Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) can grow as structured biofilm in different surfaces, including oral mucosa and denture surfaces. Such biofilms can be released into the oral fluids and aspirated, causing systemic infections such as aspiration pneumonia. This study evaluated the efficacy of two disinfectant solutions and microwave irradiation in disinfecting acrylic specimens contaminated with MRSA biofilm. Thirty-six acrylic specimens were made, sterilized and contaminated with MRSA (10^7 cfu/mL). After incubation (37 °C/48 h), the specimens were divided into 4 groups: not disinfected (positive control); soaking in 1% sodium hypochlorite for 10 min; soaking in 2% chlorhexidine gluconate for 10 min; and irradiating by microwave for 3 min at 650 W. The viability of cells was evaluated by XTT reduction method. All specimens from the positive control group showed biofilm formation after 48 h incubation. The mean absorbance value of the control specimens was 1.58 (OD at 492 nm). No evidence of biofilm formation was observed on specimens after the disinfection methods. Disinfection by soaking in 1% sodium hypochlorite and 2% chlorhexidine gluconate and irradiating by microwaves resulted in 100% reduction of MRSA biofilm metabolism. The use of chemical solutions and microwave irradiation was shown to be effective for eradicating mature MRSA biofilms on acrylic resin specimens.

**Introduction**

The oral cavity is a complex environment composed of tissues with different origins, structures and functions. A myriad of commensal bacteria, fungi and viruses colonize these sites. This is particularly important in immunocompromised and elderly patients, whose oral hygiene is poor (1). Among these microorganisms, \textit{Staphylococcus aureus} strains have been isolated from oral cavity, saliva and oral appliances, such as complete dentures (2). With the emergence of methicillin resistance, \textit{S. aureus} has received even more attention as bacteremia involving methicillin-resistant \textit{S. aureus} (MRSA) has been considered a global public health problem. A high percentage of nosocomial infections in patients in intensive care units are due to MRSA, which has been related to more severe and virulent infections and to a higher mortality rate (3).

Investigations have demonstrated that MRSA is able to adhere on a wide variety of substrates, including denture acrylic surfaces (4). Thus, the dentures may also function as a reservoir of this pathogen, favoring the oral colonization by MRSA. This microorganism can also readily form biofilms, which are structured communities of microorganisms enclosed in a self-produced hydrated polymeric matrix. Biofilm formation by MRSA has important clinical repercussions because the biofilms are more resistant to the host immune response and more tolerant to antimicrobials. This frequently compromises the effectiveness of therapies by giving rise to persistent and relapsing infections. Moreover, fragments of biofilm may be dislodged from denture surfaces and carried further into the lung, resulting in systemic infections such as aspiration pneumonia (5). A high prevalence of respiratory pathogens on the surface of removable dentures has been reported, suggesting that poor denture hygiene may be related to the development of aspiration pneumonia (6). In fact, a clinical study showed that the incidence of pneumonia and death decreased in patients receiving an intensive oral healthcare program (7).

Considering the information above, a strict routine of oral and denture hygiene program is essential to prevent the spread and recurrence of local and systemic infections associated with MRSA. Usually, denture hygiene of dependent elderly individuals is extremely poor, mainly because of manual dexterity impairment (1). Therefore, different methods of denture disinfection have been proposed to inactivate the microorganisms from the denture surface and improve the quality of oral hygiene of these patients. In this context, several chemical agents have been recommended and, among them, sodium hypochlorite...
NaOCl is considered useful as denture cleanser solution because it inactivates bacterial plaque, removes stains, and inhibits calculus formation on dentures (8–10). CHX is another agent commonly recommended for denture disinfection because it possesses a broad spectrum of antimicrobial activity (8–10). In addition to these chemical methods of disinfection, physical methods have also been recommended for denture disinfection. In this context, microwave irradiation received substantial attention for denture disinfection. Several in vitro studies (12, 13) showed that microwave irradiation for 3 min at 650 W is an effective method for killing a wide variety of microorganisms, including Staphylococcus aureus and intrinsically resistant species of Candida (C. glabrata and C. krusei). Moreover, an in vivo study (2) showed that this disinfection method was effective for inactivating S. aureus on denture biofilms of 30 individuals. Despite its effectiveness against several microorganisms, the literature does not address the effectiveness of microwave irradiation on MRSA in vitro biofilms.

This in vitro study evaluated the effectiveness of two chemical solutions (1% NaOCl and 2% CHX) and microwave irradiation (3 min at 650 W), for inactivating mature MRSA biofilms. The tested hypothesis was that there would be no differences among the disinfection methods.

Material and Methods

Preparation of Acrylic Resin Specimens

Thirty-six specimens were fabricated from an acrylic denture base resin (VipiWave; VIPI Indústria e Comércio Exportação e Importação de Produtos Odontológicos Ltda., Pirassununga, SP, Brazil) using a conventional flasking and pressure-pack technique. The resin disks were fabricated from a stainless steel mold with a breakaway compartment (10 x 2 mm). This metal mold was invested in the flask in dental stone sandwiched between two glass-slabs. For each specimen, 1 g of powder and 0.47 mL of monomer liquid were mixed and processed according to the manufacturer’s instructions. The mixture was packed into the molds, a trial pack was completed and excess material was removed. A final pack was performed and held for 15 min. The denture base acrylic resin was processed in a 500 W domestic microwave oven for 20 min at 20% power, followed by 5 min at 90% power. The flasks were allowed to bench cool at room temperature, the specimens were deflasked, and excess flash was removed with a sterile bur (Maxi-Cut; Les Fils d’August Maillefer SA, Ballaigues, Switzerland). After 48 h of storage in distilled water at 37 °C, all specimens were sterilized with ethylene oxide (12, 13).

Bacterial Culture and Suspension

The standard strain of MRSA used in this study was obtained from the American Type Culture Collection (ATCC 33591). The isolate was maintained in Tryptic Soy Broth – TSB (Acumedia Manufactures Inc., Baltimore, MD, USA) medium and frozen at -70 °C until use. To prepare the bacterial inoculum, a loopful of the stock culture was streaked onto Mannitol Salt Agar (Acumedia Manufactures Inc.) and incubated at 37 °C for 48 h. Two loopfuls of this culture were transferred to 20 mL of TSB and incubated under agitation at 37 °C overnight (12). Cells of the resultant culture were harvested and washed twice with phosphate-buffered saline (PBS, pH 7.2) by centrifugation at 5,000 g for 5 min. MRSA suspensions were resuspended in TSB and spectrophotometrically standardized at an optical density at 600 nm to a concentration of 10^7 cells/mL (12).

Biofilm Development

Biofilms were developed on the acrylic resin specimens placed inside pre-sterilized flat-bottomed 24-well microtiter plates. Aliquots of 2 mL of the standardized MRSA cell suspension were transferred into each well containing one specimen. The plates were incubated for 90 min at 37 °C in an orbital shaker at 75 rpm (adhesion phase). Thereafter, the non-adherent cells were removed from the specimens by gently washing twice with 2 mL PBS. For the biofilm phase, 2 mL of sterile TSB was added to each well and the plates were incubated shaking for 48 h (37 °C at 75 rpm) as described above. At 24 h incubation, the TSB medium was removed, specimens were washed twice with PBS, and an equal volume of fresh TSB was added. The microtiter plates were then incubated for further 24 h, resulting in a final incubation time of 48 h.

Disinfection Procedures

The 36 specimens were randomly divided into 4 groups (n=9): positive control and experimental groups. In the positive control group, specimens were immersed in distilled water for 10 min. In the experimental groups, the specimens were disinfected by soaking in 1% NaOCl and 2% CHX followed by microwave irradiation. Briefly, contaminated specimens of NaOCl and CHX groups were individually transferred to pre-sterilized flat-bottomed 24-well microtiter plates and soaked in 2 mL of 1% NaOCl (Labimpex Indústria e Comércio de Produtos para Laboratório Ltda., Diadema, SP, Brazil) or 2% CHX (Deg Importação de Produtos Químicos Ltda., São Paulo, SP, Brazil), respectively, for 10 min. In the microwave irradiation group, the specimens were transferred to a beaker with 200 mL of sterile distilled water. Each beaker was placed on the rotational plate in a domestic microwave oven and irradiated at 650 W for 3 min (12, 13).
**XTT Assay**

The effect of the disinfection procedures on the biofilm viability was evaluated by XTT reduction assay. This method correlates well with other quantitative techniques such as ATP and CFU assays and thus it has been widely used to evaluate fungal adhesion and biofilm formation. XTT (Sigma, St. Louis, MO, USA) was prepared in ultrapure water at a final concentration of 1 mg/mL. The solution was filter sterilized and stored at ~70 °C until use. Menadione solution (Sigma) was prepared in acetone at 0.4 mM immediately before each assay. Control and disinfected specimens were transferred to well microtiter plates, washed twice with 2 mL PBS, and re-transferred to new wells with a mixture of 1580 µL PBS with 200 mM glucose, 400 µL XTT and 20 µL menadione in each well. The plates were incubated for 3 h in the dark at 37 °C. An aliquot of 1 mL of the solution of each well was transferred to an eppendorf and centrifuged at 5,000 g for 2 min. The colorimetric change of the supernatant was measured using a microtiter plate reader (Thermo Plate - TP Reader) at 492 nm. All experiments were performed in triplicate on three independent occasions.

**Statistical Analysis**

The effect of the disinfection methods on the metabolic activity of the biofilm was measured as the percentage reduction (%) in relation to the XTT absorbance values of the control. The results obtained from the positive control and experimental groups were transferred to a computer spreadsheet program (Excel® 2007, Microsoft Corporation, USA) and descriptive statistics was employed.

**Results**

All positive control specimens contaminated with MRSA showed biofilm formation after 48 h incubation. The mean absorbance (OD at 492 nm) obtained from the positive control was 1.58. Disinfected specimens contaminated with MRSA showed no evidence of biofilm formation, regardless of the disinfection protocol. All methods of disinfection reduced 100% of the biofilm viability (absorbance) when compared to the positive control. The viability of MRSA biofilm in relation to absorbance, percentage reduction and standard deviation for all evaluated groups are presented in Table 1. The standard deviation was zero for all disinfected groups, in which no metabolic activity was observed.

**Discussion**

The hypothesis that there would be no differences among the disinfection methods evaluated in the present study was accepted. The results showed that all methods of disinfection reduced 100% of the biofilm viability when compared to the positive control. These findings agree with recent data from a previous in vitro study, in which complete dentures contaminated with planktonic MRSA were disinfected by the same disinfection protocols (14). Despite the similar results, there are some differences in the methodology used in these two microbiological studies. The most striking difference is that Altiery et al. (14) tested the disinfection protocols against planktonic MRSA cells rather than mature MRSA biofilms. Several researchers also demonstrated that planktonic MRSA were killed rapidly by means of antimicrobial agents (9,10). However, others showed that the established MRSA biofilms were more resistant to killing by these agents (9). A special characteristic of biofilms is their high tolerance to antibiotics and disinfectants as compared to planktonic cells. It has been suggested that the poor penetration of the antimicrobial agents into the complex biofilm matrix is not the only hurdle to overcome in the eradication of biofilm. Bacteria within the biofilm can also differentiate into “protected phenotypic states”, allowing these sessile bacteria to further resist standard antibiotic strategies (15). In addition to their increased resistance after the maturation process, the detachment of a cell from the biofilm mass may initiate a new cycle of biofilm formation elsewhere, perpetuating the infection. The results of this study presented effective methods for the eradication of mature MRSA biofilms, which can be useful in reducing the burden of disease associated with MRSA biofilms worldwide.

In the present study, chemical disinfection by 1% NaOCl and 2% CHX was effective against MRSA biofilm. These results are consistent with findings in the literature. The results also showed that there were no differences among the chemical methods and microwave disinfection. Denture disinfection strategies by means of chemical solutions have been frequently recommended due to their effectiveness. Of the available chemical disinfectants, both NaOCl and CHX have been used for denture disinfection due to their ability to reduce the microbial growth and adherence to dental materials. NaOCl is used as a traditional denture disinfectant because of its effectiveness in killing a wide range of microorganisms (8). In the present study, 10 min

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**Table 1. Mean of absorbance (OD) and percentage reduction (%) from positive control and experimental groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>XTT OD (SD)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>1.58 (0.27)</td>
<td>NA</td>
</tr>
<tr>
<td>1% sodium hypochlorite</td>
<td>0.00 (0.00)</td>
<td>100</td>
</tr>
<tr>
<td>2% chlorhexidine gluconate</td>
<td>0.00 (0.00)</td>
<td>100</td>
</tr>
<tr>
<td>Microwave irradiation</td>
<td>0.00 (0.00)</td>
<td>100</td>
</tr>
</tbody>
</table>

SD: standard deviation; NA: not applicable.
of immersion in 1% NaOCl solution reduced 100% of the MRSA biofilm viability on acrylic specimens when compared to the positive control. For MRSA biofilm inactivation, Lee et al. (4) reported that MRSA biofilms were difficult to eradicate unless 2% NaOCl was used. This could be explained by the time of biofilm maturation used in their study (120 h) and the lower immersion time (1 min). Despite being an effective disinfectant, NaOCl has been frequently related to corrosive effects on the frameworks, color changes of denture acrylic resin and changes in the flexural strength of denture base resins (16,17).

As seen for 1% NaOCl, 2% CHX reduced 100% of the biofilm viability after 10 min of immersion. While NaOCl dissolves mucin and other organic substances of the biofilm, causing dissolution of the polymer structure (11), CHX acts on the cellular membrane, causing loss of intracellular material, respiratory inhibition, and cytoplasmic coagulation (18). Another study demonstrated the efficacy of CHX against a wide range of bacteria from the genera Streptococcus, Pseudomonas, Bacillus, Acinetobacter, Escherichia, and Staphylococcus, including MRSA (10). Smith and Hunter (9) established a protocol of disinfection in which 24 h of immersion at 4% concentration solution was effective against planktonic cells of MRSA. Other studies also found that 2% CHX was effective to eradicate planktonic cells of MRSA (10) and MSSA biofilm (8), after 3 and 10 min of immersion, respectively. Despite the difference in concentration and time of immersion, these results are consistent with those found here, where the metabolism of mature biofilms was completely inhibited. Nevertheless, the use of CHX solution has been related to some side effects, such as discoloration of tooth surfaces, labial and buccal mucosa, tongue (19) and denture acrylic resin (17). Some components of these solutions can also penetrate into the material and may not be completely removed by rinsing (20), which may have toxic effects when applied in clinical practice as a routine disinfection method (21).

The ability of bacteria to develop resistance to antimicrobial agents is a critical challenge. Resistance of MRSA to antiseptics, including CHX, has been demonstrated (22). In addition, some problems have been encountered with the use of these chemical solutions since they may affect unfavorably the physical properties of denture acrylic resins (16,17). Such disadvantages are clearly a concern, since a disinfection method should be effective without having any detrimental effect on denture materials. In order to overcome these limitations, the use of alternative methods of disinfection has been encouraged. Microwave irradiation has been considered a feasible alternative method for denture disinfection because it needs no special storage, has no expiration date and, mainly, given that it is a physical method of disinfection, the emergence of resistant microorganisms would be avoided. In the present study, 3 min of microwave irradiation at 650 W reduced 100% of the MRSA biofilm viability when compared to the positive control. The killing action of this protocol was previously demonstrated against methicillin-susceptible S. aureus (MSSA), either in vitro (12) or in vivo studies (2). In a more recent in vitro study, MRSA adhered to simulated dentures was also killed by 3 min of microwave irradiation at 650 W (14). Nevertheless, to the authors’ knowledge, this is the first study reporting the effectiveness of this method in killing mature MRSA biofilms. Two potential explanations have been considered about microbial killing action of microwave irradiation: the thermal effect and non-thermal effect. The microorganisms can absorb microwave thermal heat at a much greater rate than the surrounding liquid medium or the microwaves can cause dielectric relaxation, ionic conductivity and biopolymer alteration (23). Injury of S. aureus cells exposed to microwave irradiation at sublethal temperature has shown to be greater than after conventional heating (24). The exposition of Bacillus subtilis and Escherichia coli suspensions to microwave irradiation caused reduction on viable cell counts and increased the leaching of DNA and protein, suggesting cell membrane cleavage (25). Similarly, a previous study demonstrated (23) that C. albicans cells could be irreversibly damaged and inactivated by microwave irradiation at 650 W during 6 min. A combination of these mechanisms is probably responsible for the effect of microwave irradiation observed in the present study.

The adverse consequences associated with MRSA colonization suggest that even a partially effective decolonization could be useful in reducing the burden of disease associated with MRSA biofilms. The outcomes produced by the three disinfection methods proposed in this study suggest that they could be used by private dental offices, institutions and hospitals in which elderly denture wearers with special needs are treated. Patients in these conditions usually have poor oral health due to difficulties in maintaining a sufficient level of personal oral hygiene and difficulties in accessing professional dental care, rendering them more susceptible to MRSA-related infections. The limitations of this in vitro study were mainly related to the fact that it evaluated only the metabolism of the MRSA biofilm. Thus, it could be of interest to evaluate other complementary parameters of the biofilm behavior after the disinfection procedures, such as a quantitative and qualitative biomass analysis and viability of the cells.

**Resumo**

*Staphylococcus aureus* resistente à meticilina (MRSA, do inglês methicillin-resistant *Staphylococcus aureus*) pode crescer como biofilme estruturado em diferentes superfícies, incluindo mucosa bucal e superfícies de próteses.
Eradicação de biofilme de MRSA sobre corpos-de-prova de resina acrílica.

100% de redução do metabolismo do biofilme de MRSA. O uso de soluções gluconato de clorexidina 2% e irradiação em microondas resultou em Nenhuma evidência de formação de biofilme foi observada em todas as amostras desinfetadas. A desinfecção em hipoclorito de sódio 1%, gluconato de clorexidina 2% e irradiação em microondas resultou em 100% de redução do metabolismo do biofilme de MRSA. O uso de soluções químicas e irradiação em microondas mostrou-se eficaz na eliminação do biofilme maduro de MRSA sobre corpos-de-prova de resina acrílica.

**References**


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