Assessing the Degree of *Candida Albicans* Adhesion to Two Types of Orthotic Appliance Materials. An *In Vitro* Study

A Thesis

Presented to the Faculty of Tufts University School of Dental Medicine

in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Dental Research

By

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August 2015

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ABSTRACT

Purpose:

The aim of the study was to assess the amount of *C. albicans* adhered to two types of orthotic appliance materials before and after modification with the use of a self-bonding polymer, (SBP, KISS-COTE). In addition, the effectiveness of multiple cleaning methods for the removal of *C.albicans* of those materials was also evaluated.

Materials and Methods:

Two types of materials were examined, acrylic and vinyl materials. Both materials were modified or not with KISS-COTE polymer. Twenty-four samples of each subgroup were tested after being incubated in the *C.albicans* suspension. Samples were stained with Gram's crystal violet, examined under a light microscope, and digitally photographed. The percentages of adhered *C.albicans* were calculated by using Image J software. As a second phase, specimens were cleaned by four cleaning methods: water, ultrasonic cleaner, toothbrush and Polident tablets. Percentages of the remaining *C.albicans* were analyzed, as described above.

Results:

The comparison of percentages of *C.albicans* on acrylic without KISS-COTE subgroup (mean=94.52) and vinyl without KISS-COTE subgroup (mean=93.80) was not statistically significant (P=0.11). The comparison between the groups of KISS-COTE modified surfaces was statistically significant, with vinyl group exhibiting more adherence (vinyl with KISS-COTE median=81.37, acrylic with KISS-COTE median=59.9) (P<0.001). There was a statistical significant difference when compared acrylic with KISS-COTE group and vinyl with KISS-COTE group to their controls (P<0.001). All cleaning methods removed *C.albicans* from the appliances

when compared to the control group (water) ($P<0.008$). Brushing showed the best degree of removal compared to the other cleaning methods.

Conclusions:

The amount of *C. albicans* adhering to acrylic material without KISS-COTE compared to vinyl material without KISS-COTE showed no significant difference. However, there was a significant difference between the two materials with modified surfaces. Brushing method was the most effective in removing *C. albicans* from all tested materials.

DEDICATION

To the generous hearts of my family and to my baby.

ACKNOWLEDGMENTS

I would like to express my appreciation and sincere gratitude to my Principal advisor Dr. Driss Zoukhri, whose guidance and assistance helped me greatly in finishing this study.

I owe my profound gratitude and appreciation to my advisor Dr. Tofool Alghanem for her continuous support and encouragement. This research may have never been completed without her assistance and kindness.

I am extremely Thankful to my great mentor Dr. George Maloney, whose encouragement and support helped me throughout the study and the Orofacial pain program as well. With a grateful respect I acknowledge Dr. Noshir Mehta who generously gave of his time and knowledge to this study as well as to us as residents in the Orofacial pain program.

I respectfully acknowledge the outstanding statistician Dr. Matt Finkelman who showed selfless generosity and gave us the gift of education. I greatly appreciate the support and understanding of Dr. Shuchi Dhadwal in the Orofacial pain department.

My Sincerest gratitude to my eldest brother Adil and my brother in-law Ali, who sheltered me with their hearts and supported me throughout my life. My words can't express how I am thankful to the kind heart of my eldest sister for her continuous prayers and love.

Lastly, I would like to thank my husband who held my hand through this journey and believed in me when I didn't believe in myself.

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Introduction

Intraoral orthopedic appliances in dentistry

Acrylic intraoral orthopedic appliances have a wide range of uses in the field of dentistry and each is designed differently according to its purpose. Karolyi first invented intraoral orthopedic appliances in 1901 as a method to prevent bruxism [1]. While oral appliances were first invented to treat bruxism and prevent the dental and soft tissue trauma it causes, as well as those attributable to sports injuries and cheek biting [1], they are now used in orthodontics as clear aligners and retainers, by general dentists as mouth guards, as sleep appliances to treat obstructive sleep apnea (OSA), and in Orofacial pain management, where they are used widely and are typically the first choice in the treatment of temporomandibular disorders (TMD) [1]. They are also used to manage the symptoms of motor disorders, such as Parkinson's disease and oral tardive dyskinesia [1], as well as to protect sensitive teeth in sinusitis patients, and for the treatment of patients with chronic migraine headaches [1]. Some studies have shown that migraines may be caused by muscular tightness and, although the use of oral appliances in such patients may help reduce the frequency and intensity of migraine episodes, there are, unfortunately, not enough evidence-based studies to prove that.

Nazarali et al. studied the efficacy of mandibular advancement appliances (MAAs) in treating OSA in a pediatric population. They concluded that, due to limited evidence, they could not say that MAAs were effective in treating the condition. However, they found that the use of MAAs led to short-term improvement in OSA [2].

Intraoral orthopedic appliances have also been used effectively for years in the treatment of all subgroups of TMDs, such as myofascial pain, disc displacement, and arthritic changes of the

condylar heads [1, 3]. However, the mechanism of action of these appliances in decreasing the pain associated with TMD is unknown [1]. There are many hypotheses about how intraoral orthopedic appliances work[1], including repositioning the mandibular condyle, recapturing the articular disc, reducing the activity of the masticatory muscles, preventing parafunctional habits, or modifying the occlusion [1]. It has also been proposed that intraoral orthopedic appliances are effective in treating TMD because they restore the lost vertical dimension of occlusion resulting from tooth wear or lost posterior teeth [4]. However, the literature has confirmed none of those theories [1].

TMD specialists have used different types of intraoral orthopedic appliances to treat TMD, including hard and soft acrylic stabilization appliances, anterior repositioning appliances, and anterior bite appliances [3]. However, many studies have demonstrated that these have varied efficacy in treatments related to TMD and its associated pain. A meta-analysis by Fricton et al. concluded that, in many studies, hard stabilization appliances gave better results compared to soft appliances or no treatment[3], while in two studies, soft stabilization appliances led to greater improvement in TMD symptoms compared to no treatment [3]. We can say that the choice of type and design of intraoral orthopedic appliances depends on the patient's symptoms and diagnosis. For example, patients with TMD symptoms that result from nocturnal parafunctional habits are given hard, rather than soft orthotic appliances. While soft orthotic appliances may be beneficial in patients with other TMD diagnoses, they may aggravate nocturnal parafunctional habits.

Recently, because of their aesthetic appearance, there has been a great increase in the use of Invisalign appliances in both young and adult populations [5]. Clear aligners are preferred in

over 90% of patients compared to clear brackets, which are acceptable by only 50% of patients [6]. Many types of mouth guards are given to patients to prevent bruxism. The materials and designs used vary depending on the case, patients' expectations, and providers' training.

TMD and its associated pain can be treated by different modalities [7]. Some of those are conservative, reversible modalities, while others involve more aggressive, irreversible methods [7]. In our study, we tested two types of materials used for the fabrication of the intraoral appliances used to treat TMD patients, acrylic material and vinyl material.

C. albicans and stomatitis

Because prolonged use of these acrylic appliances in the oral cavity is required for the conditions described above, they are subject to carrying various types of microorganisms that reside naturally in the oral cavity. One of these is *Candida albicans*,(*C. albicans*) a fungal nosocomial pathogen that has an affinity to attach to the mucosal surfaces of the human body [8]. This pathogen can infect body surfaces, such as the oral mucosa, or cause even more major systemic infections, such as systemic mycoses [9]. It is one of the four most common causes of blood stream infections [8]. In addition to blood stream infections, it can lead to other diseases, especially in immunocompromised patients, such as those with AIDS and diabetes mellitus. One of those diseases is oral thrush or stomatitis [8]. Oral stomatitis causes inflammation of the oral mucosa in contact with dentures [10]; the mucosal surfaces of the palate and alveolar ridges also typically develop erythema [10]. Although immunocompromised patients are more vulnerable to stomatitis, there are other risk factors that predispose healthy individuals to the disease, including wearing complete dentures, maxillary partial dentures, dentures during sleep, having poor denture hygiene, using

antibiotics, Vitamin A, folate, and iron deficiencies, low salivary gland flow, dry mouth medications, and smoking.

C. albicans attacks body surfaces by means of three different behaviors: invasive growth, thigmotropism, and the formation and accumulation of biofilm [8]. The latter behavior is especially important in the dental field, as it is the mechanism by which *C. albicans* grows on the oral mucosa, dentures, and oral appliances. 76% of denture wearers develop the condition as a result of this fungus. To date, there are insufficient data regarding the effects of *C.albicans* on different acrylic materials, and thus, research on this subject is needed.

C. albicans **biofilm**

A biofilm is defined as a three-dimensional group of microorganisms in an exopolymeric matrix of carbohydrates, proteins, and unidentified components, together with cells that have phenotypic properties that cause attachment to surfaces. Biofilm forms in three different stages: attachment of the cells to the surface; proliferation of microcolonies on the surface, and finally, the growth of those cells on the surfaces attacked [8, 11]. During the final stage, which includes the growth of the fungal cells, the production of hyphae (filamentous organisms) and secretion of exopolymeric matrix occur [8].

C. albicans biofilm has been found to form not only on dental enamel surfaces [12]; it also has the ability to form on natural heart valves [13] and implanted medical devices, such as prosthetic heart valves [8]; the latter infections are usually resistant to antimicrobial therapy [8].

According to a study by Hahnel et al., approximately 65% of patients who wear dentures develop stomatitis at some point as a result of microbial accumulation, especially that of *C. albicans* [14]. Thus, patients with TMD who use orthotic appliances regularly are at risk of developing stomatitis. The literature has shown that *C. albicans* is more likely to adhere to soft than hard denture liners because of the porous nature of the former [14]. In this study, we sought to determine whether or not soft denture liners or orthotic appliances are more susceptible to *C. albicans* adhesion.

Some *in vitro* studies have stated that the contamination of denture acrylic resin occurs rapidly and results in strong attachment of the fungal cells to the dentures [9, 15-17]. This is also the case for most intraoral devices. Gram's crystal violet is usually used to identify the adhered fungal cells to surfaces. The Crystal violet tends to stain the attached fungal cells and give them the required contrast to be easily seen under the light microscope due to size and refractivity[18].

KISS-COTE polymers

KISS-COTE polymers (KISS-CARE® Concentrated Gel, KISS-COTE Inc., Tampa, FL, USA) are self-bonding polymers (SBP) that improve the quality of surfaces to which they are applied. KISS-COTE polymers are purported to have the ability to modify the surfaces to become more like silicone based materials, which is highly resistant to changes in acid and pH. KISS-COTE is a self-bonding, non-stick, non-wetting monomolecular coating with an intrinsic adhesive component. It has been used in different areas in dentistry, including both intraoral and extra-oral applications. It is also believed that KISS-COTE polymers prevent dental surfaces from chemical attack and plaque accumulation.

n a randomized blind clinical study, Park et al. studied the effect of SBP on the gingival index (GI) and plaque index (PI)[19]. They used SBP and control groups. At follow up visits, they found that the PI in the SBP areas was statistically significantly lower than at baseline [19]. They also found that the GI values were significantly lower in the SBP group compared to the control group during a two weeks follow up visit [19].

Another study by Park et al. showed that the use of KISSCARE polymer decreased the degree of denture staining [20]. This study showed that dentures coated with the polymer showed only slight discoloration compared to control dentures [20]. They also claimed that the application of SBP directly to the teeth or any other dental materials would protect them from moisture, and chemical and biologic changes. Further, they stated that SPB reduced the degree of microbial contamination of the intraoral dental surfaces and the surfaces of the dental prosthesis and that it decreased the maintenance of dental prostheses and increased the time required between dental visits. A study by Sampaio-Maia et al. concluded that some denture adhesives had *C. albicans* contamination, while others had an inhibitory effect on the formation of *C. albicans* biofilm [21]. In another study, they found that the adhesion of *C. albicans* biofilm to Poly Methyl Methacrylate denture material was reduced significantly by modification of the surface charges and application of SBP [22]. KISS-COTE polymers are considered safe, non-toxic materials. It is also believed that they improve the surfaces to which they are applied by making them non-stick, and thus, easier to clean and maintain.

Cleaning methods

C. albicans adhered to orthotic materials can be removed by different cleaning methods, which reduces the chance of developing stomatitis. Most patients use regular cleaning methods

like brushing the orthotic appliances with a toothbrush and tap water. Others use denture dentifrice tablets to clean their appliances or simply rinse them under the running tap water. Polident denture dentifrice tablets include such components as titanium dioxide, silicon, and silica dioxide, which increase the effectiveness of the cleaning [23, 24]. Another method of cleaning is the ultrasonic cleaner that is used in the dental office during regular check-up appointments. In order to prevent the formation of *C. albicans* biofilm on dentures, routine denture cleaning should be performed [25].

Some studies, such as that of Paraskevas et al., showed that the use of a toothbrush with dentifrice removed 50% of the *C. albicans* biofilm. They mentioned that the brushing and its mechanical action were more important in removing the adhered *C.albicans* than was the dentifrice [26]. Another study concluded further that the abrasive action of the dentifrice when used with brushing resulted in wear to and roughness of the surfaces of the dentures [27], which may lead to a greater susceptibility to biofilm adhesion and fungal colonization [25].

In this study we compared the degree of attachment of *C. albicans* on the surface of two different orthotic materials (acrylic and vinyl). We also studied the effect of application of KISS-COTE® polymers on the surfaces of those materials on the attachment of *C. albicans* cells. Based on the review of the literature, there are only studies of the degree of the attachment of *C. albicans* on the denture materials. In the second part of the study, we studied the most effective method of removing the *C. albicans* from different acrylic appliance materials. Samples from each of our four subgroups were subjected to four cleaning methods. The amount of *C. albicans* adhering on the samples was measured afterwards to quantify the efficacy of each cleaning method.

Aim and Hypothesis

The goal of the study was to compare the adhesion of *C. albicans* to different appliance materials (acrylic vs. vinyl) under varied conditions (KISS-COTE vs. non KISS-COTE). The materials used were 1.5mm acrylic material (Great Lakes Orthodontics, Ltd.) and 1.5mm vinyl material (Great Lakes Orthodontics, Ltd.). These materials were also compared after adding KISS-COTE polymers to their surfaces based on the percentage of *C. albicans* adhering to them to evaluate the effectiveness of KISS-COTE polymers in reducing the adhesion of *C.albicans.* Thereafter, the effectiveness of multiple cleaning methods was compared. This comparison was based on the amount (percentage) of *C. albicans* that remained on the surfaces of the materials after cleaning.

We hypothesized that the vinyl orthotic appliance material would accumulate more *C. albicans* compared to the acrylic material due to its porous nature. We also hypothesized that both appliance materials that had KISS-COTE polymers applied to their surfaces would show less *C. albicans* adhesion by comparison to those without KISS-COTE polymers. In the second part of the study, we hypothesized that tooth brushing would remove a higher mean amount of the *C. albicans* compared to other cleaning methods including tap water, ultrasonic cleaner, and Polident denture cleaning tablets.

Significance

This study may help providers to choose the material that is less susceptible to *C. albicans* attachment for patients at risk of developing stomatitis. If proven in the study that the use of KISS-COTE polymer in oral appliances reduces the fungal cells attachment, then it might help clinicians to reduce the risk of *C. albicans* adhesion and thus reduce the chance of oral

stomatitis, especially in patients with long term use of orthotic appliances or dentures and those with high susceptibility to oral stomatitis like those with bad oral hygiene or in immunocompromised patients. It will also provide us with the most effective method to remove *C.albicans* of the orthotic appliances.

Materials and Methods

Materials:

The study was conducted at Tufts University School of Dental Medicine (TUSDM). Two main orthotic appliance materials were tested: acrylic orthotic appliance material (1.5mm) obtained from Great Lakes ORTHODONTICS, LTD and vinyl orthotic appliance material (1.5mm) obtained from Tough elastic foil, TIEFZIEHTECHNIK, Transfermasken, Fluoride U. Medikamententräger. Two subgroups were designed to be modified by adding KISS-CARE concentrated gel purchased from KISS-COTE, Inc., Tampa, FL. Frozen stock of the standard strain of *C.albicans* (ATCC MYA-2876™) (SC5314) was from American Type Culture collection, Manassas, VA 20108 USA. Difco™ YPD Agar and Difco™ YPD Broth used to prepare the fungal suspension were obtained from Becton, Dickinson and Company Sparks, MD 21152 USA. Phosphate buffered saline PBS (Sterile) used to maintain the required osmolality of fungal cells during the experiment was from Boston BioProducts, 159 Chestnut Street, Ashland, MA 01721. Gram's crystal violet stain, stains the attached fungal cells to give them the required contrast to be easily seen under the light microscope, was purchased from Sigma-Aldrich, USA. Tartar & stain remover used with the Quantrex ultrasonic cleaner was obtained from Henry Schein Inc., Melville, NY, USA. Antibacterial 3-minute denture cleanser from Polident and manual soft bristles toothbrush from Oral-B Pro-Health were also used to study various cleaning methods.

Methods:

Sample size calculation for part 1

A power calculation was conducted using nQuery Advisor (Version 7.0). The calculation assumed that the mean percentage of surface area covered with *C. albicans* would be 5.39% for the acrylic with KISS-COTE group[21] and 8.80% for the acrylic without KISS-COTE group [21]. We also assumed that the within-group standard deviation would be 5.72% [21] and that the means of the vinyl groups would be 50% higher than the respective acrylic groups. Under these assumptions, a sample size of n=24 per group was adequate to obtain a Type I error rate of 5%, a power of 95% for comparing the KISS-COTE condition versus the without KISS-COTE condition, and a power of 85% for comparing the acrylic condition versus the vinyl condition.

Sample size calculation for part 2

A power calculation was conducted using nQuery Advisor (Version 7.0). Assuming that the percent removal would be 98% for the brushing group [25], 90% for the denture dentifrice tablet group [25], 90% for the ultrasonic cleaner group, and 1% for the water group, as well as a within-group standard deviation of 1%, a sample size of n=6 per group was adequate to obtain a Type I error rate of 5% and a power greater than 99%.

Randomization

Once the results from the comparison of percentages of *C.albicans* of all groups were obtained, samples were randomized to their respective cleaning methods via a randomized block design. The blocking factor was the percentage of surface area covered with *C.*

albicans (which was found previously). The purpose of the blocking was to achieve a balanced level of initial *C.albicans* coverage across the four groups in the cleaning methods. The randomization was performed using the statistical software package R (Version 3.1.2).

1- Study groups

Two main groups (divided into four subgroups) of orthotic appliance materials were tested (Figure 1).

Subgroup A: hard acrylic material without KISS-COTE

Subgroup B: hard acrylic material with KISS-COTE

Subgroup C: soft vinyl material without KISS-COTE

Subgroup D: soft vinyl material with KISS-COTE

2- Sample preparation

A total of ninety-six samples (twenty-four in each subgroup) were fabricated on a prepared stone cast (made of dental stone). Samples were prepared using sheets of acrylic and vinyl orthotic appliance materials in a flasking pressure machine (Biostar, Great Lakes). Using dremel (4000-2/30 120-Volt variable speed rotary tool), (1cm x 1cm and 1.5mm thickness) square samples were cut of acrylic and vinyl orthotic appliance materials. The samples were then finished using dental polishing burs and polished using pumice and a rubber wheel per the manufacturer's recommendations .The KISS-COTE polymer subgroups (subgroups B and D) were covered on both sides with KISS-CARE concentrated gel (KISS-COTE®, Inc., Tampa, FL) by applying one drop of the gel (approximately 5 mg) to each sample. The polymer was

then spread evenly on both sides of each sample using the fingertips. All prepared samples were stored under ultraviolet light in the lab.

3- Preparation of the yeast suspension

A frozen stock of the standard strain (SC5314) of *C. albicans* was used to prepare the fungal suspension. The microbial suspension was first prepared by placing 5ml of sterile distilled water in a test tube. Then 0.5ml of the distilled water was added to re-suspend the pellet of the frozen stock of *C. albicans* and allowed to stand for 3hrs at room temperature. In order to provide a suitable medium for *C.albicans* to grow, YPD agar was prepared. The preparation was done by adding 32.5g of YPD agar powder (Difco^{TM} YPD Agar) to a flask containing 500ml of distilled water. The flask was covered with aluminum foil and placed in the autoclave at 112^0C for 15 min. The agar then cooled to 30^0C for 45 min before being removed from the autoclave. Thereafter, the YPD agar was poured into petri dishes and allowed to cool at room temperature. The petri dishes were placed upside down in a plastic bag and allowed to cool in the refrigerator for a few hours. Using an inoculation loop, *C. albicans* suspension was streaked over the YPD agar plates. The petri dishes incubated for 24hrs at 30^0C. Fungal colonies growth was observed after overnight incubation.

Broth was prepared by adding 25g of the YPD powder to 500ml of distilled water. This was covered with aluminum foil, placed in the autoclave at 112^0C for 15 min, and then allowed to cool in the autoclave for 45 min. After it reached room temperature, 5 ml of the broth was placed in several centrifuge tubes. Using the inoculation loop, a single colony of the grown *C. albicans* cells on the YPD agar plates was cultured in 5ml of YPD broth. Then they were incubated at 30^0C for 48hrs under aerobic conditions.

4- Development of *C. albicans* cultures

C. albicans was created on all specimens with the following steps. The prepared cultures of *C.albicans* were centrifuged for 4 min at 1000 rpm (50 x g). The YPD medium was removed; an equal volume (5ml) of sterile phosphate buffering saline (PBS) was added to the pellet and re-suspended. Samples were sanitized using 70% ethanol before being placed in the *C. albicans* suspension in twelve well flat-bottomed plates. Each specimen was placed individually in each well. To guarantee getting a uniform *C.albicans* adhesion across all samples, an amount of four hundred µl of *C. albicans* suspension was added to the samples in each well. Well plates then incubated for 24hrs at 37^0C to provide adhesion of *C.albicans* to specimens (adhesion phase). After 24hrs of incubation, 2 ml of PBS was used to wash the non-adherent fungal cells of each sample. Subsequently, all samples were placed in 10% formalin for 15 min. This step fixed the *C. albicans* cells and maintained their characteristics. Disks were then rinsed twice with PBS to remove excess formalin. The adherent fungal cells on each disk was stained with 400 µl of 1% Crystal violet solution. The standard Crystal violet solution was prepared by adding 1ml of Gram's crystal violet (Sigma-Aldrich, USA) to 99 ml of 10% ethanol to reach the final concentration of 1%. Samples were immersed in the stain for 15 min. Thereafter disks were washed with water until the water ran clear and then dried at room temperature.

5- Fungal cell quantification

The percentages of *C.albicans* formed on acrylic and vinyl samples were analyzed. After the samples were stained, washed and dried, we measured the percentage of the stained *C. albicans* attached to each. Staining with Gram's crystal violet identified the surface area of the

C. albicans attached to the surfaces of the samples as it gives them the required contrast to be easily seen under the light microscope. Samples were examined under a light microscope, after which four digital photographs of each sample were taken; each photograph represented one quadrant of each sample. The percentage of the surface area of *C. albicans* adhering to the samples was measured by analyzing the photographs with Image J software (Figs. 2,3).

6- Cleaning methods

All twenty-four samples were then assigned randomly to four cleaning methods (n=6 for each method): tap water (control); an ultrasonic cleaner; manual toothbrush, and Polident denture cleaning tablets.

a) Brushing with a toothbrush

The toothbrush used in this method was a manual soft bristles toothbrush (Oral-B Pro-Health). Each sample assigned to this group was dipped momentarily in tap water then cleaned with the toothbrush with 3 gentle strokes. This resulted in one brushing session for each individual specimen, which is equivalent to 90 reciprocal strokes in the mechanical tooth-brushing machine.

b) Polident denture dentifrice

Polident antibacterial tablets were placed in 200 ml of tap water; samples were placed in the Polident suspension for 3 min according to the manufacturer's recommendations. Specimens were removed from the Polident solution and placed on bench top to dry at room temperature.

c) Ultrasonic cleaner

Each sample was placed in a Ziploc bag with 100 ml of a stain remover. Bags then were placed in the ultrasonic cleaner for 3 min (based on the clinical standard of care). Because the samples were placed in a stain remover, the Gram's crystal violet stain was wiped off the samples after removal from the ultrasonic cleaner. Therefore, samples in this cleaning method were re-stained after being removed from the ultrasonic cleaner.

d) Tap water (control)

Samples were immersed in 200 ml of tap water for 3 min. They were placed on bench top to dry at room temperature before being examined under the light microscope. Samples from this group were further used to test the effect of a combined method of brushing and Polident tablets. Specimens were immersed in a suspension of Polident for 3 min then brushed with a toothbrush using the standardized method (3 gentle strokes over each sample).

The percentage of the *C. albicans* that remained on each sample was visualized under the light microscope; digital photographs were taken and analyzed using Image J software.

Data presentation and statistical analyses

Descriptive statistics (means and standard deviations) were computed. We planned initially to use two-way ANOVA for part 1 and one-way ANOVA for part 2. Due to the fact that the

data in part 1 were normally distributed in some groups but not others, we used the independent-samples t-test and Mann-Whitney U test as appropriate to compare groups to one another in this part. For part 2, due to non-normality of the data, the Kruskal-Wallis test (followed by the Mann-Whitney U test with Bonferroni correction for post-hoc tests) was used for the comparison of the cleaning groups. Because both parametric and non-parametric statistical tests were used in part 1, means, medians, standard deviations, and inter-quartile ranges are all presented for part 1 results. Because only non-parametric statistical tests were used in part 2, only medians and inter-quartile ranges are presented for part 2 results. P-values less than 0.05 were considered significant, with the exception of tests in which the Bonferroni correction was used. SPSS Version 22 was used in the analysis.

Results

In order to compare the affinity of the materials of all subgroups to *C.albicans* attachment, digital pictures of the samples taken of the light microscope were analyzed using Image J software. Figures 2 and 3 represent digital photographs taken of the stained fungal cells seen under the light microscope to be analyzed by Image J software. Image J software provided us with the percentages of the adhered fungal cells on each sample. First, acrylic without KISS-COTE group was compared to vinyl without KISS-COTE group in order to evaluate the affinity of adherence to both materials. Secondly, acrylic with KISS-COTE and vinyl with KISS-COTE were compared to their controls to evaluate the effectiveness of KISS-COTE polymers. We finally compared the modified groups to each other. We started by comparing the percentages of *C.albicans* attached to acrylic without KISS-COTE subgroup to the percentages of the biofilm attached to vinyl without KISS-COTE subgroup. The groups

were compared based on the percentages of *C.albicans* adherence. Acrylic without KISS-COTE group had a similar amount of *C.albicans* adherence compared to vinyl without KISS-COTE group. Modified groups with KISS-COTE showed a lower level of *C.albicans* adhesion. Table 1 summarizes the medians, means, SD and IQR of acrylic without KISS-COTE subgroup, acrylic with KISS-COTE subgroup, vinyl without KISS-COTE subgroup and vinyl with KISS-COTE subgroup.

Statistical tests including Mann-Whitney U tests and independent samples t-tests were performed to evaluate the degree of adhesion among subgroups (acrylic without KISS-COTE subgroup, vinyl without KISS-COTE subgroup, acrylic with KISS-COTE subgroup, and vinyl with KISS-COTE subgroup). The Mann-Whitney U test was used to compare the acrylic without KISS-COTE subgroup to the vinyl without KISS-COTE subgroup. The percentage of *C.albicans* adhering to acrylic samples without KISS-COTE was not statistically significant when compared to vinyl samples without KISS-COTE ($p = 0.11$). We also evaluated the effectiveness of KISS-COTE polymer in reducing *C.albicans* attachment in both acrylic and vinyl materials. Table 1 summarizes the medians of the percentages of *C.albicans* on acrylic with KISS-COTE and vinyl with KISS-COTE subgroups. The comparisons were made between the subgroups with modified and non-modified surfaces of the same types of materials. Mann-Whitney U tests and independent samples t-tests were used to assess whether there was a statistically significant difference on the adhesion of *C.albicans* among all the subgroups (acrylic without KISS-COTE subgroup, vinyl without KISS-COTE subgroup, acrylic with KISS-COTE subgroup, and vinyl with KISS-COTE subgroup).

The comparison of the acrylic samples modified with KISS-COTE polymers to acrylic samples without KISS-COTE polymers using Mann-Whitney U test was statistically significant with a p-value <0.001. Independent samples t-test was used to compare vinyl with KISS-COTE group to vinyl without KISS-COTE group; the results showed a statistically significant difference between the groups (p-value <0.001). The percentage of *C.albicans* adhering to acrylic samples with KISS-COTE was statistically significant when compared to the percentage of *C.albicans* adhering to vinyl samples with KISS-COTE using independent samples t-test (p-value < 0.001).

Further experimentation took place to determine the best method to remove *C.albicans* of both materials. Each subgroup was subjected to four different cleaning methods: water, ultrasonic cleaner, toothbrush and denture cleaning tablets (Polident).

The medians of the percentages of the amount of remaining *C.albicans* in the acrylic samples without KISS-COTE after application of the cleaning methods are summarized in Table 2. Water group had the highest amount of remaining *C.albicans* while brushing group had the lowest amount of remaining *C.albicans*. The other cleaning groups (ultrasonic and Polident had higher levels of remaining *C.albicans* compared to brushing group. Kruskal-Wallis test for the comparison of cleaning groups was statistically significant (p-value<0.001). The median of the control group (cleaning with water) was (median [IQR]=93.48[3.48]). When the ultrasonic group was compared to the water group, the ultrasonic group was statistically significantly more effective in removing *C.albicans* compared to water (p-value=0.002). The result of the comparison of the toothbrush group with the water group showed that brushing statistically significantly reduced *C.albicans* when compared to water (p-value=0.002). Similar results were obtained when comparing Polident solution to the water group where Polident

solution was statistically significantly more effective in reducing *C.albicans* than water (p-value=0.002). The results of the statistical analyses suggested that all cleaning methods had a better degree of reduction of the adhered *C.albicans* when compared to the water group. As the water was the least effective method in the removal of the *C.albicans*, samples of this group were subjected to another cleaning method, brushing after immersion in the Polident solution. In assessing whether the mechanical cleaning using the toothbrush was more effective than the chemical cleansing method using Polident solution, the Mann-Whitney U test showed that brushing with water compared to the immersion in the Polident was statistically significant (p-value=0.002). When brushing with a toothbrush was compared to the ultrasonic cleaner, the difference was not statistically significant when the Bonferroni correction was used to adjust for multiple comparisons (p-value= 0.009). Similar results were obtained for the comparison of the ultrasonic cleaner and the Polident group (p-value= 0.009).

Further statistical analyses were conducted to evaluate the effectiveness of brushing with Polident method. When brushing with Polident was compared to the immersion in the Polident solution, the result was statistically significant. Brushing with water and brushing after the immersion in the Polident solution were more effective in removing *C.albicans* compared to all other cleaning methods (water, ultrasonic cleaner, Polident). There was no significant difference in the reduction of *C. albicans* among brushing groups.

The medians of the percentages of the amount of remaining *C.albicans* on the vinyl samples without KISS-COTE after applying the cleaning methods are summarized in Table 2. The median of the control group (cleaning with water) was (median [IQR]=94.64[3.63]). The comparison of ultrasonic cleaner to the water showed no statistically significant difference between the groups (p-value =0.699). Comparison of the toothbrush group with the water

group showed that brushing statistically significantly reduced the adhered *C.albicans* when compared to water (p-value=0.002). The Polident solution was also found to statistically significantly be more effective in reducing adhered *C.albicans* than water (p-value=0.004). All cleaning methods showed a better degree of reduction of the adhered *C.albicans* when compared to the water group. Samples of this group were also used to test the effectiveness of combining brushing with Polident solution in the removal of the adhered *C.albicans*.

There was a significant amount of reduction of *C.albicans* in the toothbrush group when compared to the Polident group (p-value =0.002). When brushing was compared to the ultrasonic cleaner, the difference was statistically significant (p-value= 0.002). However, the comparison of the amount of reduction of *C.albicans* using the ultrasonic cleaner and the Polident was not statistically significant when using the Bonferroni correction (p-value =0.026, Table 2). The results of the statistical analysis that compared the ultrasonic cleaner and the Polident to the brushing method were statistically significant (p-value = 0.002). Brushing was the more effective method in the removal of *C.albicans* compared to the other cleaning methods.

Statistical tests showed that brushing with water and brushing after the immersion in the Polident solution were more effective in removing *C.albicans* compared to all other cleaning methods (water, ultrasonic cleaner, Polident). There was no significant difference in the reduction of the adhered *C.albicans* among brushing groups.

When acrylic with KISS-COTE group and vinyl with KISS-COTE group were subjected to all four cleaning methods, similar results to those obtained with non KISS-COTE groups were achieved (data not shown).

Discussion

This research was conducted to test the adhesion of *C.albicans* on orthopedic appliance materials that have different characteristics. The conditions of the experiment among all subgroups (thickness of samples, concentration of *C.albicans*, amount of *C.albicans* and incubation time) were standardized. Intraoral orthopedic appliances are used widely in dentistry. However, it has not been proven in previous studies if those specific materials of the orthotic appliances have the tendency to get adhesion by *C.albicans* on their surfaces. Park et al. studied the tendency of adhesion of *C.albicans* on three different materials (pure poly methyl methacrylate –PMMA-, modified PMMA and PMMA coated with SBP) used for fabrication of dentures [22]. They reported that the modified surfaces and the surfaces coated with SBP had a significantly lower amount of *C.albicans* compared to the control[22].

The results of our study showed that there was no significant difference between acrylic without KISS-COTE material and vinyl without KISS-COTE material. However, the comparison of modified materials with KISS-COTE polymers to their controls was statistically significant. Also the comparison of the modified groups to each other was statistically significant. We concluded that the effectiveness of KISS-COTE polymer varies based on the materials they are applied to as it was more effective in reducing *C.albicans* adhesion when applied to acrylic material compared to vinyl material. That is probably due to the difference in the chemical components of the materials and how the polymers react with those components.

C.albicans is one of the most predominant fungal pathogens in the oral cavity[28]. It is known as the primary cause of stomatitis among denture wearers [28]. Fungal cells have high affinity to attach to different types of dental materials by almost the same behavior that they take to attach to oral tissues [29]. *C.albicans* is one of the most important fungal cells that form

biofilm over mucosal surfaces and surfaces of fitting surfaces of the dentures. This attachment of fungal cells cannot be easily removed from the surfaces of dental materials [29]. Chemical and mechanical cleaning methods were recommended in some studies to minimize the adhesion of *C.albicans*[29, 30].

The present study evaluated multiple cleaning agents in order to determine the most effective method to remove *C.albicans*. The samples were fabricated from 1.5 mm acrylic material and 1.5 mm vinyl material according to the manufacturers' recommendations. The procedures of the preparation of the orthotic appliance materials may have contributed to the increased adhesion of *C.albicans* to the surfaces. All study groups were subjected to four different cleaning methods in order to study their effectiveness in removing the adhered *C.albicans.* The statistical analysis revealed that the mechanical brushing with water among all groups was the most effective method in removing *C. albicans*. The comparison of the other methods showed that the cleaning with tap water was the least effective among all groups, followed by the Polident and then the ultrasonic cleaner. The mechanical brushing with water and brushing after the use of Polident were the most effective methods for the removal of the adhered *C.albicans*.

Park et al. concluded that the use of SBPs was effective in reducing the degree of staining to the denture base materials compared to the control [20]. The results of the statistical analysis in our study revealed that the KISS-COTE groups had lower amounts of adhesion on their surfaces compared to the non KISS-COTE groups. The vinyl with KISS-COTE group had a statistically significantly higher amount of adhesion compared to acrylic with KISS-COTE group. Considering the increase of the susceptibility of orthotic appliance material to the

adhesion of *C.albicans* based on our results, adding the SBPs (KISS-COTE polymers) will help in reducing the degree of attachment of *C.albicans* to those materials.

The daily cleaning of the intraoral appliances is recommended to all patients that use intraoral appliances as homecare of the appliances. In a study by Pellizzaro et al., they stated that the brushing with dentifrice showed a higher degree of reduction of *C.albicans* biofilm (98%) from denture base resin material compared to brushing with water that removed around 96% of the biofilm [25]. However, there was no significant difference between the two methods [25]. Brushing with dentifrice proved to be effective in the removal of *C.albicans* from denture materials in a previous study [26].

Our study showed that brushing was effective in removing up to 92% of the *C. albicans* from the orthotic appliance materials while brushing with Polident removed up to 91% of the adhered *C. albicans*. Mechanical brushing with water was not statistically significantly different when compared to brushing with Polident.

The limitations of our study include that it was an *in vitro* study that lacks the real environment of the intraoral cavity and factors that could affect the growth of the *C.albicans.* The lack of the curvature of the alveolar ridge of the oral cavity may have affected the attachment of *C.albicans* in our experiment. Even though the analysis using Image J software provides the accurate percentage of the covered surface area, it can still be considered as a subjective method.

Conclusion

Within the limitations of our study, there was no significant difference in the amount of adhesion of *C. albicans* on acrylic without KISS-COTE or vinyl without KISS-COTE orthotic

appliance materials. However, there was a statistically significant difference in the amount of *C. albicans* adhered to modified materials (acrylic and vinyl) when compared to their controls.

Our study also showed that KISS-COTE polymer could be used as a lining to the orthotic appliances to reduce the amount of *C. albicans* adhesion. We also conclude that the use of the brushing technique either with water or Polident was the most effective way to remove *C. albicans* from the orthodontic appliances. Future *in vivo* studies are necessary to provide more clinically applicable conclusions, as our study was an *in vitro* study and lacks the real conditions of the oral cavity.

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APPENDICES

Appendix A: Tables

Appendix B: Figures

Table 1 Percentage of *C. albicans* adhering to different materials (n=24 per group).

Material	KISS-COTE	Mean	SD	Median	IQR
Acrylic	Yes	61.80	6.85	59.92	9.94
	No	94.52	3.70	95.65	4.89
Vinyl	Yes	80.71	8.29	81.37	12.15
	No	93.80	2.39	94.43	3.10

Comparison of acrylic without KISS-COTE vs. acrylic with KISS-COTE (Mann-Whitney U test): $p < 0.001$; comparison of acrylic with KISS-COTE vs. vinyl with KISS-COTE (independent samples *t*-test): p-value < 0.001; comparison of vinyl without KISS-COTE vs. vinyl with KISS-COTE (independent samples *t*-test): p-value < 0.001; comparison of acrylic without KISS-COTE vs. vinyl without KISS-COTE (Mann-Whitney U test): p-value =0.11

Table 2 Percentage of *C. albicans* remaining on acrylic and vinyl samples without KISS-COTE following cleaning (n=6 per group).

	Acrylic Appliance		Vinyl Appliance	
	Median	**IQR**	**Median**	**IQR**
Water	93.48[A]	3.48	94.64[A]	3.63
Ultrasonic	53.05[BD]	34.57	93.90[AB]	7.94
Toothbrush	17.54[BC]	14.12	31.10[C]	23.51
Polident	79.05[D]	12.06	88.46[BD]	17.21

Groups with the same letter did not exhibit a statistically significant difference (Mann-Whitney U test with Bonferroni correction).

Figure 1 Study groups.

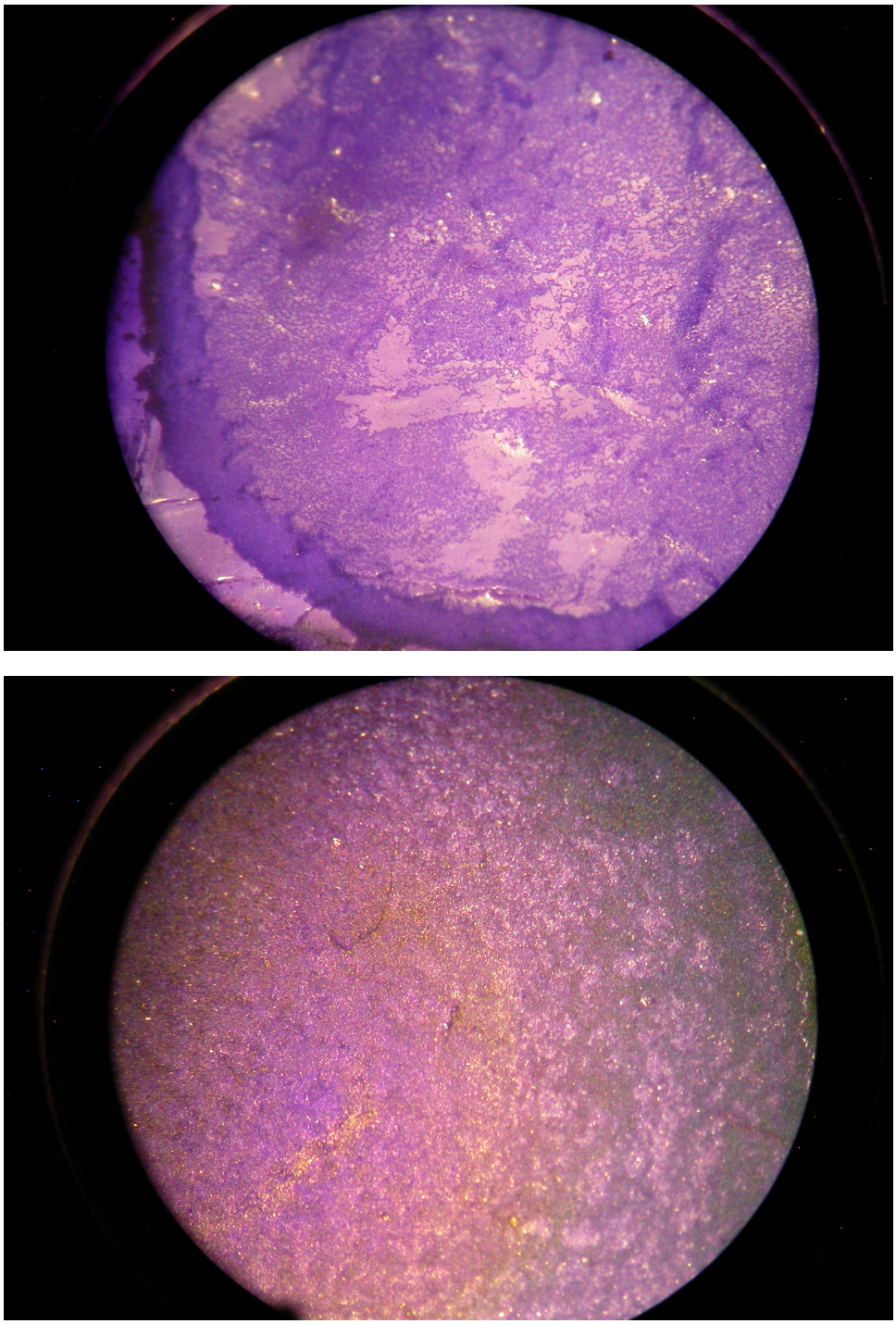

Figure 2 Digital images taken from the light microscope. Arranged from the top; acrylic without KISS-COTE sample, vinyl without KISS-COTE sample.

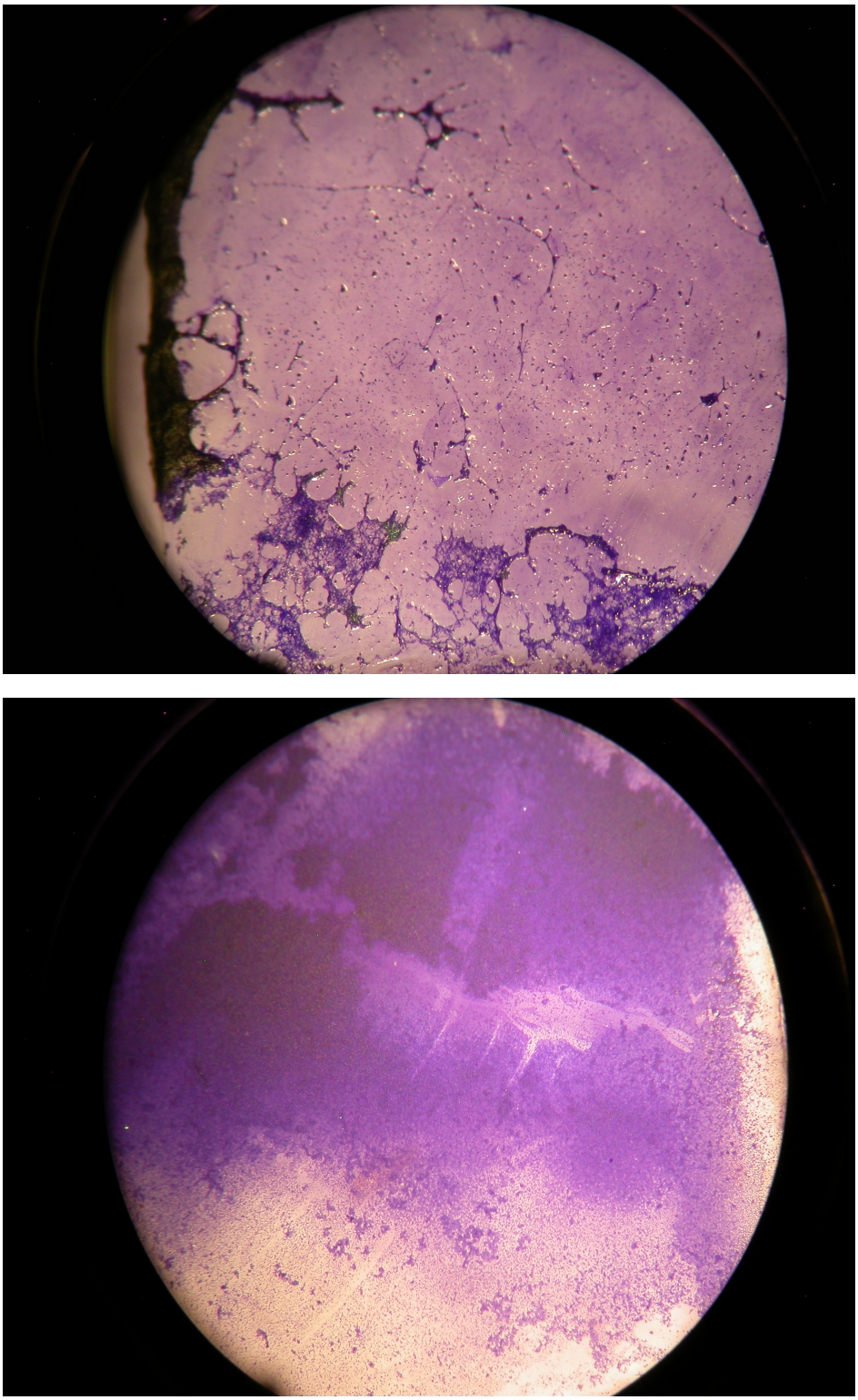

Figure 3 Digital images taken from the light microscope. Arranged from the top; acrylic with KISS-COTE sample, vinyl with KISS-COTE sample.

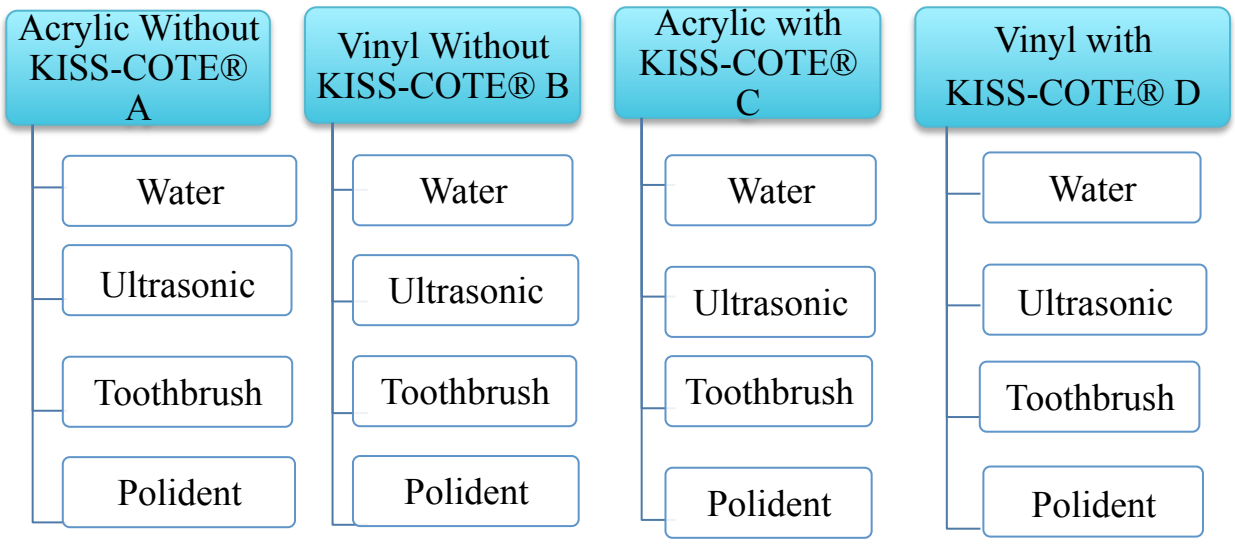

Figure 4 Cleaning Methods.