The antifungal properties of chlorhexidine digluconate and cetylpyridinium chloride on oral Candida

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A B S T R A C T

Introduction: C. tropicalis and C. krusei have emerged as virulent species causing oral infections. Both have developed resistance to commonly prescribedazole antifungal agents.
Objective: The study aimed to determine the effect of mouth rinses containing chlorhexidine digluconate (CHX), cetylpyridinium chloride (CPC) and their combination (CHX–CPC) on the growth of these strains.
Methods: The minimal inhibition concentrations (MIC) of the mouth rinses were determined. The growth curves of the strains produced under the mouth rinse-treated and untreated conditions, as well as alterations to the morphology of the growth colonies and cells following the treatments were compared and analysed.
Results: The MICs of CPC compared to CHX mouth rinses were found to be lower for both Candida sp. In the mixed formulation, CPC doubled the inhibitory effect of CHX towards both Candida sp., while CHX quadrupled the activity of CPC towards C. tropicalis. The growth colonies also appeared coarse, wrinkled and dried.
Conclusion: The profound effects shown may suggest the fungicidal activities of the mouth rinses incorporated with CHX, CPC or their combination on both C. tropicalis and C. krusei. Gargling using mouth rinses with such fungicidal activity would enhance a rapid reduction in the candidal population of patients with fungal infection.

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1. Introduction

The genus Candida forms part of the normal oral flora. With a low carriage rate of about 2% in the mouth of asymptomatic adults the possible pathogenic role of Candida is usually underestimated. Under a number of predisposing conditions, the number of Candida may increase dramatically resulting in tissue penetration and inflammation of the oral mucosa. Superficial candidal infection of the oral cavity is often seen as a sign of impaired local or systemic defense mechanism. Several non-Candida albicans Candida (NCAC) are now known to be of major medical importance in humans in that they can contribute to opportunistic infections in immune-compromised, neonatal and terminally ill patients, especially those with mucositis in the oral cavity. Oral mucositis is inflammation of the mucosa of the mouth which ranges from redness to severe ulceration. Patients with damaged oral mucosa and reduced immunity resulting from chemotherapy and radiotherapy are also prone to opportunistic infections in the mouth.

Oral decontamination using antifungal and antibacterial rinses is one of the five approaches often used to manage oral mucositis. Antifungal prophylaxis often includes the use of nystatin, fluconazole and amphotericin rinses, while antibacterial prophylaxis includes the use of fluoride rinses and gels. Despite these regimes however, none of the treatments according to an overview by Treister and Woo, specifically

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showed a reduction in the risk of developing oral mucositis. Candida tropicalis and Candida krusei are two examples of the NCAC that have emerged as virulent species causing candidal infection in the oral cavity. Both these NCAC have been reported to developed resistance to commonly prescribed azole antifungal agents, such as fluconazole. Chlorhexidine digluconate and cetlypiridinium chloride are two antiseptics often incorporated in mouth rinses and used as prophylaxis for both chemotherapy and radiotherapy induced mucositis. Evidence to support their effectiveness as antifungal agents however, is lacking. Therefore, the objective of the study was to determine the minimal inhibitory concentrations (MIC) towards C. tropicalis and C. krusei of mouth rinses containing chlorhexidine digluconate (CHX) and cetlypiridinium chloride (CPC) and the combination of CHX-CPC. The growth curves of C. tropicalis and C. krusei in the presence of mouth rinses were used to elucidate their mechanism of action as antifungal agent.

2. Materials and methods

2.1. Test materials

Mouth rinses used in the study were available commercially and contains the following active ingredients: 0.12% chlorhexidine digluconate (CHX); 0.053% cetlypiridinium chloride (CPC); combination of 0.12% chlorhexidine digluconate and 0.053% cetlypiridinium chloride (CHX–CPC). Two NCAC strains used were clinical samples of C. tropicalis and C. krusei obtained from the microbial stock collection of the Department of Oral Biology, Faculty of Dentistry, University of Malaya. C. tropicalis and C. krusei were revived and cultured in yeast extract peptone dextrose (YEPD) broth and agar (Difco), respectively.

2.2. Preparation of growth suspension

A 100 μL stock culture of C. tropicalis and C. krusei was inoculated into two separate sterile culture tubes containing 5 mL of broth. Following an overnight incubation at 37 ℃, the growth turbidity of the cell suspension was spectrophotometrically measured and the optical density (OD) standardised to a reading of 0.144 at a wavelength of 550 nm. A cell suspension with this OD represented a population of 1 x 10^6 cell/mL.

2.3. Determination of the minimal inhibitory concentrations of the mouth rinses against C. tropicalis and C. krusei

0.5 mL of YEPD broth was dispensed into 10 sterile test tubes designated as T1 through T10. 0.5 mL of the CHX-containing mouth rinse was added into T1 and T2. Following thorough mixing, 0.5 mL from T2 was pipetted out into T3 and these steps were repeated until T9. However, after thorough mixing, 0.5 mL of T9 was discarded. The test tubes were then inoculated with the candidal suspension; 0.5 mL of C. tropicalis suspension was added to T2 through T10. T1 which did not contain the candidal suspension represented the positive control while, T10 which did not contain the mouth rinse represented the negative control for the experiment. The final concentrations of the active ingredients in the series of serially diluted mouth rinse were as in Table 1. Tubes T1–T10 was then incubated overnight at 37 ℃. Observation for growth was done the following day based on the turbid appearance in each tube. The concentration of the active ingredient in the test tube showing no turbidity was taken as the minimal inhibitory concentration (MIC) of the mouth rinse against C. tropicalis. Similar procedure was carried out to determine the MIC of the mouth rinses against C. krusei.

2.4. Determination of the growth curves of C. tropicalis and C. krusei

(i) Normal growth curves: 0.05 mL of C. tropicalis and C. krusei suspensions were added into separate culture tubes containing 5 mL of YEPD broth. The OD of the suspension was measured to represent the reading at 0 min (t0). The tubes were then incubated in a shaking water bath at 37 ℃. The increase in the turbidity of the suspensions which indicated the growth of the cells was monitored every 15 min intervals. Graphs of OD versus time were plotted, each for C. tropicalis and C. krusei. Based on the curves, the time required for C. tropicalis and C. krusei to reach the middle of the log phase was determined as t1. All determinations were carried out in duplicate and repeated twice to ensure reproducibility of results.

(ii) Mouth rinse-treated growth curves: Similar procedure was performed to determine the growth curves of C. tropicalis and C. krusei under the mouth rinse-treated growth conditions. This time however, a volume of the mouth

<table>
<thead>
<tr>
<th>Active ingredient (μg/ml)</th>
<th>T1 (positive control)</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T7</th>
<th>T8</th>
<th>T9</th>
<th>T10 (negative control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHX</td>
<td>600.0</td>
<td>600.0</td>
<td>300.0</td>
<td>150.0</td>
<td>75.0</td>
<td>37.5</td>
<td>18.8</td>
<td>9.0</td>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td>CPC</td>
<td>265.0</td>
<td>265.0</td>
<td>132.5</td>
<td>66.0</td>
<td>33.0</td>
<td>16.5</td>
<td>8.0</td>
<td>4.0</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>CHX-CPC</td>
<td>600.0</td>
<td>600.0</td>
<td>300.0</td>
<td>150.0</td>
<td>75.0</td>
<td>37.5</td>
<td>18.8</td>
<td>9.0</td>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td>chxCPC</td>
<td>265.0</td>
<td>265.0</td>
<td>132.5</td>
<td>66.0</td>
<td>33.0</td>
<td>16.5</td>
<td>8.0</td>
<td>4.0</td>
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<td>0</td>
</tr>
</tbody>
</table>
rinse containing the active ingredient (CHX, CPC and CHX–CPC) was added into the growth medium at \( t_0 \). The volume added was determined by the MIC of the rinses determined earlier. Calculations were made so that the final concentration of active ingredient in the 5 mL growth medium equals the MIC of the respective mouth rinses. Similarly, the growth of the cells was monitored at every 15 min intervals over a period of 480 min. With regards to the combined formulation (CHX–CPC), two separate tests were carried out at two different concentrations of active ingredients. This was to take into account that the combined formulation contains CHX and CPC at two different concentrations (0.12% and 0.053%, respectively). The first which was designated as CHXcpc made use of the MIC value for CHX while, the second that was designated as chcxCPC, made use of the MIC value for CPC. Graphs of OD versus time (min) were plotted for each of the mouth rinse-treated growth conditions for both C. tropicalis and C. krusei. All determinations were carried out in duplicate and repeated twice to ensure reproducibility of results.

2.5. Determination of the effect of growth treatments on the morphology of C. tropicalis and C. krusei

C. tropicalis and C. krusei were cultured under both the untreated and mouth rinse-treated conditions. As \( t_1 \) was reached, a 100 \( \mu L \) aliquot of the growth suspension was pipetted out and evenly streaked on to YEPD agar plates. Following an overnight incubation, the colony forming units (cfu) were observed under a light microscope and their characteristics which include the diameter, margin and texture were recorded. An isolated colony was also taken and smeared on to glass slide. Gram staining procedure was performed and the slides were examined under a light microscope.

3. Results

3.1. Minimal inhibitory concentrations (MIC)

The MICs of CHX, CPC and CHX–CPC containing rinses for C. tropicalis and C. krusei were tabulated in Table 2. The MICs of CPC were lower than those of CHX for both C. krusei (66.0 and 75.0 \( \mu g/mL \), respectively) and C. tropicalis (33.0 and 150.0 \( \mu g/mL \), respectively). In the mixed formulation, CPC doubled the inhibitory effect of CHX towards both Candida sp. while CHX quadrupled the activity of CPC towards C. tropicalis. The activity of CPC towards C. krusei was not enhanced in the presence of CHX (Table 2).

3.2. Normal growth curves of C. tropicalis and C. krusei

The normal growth curves of C. tropicalis and C. krusei exhibited sigmoidal growth pattern (Fig. 1). The lag phases of both Candida sp. extended for a period of 120 min before the log phases began. The log phases then extended over a period of 300 min and 270 min, respectively for C. tropicalis and C. krusei. Between the two Candida however, the maximum cell population attained by C. tropicalis was much higher compared to C. krusei as indicated by the high OD of the cell population. Between the two, the stationary phase for C. krusei started slightly earlier than C. tropicalis.

3.3. Mouth rinse-treated growth curves of C. tropicalis and C. krusei

Descriptively it was observed that the growth curves of C. tropicalis and C. krusei were significantly affected by the addition of the mouth rinses into the growth media. The presence of the active agent either in the form of CHX, CPC or CHX–CPC in the mouth rinses had almost (~90%) depleted the growth of the Candida sp. Strong growth inhibitory activity was indicated by the loss of the log phases from the growth curves. CHX mouth rinse however displayed a slight increase in the cell population of C. krusei at the early stage (30–90 min) of incubation (Fig. 2a). However, the maximum population attained was very minor and accounted for only about 20% growth compared to the maximum population of cells under the untreated condition. The 80% drop in cell population provided an indication of the strong growth suppression effect of CHX on C. krusei. Based on the growth pattern, the action of CHX on C. krusei was fungistatic.

Similar to the effect showed on C. krusei, all the three mouth rinses also exhibited strong growth inhibitory effect on C. tropicalis. In this case however, there were no log phases observed and the growth was more than 90% depleted or

![Fig. 1 – The normal growth curves of C. krusei and C. tropicalis. OD; optical density measured at 550 nm.](image-url)
deviated from the normal curve pattern of the untreated cells. Descriptively it was observed that the growth inhibitory activity of CHX, CPC and CHX–CPC mouth rinses on C. tropicalis was very significant (Fig. 2b).

3.4. Colony and cell characteristics of C. tropicalis and C. krusei

The normal C. krusei were white to cream, opaque colonies but were rough with myceliated edges (Fig. 3a(i)).5,6 The normal C. tropicalis colonies also exhibited elevated white to cream colonies that was smooth and opaque with entire margin (Fig. 3b(ii)). Both cells reacted positively to Gram’s staining with large and rounded cells and showed the presence of blastospores. On treatment with the mouth rinses, the colonies and cells showed morphological variations (Fig. 3a(ii and iii) and b(ii and iii)). The colonies appeared dry with rough surfaces and irregular margins. The presence of blastospores was increased and the cells appeared elongated.

Similar characteristics were also observed on colonies following treatment with CHX–CPC mouthrinse. Colonies following treatment with chxCPC exhibited appearance similar to treatment with CPC while, those following treatment with CHXcpc appeared similar to those that was treated with CHX.

4. Discussion

The oral cavity of patients with reduced immunity and inflamed oral mucosa is predisposed to infection by opportunistic microorganisms such as the Candida sp. In cancer patients with induced-oral mucositis, infection by Candida can compromise their response to treatment and palliative care.4 Many oral care regimens include prophylactic antibacterial or
antifungal treatments to clear the mouth of oral microflora before and during therapy. Nevertheless, cases with mucostic patients have shown that antifungal agents like nystatin and clotrimazole washes failed to show any significant reduction in its severity.\(^7\) Antibiotic lozenges have also been designed to decontaminate the oral mucosa and reduce infections in the oral cavity. Antiseptics such as CHX is one of the most common mouth rinses used as prophylaxis for both chemotherapy and radiotherapy induced mucositis.\(^8\) The effectiveness of CHX as an antimicrobial agent is however influenced by its concentration and exposure time. Better substantivity of antimicrobial activity in saliva have been reported following exposure to mouth rinse incorporated with 0.2% CHX compared to 0.12% CHX. Greater substantivity was also recorded following longer exposure to this agent.\(^7\) However, despite the various approaches and many attempts, firm conclusions with regards to the best method to prevent infection in the oral cavity remains difficult to draw as many of the suggestions made were based on studies with non-standardised methodological issues.\(^6\)

Focus of the study was on mouth rinses incorporated with common active ingredients. Both CHX and CPC have been shown to possess very effective antibacterial activity with good success in controlling dental plaque development.\(^9\)–\(^11\) A formulation containing both CHX–CPC was used as test compound in the study based on reports that the combination of both active ingredients at a formulation of 0.12% CHX and 0.05% CPC had shown benefits both microbiologically and clinically.\(^12\)–\(^13\) CHX and CPC are cationic antiseptics. The former is a biguanide and the later is a quaternary ammonium compound. Both are surface active agents that bind non-specifically to charged protein and phospholipid moieties of the wall structure. This then alter and modify surface tension of the cell wall structure that eventually may lead to cell wall leakage before affecting cell metabolism.\(^14\)–\(^16\) A study carried out by Yu-Long et al.\(^17\) on Salmonella typhimurium and Escherichia coli suggested the antimicrobial activity of CPC relied on a chain of reactions resulted from interaction of its basic ions with acidic molecules of the bacteria. Such interaction destroys the cell wall, changed the cellular morphology and causes an efflux of intracellular component that subsequently inhibits its metabolism. This cascade of events eventually leads to interference of its respiration making the cell becoming more susceptible to CPC. Similar mechanism of action may thus, apply to Candida cell despite it not being a true bacterium. The effectiveness however, is dependent on the amount or concentration used in a formulation.\(^15\) CHX on the other hand was also found to have other positive effects in addition to it being an effective antimicrobial agent. Studies have shown that the presence of CHX in the oral cavity may enhance the initial adhesion of fibre-reinforced post to root canal by improving and stabilising bond strength of the adhesions\(^18\)–\(^19\) and at the same time promote the remineralisation of demineralised dentine.\(^20\) In another study, a significant antibacterial activity was noted when a self-etching primer was incorporated with CHX.\(^21\)

In this study C. tropicalis and C. krusei were found susceptible to mouth rinses containing CHX, CPC and CHX–CPC. This is in accordance with reports by other research groups working on oral microorganisms.\(^22\)–\(^23\) The antifungal activity of the mouth rinse containing CPC was found to be more effective against C. krusei than C. tropicalis with MIC values of 33.0 ± 0.0 and 66.0 ± 0.0 µg/mL, respectively. In contrast, the antifungal activity of the CHX mouth rinse was more effective on C. tropicalis than C. krusei. The MIC for the former was at 75.0 ± 0.0 µg/mL while for the later at 150.0 ± 0.0 µg/mL, which was 2-fold higher. Interesting results were obtained with respect to the antifungal activities of the combined formulation CHX–CPC. The incorporation of CPC seemed to have an enhanced effect on the antifungal activity of CHX and vice versa. For C. tropicalis it was shown that the amount of CHX and CPC required to minimally inhibit the growth had greatly reduced by 2-fold (37.7 µg/mL) and 4-fold (16.5 µg/mL), respectively. As for C. krusei however, the incorporation of CHX produced no effect on the antifungal activity of CPC and the MIC was maintained at 33.0 µg/mL (Table 2). The susceptibility of CHX however, was enhanced by 2-fold with the addition of CPC. Such effect on combined mouth rinse formulation on other oral microorganisms has also been reported.\(^24\)–\(^25\)

Considering the different susceptibilities of both Candida towards CHX and CPC, the concentration of CHX–CPC mouth rinse used in the growth curve study was evaluated separately at two different concentrations. In CHXcpc, the growth inhibiting strength was set at the MIC of CHX while, that of ChxCPC was set at the MIC of CPC. Cell growth is observed when there is an increase in the cell number or population.\(^26\) A cell should grow optimally when every growth requirement is provided.\(^27\) It was observed that under a normal untreated growth condition both C. tropicalis and C. krusei showed normal sigmoidal growth profiles with similar lag and log periods. The maximum population of cells attained on entering the stationary phase was however, higher for C. tropicalis compared to C. krusei (Fig. 1). The presence of CHX, CPC and CHX–CPC in the growth environment exhibited immediate profound effect on the growth profiles of both Candida sp. This was an indication of the fungicidal effect of CHX, CPC and CHX–CPC mouth rinses on C. tropicalis and C. krusei. CHX mouth rinse however, showed fungistatic effect on C. krusei that supported some minor cell growth (Fig. 2a). Similar observation has been reported by an earlier researcher who stated that low concentration of CHX may result in bacteriostatic and fungistatic effects on microorganisms.\(^24\) Descriptively, based on results obtained in this study the combined formulation appeared to be more effective in inhibiting the growth of C. tropicalis than C. krusei comparative to the individually CHX and CPC mouth rinses. However despite the better and rapid antifungal activity towards C. tropicalis and C. krusei (~80% inhibition of cell growth) shown by CHX–CPC, the effectiveness between the two active agents was not significantly different, an observation also shared with an earlier report by Nakamoto et al.\(^25\)

Suppression of cell growth was observed with the extension of the lag and the disappearance of the log phases (Fig. 2). The lag phase of a growth cycle is when the cells are becoming adapted to the growth environment and trying to synthesis new macromolecules required for the growth while the log phase is when the number and cellular components of the cells are increased at a maximum rate.\(^28\) The presence of the active ingredients in the growth environment of both Candida
had obviously interfered with the normal biological functions of the cells, rendering them unable to propagate and increase in population.\textsuperscript{23,28} Under this suppressed conditions, the affected Candida exhibited variant in their colony and cell morphologies from the normal characteristics (Fig. 3). The emergence of variant colony morphology especially in pathogenic strains has been associated with the increase in the frequency of phenotypic switching by the cells, an ability often observed when Candida strives to survive under a stressed growth environment.\textsuperscript{29} Under the switched state, many physiological cell activities are suppressed that supported the lost of the log phase in the growth curves (Fig. 2). A recent study on C. krusei reported that once in the switched state, the cells experienced changes in the biological property that includes adherence to hard surfaces and susceptibility towards CHX.\textsuperscript{30}

Despite the effective antimicrobial property of CPC, its usage in oral care products is limited due to the fact that its biological activity tended to be hindered by other common ingredients in dentifrices and mouth rinses.\textsuperscript{31} Based on results of this study in the combined formulation, the potency of CHX–CPC as antifungal agents was enhanced and lower IC\textsubscript{50} values were noted. Individually however, CHX was found more effective on C. tropicalis while CPC was more effective on C. krusei (Table 2). The resilience characteristic of C. krusei to both agents was similar to that exhibited towards the antifungal agent fluconazole.\textsuperscript{32,33}

As a conclusion, mouth rinse containing chlorhexidine digluconate and cetylpyridinium chloride or their combination possess fungicidal properties on C. tropicalis and C. krusei. Thus, rinsing using these mouth rinses would effectively enhance a rapid reduction in the candidal population of patients with fungal infection. The concentrations of these active agents in mouth rinses however, can be reduced to the MIC level as excessive amount may severely affect the ecological balance of the normal flora in the oral cavity.

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