *Candida albicans* by antifungal agents. *Oral Surg Oral Med Oral Pathot Oral Radiol Endod* 1996;81:44—9.
7. Anil S, Ellepola ANB, Samaranayake LP. The impact of chlorhexidine gluconate on the relative cell surface hydrophobicity of oral *Candida albicans*. *Oral Dis* 2001;7:119—22.
8. Pizzo G, Giuliana G. Antifungal activity of chlorhexidine containing mouthrinses: an *in vitro* study. *Minerva Stomatol* 1998; 47(12):665—71.
9. Salem AM, Adams D, Newman HN, Rawle LW. Antimicrobial properties of 2 aliphatic amines and chlorhexidine *in vitro* and in saliva. *J Clin Periodontol* 1987;14:44—7.

10. Duguid JP. Staining methods. In: Collee JG, Fraser AG, Marmion BP, Simmons, eds. *Mackie and McCartney Practical Medical Microbiology*, 14th ed. New York: Churchill Livingstone, 1996:796—9.
11. Mahon CR, Lehman DC, Mannselis G. *Textbook of Diagnostic Microbiology*, 3rd ed. St Louis, Missourie: Saunders, 2007.
12. Giuliana G, Pizzo G, Milici ME, Giangreco R. *In vitro* activities of antimicrobial agents against *Candida* species. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1999;87:44—9.
13. Ellepola ANB, Samaranayake LP. Adjunctive use of chlorhexidine in oral candidoses: a review. *Oral Dis* 2001;7: 11—7.