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Effects of Various Decontamination Protocols on the Surface Microbial Load of Conventional and 3D Printed Surgical Guides for Dental Implants

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Effects of Various Decontamination Protocols on the Surface Microbial Load
of Conventional and 3D Printed Surgical Guides for Dental Implants

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APPROVAL PAGE

Master of Dental Science Thesis

Effects of Various Decontamination Protocols on the Surface Microbial Load of Conventional
and 3D Printed Surgical Guides for Dental Implants

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D. ABSTRACT

STATEMENT OF PROBLEM: Currently, there is limited research on the surface microbial load of 3D printed and conventionally fabricated surgical (prosthetic) guides and the effect of commonly available solutions in their ability to reduce the surface microbial load.

OBJECTIVES: The primary objective of this study was to determine if there was any difference in the microbial load sampled from the surface of surgical guides between various commonly used methods of disinfection and sterilization. The secondary objectives were to identify if there were any differences in microbial load between untreated conventional acrylic resin surgical guide samples and 3D printed surgical guide samples.

MATERIALS AND METHODS: In this 2-part study, a total of 61 surgical guides were fabricated for an edentulous maxilla of which 54 were fabricated conventionally using autopolymerizing acrylic resin (CSG) and 7 were fabricated using 3D printing technology (3DG). In the first part of the study, 36 CSGs were randomly distributed into 6 groups as follows: 1) a multi-purpose surface disinfectant spray (DS); 2) a 0.12% chlorhexidine gluconate (CHX) immersion for 15 minutes; 3) a 70% isopropyl alcohol (IPA) wipe for 3 minutes; 4) immediate use steam sterilization (“flash cycle”) for 4 minutes at 135°C (IUSS); 5) steam sterilization for 30 minutes at 121°C (SS) and 6) a multi-purpose surface disinfectant wipe (DW) for 3 minutes. One sample, from each of the 6 groups, was not subjected to disinfection while the remaining 5 samples underwent disinfection treatment. Immediately after disinfection, the surface of each CSG, including the positive control, was sampled and 0.1mL aliquots were plated individually on brain heart infusion (BHI) agar medium. The colony forming units (CFU/mL) from each plate were counted and the data was used for comparative quantitative statistical analysis. In the second part of the study, 10 3DGs were fabricated and sampled within

6 hours of fabrication without disinfectant. The CFU/mL values from untreated 3DGs were compared with CFU/mL from untreated CSGs to identify any differences in microbial load between these two modes of fabrication. In the statistical analysis, percent reduction values were determined using the median CFU/mL values from the disinfected and control samples in the first part of the study. The percent bacterial reduction for each disinfectant group was used to compare the 6 disinfectant groups. A Kruskal-Wallis test was applied to test for any between-group differences, followed by Dunn tests for pairwise-group comparisons, where the p-values were adjusted for multiple testing. Additionally, the CFU/mL values from the untreated CSG and 3DG were compared using a Wilcoxon rank-sum test ($p < 0.05$).

RESULTS: When comparing various groups, the highest reduction of CFU was seen in groups treated with (IUSS) and (SS). Out of all the chemical disinfectants, group 1 (DS) and group 6 (DW) reduced the greatest number of viable cells ($p < 0.0003$). There was no difference in microbial load when comparing these 2 groups ($p < 0.578$). Group 3 (IPA) performed significantly better if compared with group 2 (CHX) ($p < 0.024$). There was no difference in CFU reduction when comparing the surgical guides treated by IUSS or SS, as both showed no growth. Finally, the conventionally fabricated acrylic resin surgical guides had significantly more bacterial growth than surgical guides made from 3D printing technology ($p < 0.001$).

CONCLUSION: All disinfectant groups, except for the CHX group, worked effectively by reducing the microbial load in the treated samples as compared to the untreated controls. This was true for CSG and 3DG. Both of the steam sterilization groups (IUSS and SS) performed significantly better than chemical disinfection groups as they denatured all viable cells living on the surface of the experimental samples indicating that they may be the first choice in decontamination of surgical guides.. The popularly used multi-purpose disinfectant spray and

disinfectant wipes both demonstrated excellent bacterial reduction potential and were objectively quicker than the steam sterilization methods, although they failed to completely eliminate all viable organisms. As a result of the fabrication method for 3D printed surgical guides, the number viable cells on the surface of these samples was significantly lesser when compared to conventional surgical guides (CSG)

E. INTRODUCTION

Review of Literature

In the United States the rate of edentulism is expected to decrease ten percent every decade for the next thirty years, however due to an increase of an aging population the number of people who will need one or two dentures will increase through 2020.¹ This number is expected to grow in the next two decades, and in recent years dental implant therapy has been the standard of care for replacing missing teeth in edentulous populations.² Various treatment planning techniques have been employed by clinicians to aid in surgical dental implant placement. In a completely edentulous implant therapy, a duplicate of the existing prosthesis is commonly used as a surgical guide to assist the clinician during surgical implant placement. Often the patient either presents with an existing acceptable denture, or a new one is made to the desired esthetics and function.³ A surgical guide can then be fabricated, based on ideal prosthetic planning and subsequently provides a more predictable prosthetic outcome when used during time of implant surgery.³

As the surgical guide comes into contact with hard and soft tissue during the sterile surgical procedure, it poses a potential risk of pathogenic transmission. Risk of infection depends on the minimum infective dose of any given organism, as well as the virulence.⁴ In a study evaluating microbial counts of polishing pumice in a dental production laboratory, clinical area, and student teaching laboratory at the University of Leeds School of Dentistry, Witt et al reported abnormally high counts of pathogenic organisms.^{5,6} These bacteria were identified on patient dentures, pumice samples and scrub jackets. The major bacterial species identified from these samples included *staphylococcus*, *pseudomonas*, *enterobacter*, and *candida* species. Kirchner et al. conducted fifty six tests of ultrasonic units, pressure pots, polishing pans, and other working areas and identified similar species.⁷ All of these reported sites are in direct contact with surgical

guides during their fabrication and thus pose a risk for cross-contamination. A Witt et al suggested contamination appears to originate from four possible sources; tap water, the atmosphere, the appliance and the clinician.⁶ Bacterial infection is not a common reason of early implant failure or surgical complications if stringent aseptic surgical protocols are followed.⁸ However, knowing that cross-contamination inevitably occurs in many dental laboratories, it is critical for the clinician to be aware of and follow proper protocols for maintaining an aseptic and/or sterile surgical field to ensure minimal post-operative complications.

Similar studies which looked at decontamination and/or disinfection protocols existing in literature, however had some limitations.⁷ Many of the surgical guides evaluated in literature to date have been reported to be heat intolerant.⁷ Thus, a majority of the existing literature has evaluated and cited liquid disinfectants as the main disinfection mode used among clinicians today.^{6,10,11,12} These studies compared the ability of chemical disinfectants to reduce the microbial count on both heat and cold cured acrylic resin.

Kirchner et al. conducted a questionnaire of 100 clinicians (maxillofacial surgeons and general dentists) to determine what kind of sterilization, disinfection procedures were used in their practice.⁷ They found that out of 100% of responses 30% clinicians used Chlorhexidine-gluconate 0.12% (Paroex ®), 23% used ethanol 80%, (Alkopharm ®), and 7% used Pctemodome-dihydrochloride 0.1% (Octenisept ®).⁶ In the study by Smith et al. they included 70% ethanol, 5.25% Sodium Hypochlorite, and 0.12% chlorhexidine gluconate in their testing. Smith et al. concluded that immersion in 70% ethanol for 15 minutes as the most effective antimicrobial agent against both aerobic and anaerobic species, eliminating 100% of viable microorganisms.¹² In their analysis both 0.12% chlorhexidine and 0.5% sodium hypochlorite achieved no CFU on culture after disinfection.¹² These results agree with investigations by

Sennhenn-Kirchner et al. who also concluded that soaking in ethanol for 15 minutes, albeit 80% ethanol, reduced the microbial load the most of all the disinfectants tested.^{7,12} This group concluded that chlorhexidine was not as effective against *Candida* and proposed that this is due to the ability of this organism to form a biofilm thus reducing its disinfection potential.⁷

In another study, da Silva et al. evaluated 1% sodium hypochlorite, 2% chlorhexidine digluconate, 2% glutaraldehyde, 100% vinegar, sodium perborate-based denture cleanser tabs, and 3.8% sodium perborate.¹³ They found that the most effective disinfectants overall were 1% Sodium Hypochlorite, followed by 2% chlorhexidine digluconate and 2% glutaraldehyde. If looking at the effect of disinfectant on specific organisms, da Silva et al. concluded that glutaraldehyde worked best against *S. aureus*, and *C. albicans*, while Sodium hypochlorite had the greatest effect on *S. mutans*, *E. coli* and *B. subtilis*.¹¹ Kirchner et al. found alcohol most effective on *S. aureus*, *E. faecalis*, *E. Faecium*, *E. coli*, *E. cloacae*, *P. aeruginosa*, *A. baumannii* and *C. albicans* at an application of 1 minute, with a more significant effect if applied for at least 5 minutes.⁷

In the studies reviewed there were some limitations identified. Kirchner et al. utilized small samples of auto-polymerizing acrylic resin measuring 4.0cm x 0.8cm x 0.8cm in their analyses.⁷ Smith et al. investigated the microbial load on commercially fabricated and in-house laboratory fabricated acrylic surgical guides and found that both groups had microbial growth with in-house guides yielding significantly higher CFU.¹² They utilized a small number of lab fabricated and commercially fabricated surgical guides which were cut into small pieces to create multiple samples.¹² Ganesh et al. utilized 82 acrylic resin specimens of acrylic resin with a 1 degree flare designed to make the fabrication process easier, and to simultaneously evaluate changes in flexural properties.¹⁰ Da Silva et al. evaluated disinfection potential on 350 acrylic resin

specimens measuring 3 x 0.7 x 0.2cm.^{3,13} Interestingly, none of these studies used a full-size surgical guides as their representative sample, yet many regulatory agencies such as European Committee for Standardization and the Food and Drug administration require that samples mimic the conditions where the disinfectants were used. European standards require disinfectants to undergo three parts of evaluation.¹⁴ The first part takes into account the ability for the disinfectant to eliminate organisms in general, but does not factor in the environmental conditions, conditions intended for use or ability to eliminate bacterial spores. The second and third parts are more stringent because they employ quantitative suspension tests that measure bactericidal, mycobactericidal, fungicidal, viricidal activity and the ability to eliminate spores. In part 2 step 1 the disinfectants are evaluated for antimicrobial efficacy against organisms in challenging conditions such as moist soil sites, whereas part 2 step 2 assess sites intended for use such as hard surfaces seen in hospitals and clinics. Part 3 evaluation occurs in the form of a field test, under routine conditions.¹⁵

A surgical guide is known to be porous and has anatomy related irregularities and crevices which are difficult to polish, leaving behind a roughened acrylic surface which may have a marked effect on microbial adherence and biofilm formation that is well reported in literature.^{16,17} Morgan and Wilson describe roughened surface, larger surface area as well as provision of protected sites for colonization all factors influencing adhesion of *Streptococcus oralis*.¹⁶ Additionally, among these three studies, only one of the disinfectants (Glutaraldehyde) is classified as an appropriate chemical sterilant or high-level disinfectant accepted by the CDC for treatment of critical items.¹⁸

The current Centers for Disease Control and Prevention (CDC) guidelines for disinfection and sterilization categorizes dental instruments based on their potential risk for infection first

described by Spaulding in 1968.¹⁸ Critical items are used to penetrate soft tissues or bone, have the greatest risk of transmitting infection, and should be sterilized by heat.¹⁸ Semi-critical items touch mucous membranes or non-intact skin, have lower risk than critical, and if heat tolerant should be sterilized by heat.¹⁸ If intolerant to heat, should at least be treated with a high-level disinfectant. Food and drug administration (FDA)-cleared sterilant/ high-level disinfectants and Environmental Protection Agency (EPA) registered disinfectants must have clear label claims for intended use according to manufactures instructions.¹⁹ In common surgical implant placement protocols, a full thickness mucoperiosteal flap is raised to facilitate in bone contouring and at this time the surgical guides often come into direct contact with bone and blood. By definition, the CDC classifies instruments with bone contact as Critical.¹⁸ These types of instruments should be heat-sterilized, yet surveys of Implantologists have demonstrated that many clinicians are utilizing liquid immersion with intermediate-level disinfectants.^{7,10,12,13}

Multiple studies cite the inability of auto-polymerized acrylic to tolerate heat as the limiting factor when autoclaving these critical classified surgical guides.^{7,12,20} Presently there is no data supporting the notion that heat sterilization will adversely affect the surgical guide's clinical stability, utility, and treatment outcomes. In fact, one group found that the fracture toughness of auto-polymerizing resins significantly increased following conventional autoclave post polymerization at 130°C for 10 minutes ($P < .05$).²¹

As technology advances in dental treatment planning, both analog and digital approaches to dentoalveolar reconstruction are increasingly utilizing surgical guides during dental implant surgery and the proper aseptic protocols are vital to minimize post-surgical complications while following regulatory guidelines.

Rationale

At this time, no literature exists on the bacterial reduction potential of disinfectant agents commonly available in the routine dental practice on conventional acrylic and 3D Printed surgical guides. Fortunately, the rate of surgical site infections in dental implant surgery is very low, in spite of the many reports indicating opportunistic pathogens present in dental laboratories where surgical guides are being made.^{22,23} To date, there is no standardized protocol for disinfection or sterilization of laboratory fabricated surgical guides used in dental implant surgery. Current in-office practices for sterilization of surgical guides before the use in surgery include chemical disinfectants and require long soaking times to be efficacious. Conversely, heat sterilization by way of steam autoclave is commonly available, convenient and very efficacious but has been reported to induce deformation in the heat sensitive surgical guides.^{7,36} Ethylene oxide (EO) sterilization modalities are rarely used by the average dental office due to the exuberant cost, unavailability and long processing time to allow for the degassing of hazardous vapors.²⁴ Identifying a convenient, clinically acceptable means of surgical guide decontamination will improve the quality of treatment for patients and clinicians.

A report from the Center for Disease Control (CDC) described multiple factors which are important for bacterial transmission including the availability of vulnerable patients, patients undergoing recent surgery, indwelling medical devices.^{25,26} The distribution of pathogens isolated from surgical site infections have not changed in recent decades.²⁷ *Staphylococcus aureus*, coagulase negative *staphylococci*, *Enterococcus spp.*, and *Escherichia coli* are the most frequently isolated organisms. A greater focus is placed on pathogens isolated from SSI which have antimicrobial potential such as methicillin-resistant *S. aureus*, (MRSA) and *Candida albicans*.^{25,27} Many of the organisms identified in surgical site infections (SSI) are ubiquitous

within the oral cavity. *Staphylococcus aureus* and *Candida albicans* have been reported at high levels in patients with denture stomatitis.²⁸ Coagulase-negative *staphylococci* (CoNS) are part of the normal flora of human skin. *Acinetobacter*, a non-oral bacteria has been associated with infectious processes of the eye, septicemia, meningitis and pneumonia.²⁹ Bacteria often associated with osteomyelitis include *Staphylococcus aureus*, *Pseudomonas aeruginosa*.³⁰ These organisms have relatively low virulence alone, though given the proper conditions such as deep surgical incisions into subcutaneous tissue, fascia and muscle there is an increase in SSI potential.³¹ Thus, these microbes are recognized as agents of clinically significant infection of the bloodstream and other sites.³¹

Infections of biomaterials placed in the maxillofacial region are rarely associated with a conspicuous biofilm.⁹ Some of the most encountered and well characterized bacteria in biomaterial centered infections are slime-producing bacterial species such as *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.⁹ Microorganisms adhere to a surface, forming a biofilm or a slime where they are protected from the host and antibiotics.⁹ In vitro findings of Franson et al. showed that persistent survival of coagulase-negative *staphylococci* adherent to intravascular catheters in absence of conventional nutrients may support the hypothesis that these organisms are protected by their glycocalyxes until they start to proliferate.³² When selecting a protocol in the present study, it must be focus on organisms which have the realistic potential to exist in a dental laboratory and be cross contaminated onto a surgical guide fabricated for surgery. Additionally, the organisms in focus must have the ability to grow on an auto-polymerized acrylic resin surgical guide and be viable for to the investigators to identify in the sample culture.

Existing studies have all tested surrogate markers which were small, flat, smooth and did not

mimic the topography and microenvironment of a surgical guide. When describing protocols used in previous investigations, one study described using surrogate specimens at a specified dimension due to the ease of fabrication.¹⁰ It has been shown that the surface roughness of acrylic resin affects the early stages of biofilm formation by *S. oralis*.¹⁶ A flat surrogate acrylic specimen can be easily polished, altering the potential for biofilm adherence. A full sized surgical guide has areas which are difficult to polish ideally and happen to correspond with the sites that would be in contact with bone in-vivo. These roughened unpolished areas may serve small bacterial enclaves and can only be studied if the experimental samples mimic the true specimen surface topography. In the present study, we will utilize samples of the actual surgical guides which would be used in surgery, so the in-vitro surface topography mimics exactly that which would be encountered in-vivo.

Existing studies evaluated the bactericidal potential of chemical agent on surgical guides, however the agents used in their studies are not recommended by the CDC for use on items deemed critical items. These include liquid sterilization, or high-level disinfection agents so the clinical relevance is questionable.⁷ In the present study, both chemical and heat sterilization modalities were employed. Considerations for selection of sterilization modalities include, availability to the consumer, cost, toxicity to the patient and clinician, and ease of use. Considering steam sterilization is a commonly available modality in the routine dental office, it was included in the present study for comparison with chemical disinfectants in its potential to reduce microbial load.

There are many differences between conventionally fabricated acrylic resin surgical guides and surgical guides fabricated using 3D printing technology. The first consideration is the material composition. Most surgical guides fabricated using 3D Printing technology or “additive

manufacturing” are composed of Bisphenol A ethoxylate dimethacrylate and <1% Phenyl-bis (2,4,6,- trimethyl benzoyl) phosphenoxide. The polymerization reaction of this material is activated in the presence of light, in this case within the 3D printer. Conversely, guides fabricated by conventional techniques are typically composed of Poly(methyl methacrylate) and dimethyl-p-toluene.^{34,35} When comparing the two materials as scaffolds for bacterial growth, one thing to consider is the inherent material properties which can alter the bacterial adhesion potential. When comparing the two methods of surgical guide fabrication, conventionally processed surgical guides for full arch implant surgeries in edentulous patients typically utilize a denture duplication method. The denture duplication method requires the direct contact of a patient’s existing prosthesis using the impression materials in the dental laboratory. This step alone can be the portal-of-entry for cross-contamination in the dental laboratory. The conventional method often involves the placement of autopolymerized acrylic resin samples into a pressure chamber filled with water. The purpose of this step is to eliminate any irregularities in the acrylic resin, such as bubbles, which may affect the final surface texture of our samples. This environment has been reported to harbor significant quantities of bacteria.⁷ The post processing for conventional guides requires the use of pumice, lathe wheels, and other instruments. These tools are commonly used in the dental laboratory, frequently left unchanged, posing a significant risk for cross-contamination.^{7,36} Conversely, 3D printed guides are fabricated in high detail and require minimal post processing. The steps include manual removal of the supporting structures, alcohol rinse to remove residual resin, and UV light curing to ensure the resin has reached its final setting stage, thus less contact with potential sources of cross-contamination.

F. OBJECTIVES

This study investigated the surface microbial load on surgical guides fabricated in a dental laboratory under routine conditions. The investigator took every effort to maintain the same environmental conditions as those for patient care. The investigator used microbial sampling and quantification techniques to understand the quantity of viable organisms present on the surface of surgical guides. The surface microbial load was quantified and recorded using CFU/ml values in the presence and absence of disinfectants on surgical guides fabricated using conventional methods and 3D printing methods. The disinfectants selected for comparison in this study were based on commonly available agents to the average dentist. The study objectives were as follows:

1. To know if there was any difference in the microbial load after disinfection between various disinfection methods; 1) a multi-purpose surface disinfectant spray (DS); 2) a 0.12% chlorhexidine gluconate (CHX) immersion for 15 minutes; 3) a 70% isopropyl alcohol (IPA) wipe for 3 minutes; 4) immediate use steam sterilization (“flash cycle”) for 4 minutes at 135°C (IUSS); 5) steam sterilization for 30 minutes at 121°C (SS) and 6) a multi-purpose surface disinfectant wipe (DW) for 3 minutes.
2. To know if there is a difference in microbial load on surgical guide samples when comparing various methods of disinfection such as a spray, wipe, and immersion.
3. To know the difference in microbial load on surgical guide samples when comparing various methods of guide fabrication; 1) 3D printing , 2) Conventional fabrication.
4. To know if there was a difference in microbial load as covariates such as the site of sampling, objective porosity and duration of disinfection immersion changed.

HYPOTHESES

The following null hypotheses were tested:

1. There was no difference in microbial load after disinfection between various disinfection methods 1) a multi-purpose surface disinfectant spray (DS); 2) a 0.12% chlorhexidine gluconate (CHX) immersion for 15 minutes; 3) a 70% isopropyl alcohol (IPA) wipe for 3 minutes; 4) immediate use steam sterilization (“flash cycle”) for 4 minutes at 135°C (IUSS); 5) steam sterilization for 30 minutes at 121°C (SS) and 6) a multi-purpose surface disinfectant wipe (DW) for 3 minutes.
2. There was no difference in microbial load on surgical guide samples when comparing various methods of disinfection such as a spray, wipe, and immersion.
3. There was no difference in microbial load on surgical guide samples when comparing various methods of guide fabrication; 1) 3D printing , 2) Conventional fabrication.
4. There was no difference in microbial load as covariates such as the site of sampling, objective porosity and duration of disinfection immersion changed.

G. MATERIALS AND METHODS

Experimental Design

A total number of 61 surgical guides were fabricated for the purpose of this study (Table 1). The experimental analysis included a total of 43 surgical guides with 36 conventionally fabricated surgical guides (CSG), and 7 using 3D printed surgical guides (3DG). A pilot study was performed before the experiment to determine experimental design and verify testing methods (Appendix L). The investigator used microbial sampling and quantification techniques to understand the quantity of viable organisms present on the surface of surgical guides. The surface microbial load was quantified and recorded using CFU/ml values in the presence and absence of disinfectants on surgical guides fabricated using conventional methods and 3D printing methods. The first part of the study was designed to compare the bacterial reduction potential between various disinfectant types on conventionally fabricated surgical guides (CSG). The second part of the study was designed to compare the surface microbial load between CSG and 3DG immediately after lab fabrication prior to disinfection. A standardized protocol was created to sample the surface of each surgical guide sample for bacterial contamination transferred from the dental laboratory during fabrication. The surface samples were transferred to a non-selective growth medium, and the total number of colony forming units (CFU/mL) were counted and compiled in a data-set for future statistical analysis. Each surgical (prosthetic) guide was fabricated from a maxillary complete denture previously prepared for this study. This in-vitro investigation was conducted at the University of Connecticut School of Dental Medicine in the Division of Post-Graduate Prosthodontics dental laboratory and in the Department of Molecular and Cell Biology laboratory of Dr. Spencer Nyholm at University of Connecticut, Storrs Campus.

Surgical Guide Sample Fabrication and Methodology

All surgical guide samples were fabricated individually by one investigator in the dental laboratory. Irreversible hydrocolloid was mixed to a fluid consistency using the amount of water recommended by the manufacturer and placed into the flask until half full. The denture was seated into the flask, covered with the remaining irreversible hydrocolloid and allowed to set. After setting, the flask was separated, opened and the denture removed. Next, auto-polymerized acrylic ortho resin (PERM® Coltene Whaledent, multi-purpose cold cured acrylic, color fast - cross-linked) was mixed according to manufacturer specifications and poured into the void. The flasks were closed tightly and placed into a pressure-pot curing unit in 37.8°C water at 25 psi for at least 10 minutes. After removing the flask from the pressure pot, the halves were separated and the cured resin surgical guide removed. The newly fabricated conventional surgical guide was finished with acrylic burs to remove gross irregularities and polished with felt pads in a pumice polishing station. The guides were rinsed and dried using a laboratory pressure steamer. After completion all surgical guides were inspected, packaged into sealed sterile packaging, and sampled within six hours from fabrication.

The Asiga MAX 3D Printer (Whip Mix, Louisville, KY) was used to fabricate 15 3D printed surgical guides (3DG). This printer utilized digital light processing (DLP) to photopolymerize ultraviolet-photopolymerizing resin in layers. The surgical guides in the present study were manufactured at 50µm layers with clear Veriguide resin (Whip Mix, Louisville, KY), a methacrylate-based resin photopolymerized at approximately 385nm. The maxillary complete denture fabricated for this study was optically scanned using a Freedom HD desktop optical scanner (DOF Labs, Seoul, Korea). The scan data was digitized into STL (standard tessellation

language) file using the CAD technology in Asiga Composer software. Per the manufacture instructions, any bubbles were removed from the resin with a spatula prior to print initiation. Next, the .stl files were sent to the 3D printer to be printed. All post-processing steps were followed per manufacturer's protocol (Whip Mix, Louisville, KY) including removing printing supports, placing the printed guides into an ultrasonic bath with isopropanol >97% for 5 minutes and then into a UV light curing unit set to 300-700nm at 200 Watt output for 10 minutes The UV light polymerized any residual uncured resin. Once removed from the UV post-processing all surgical guides were inspected for any gross deformities, and if deemed satisfactory individually packaged into sealed sterile packaging prior to surface bacterial sampling within 6 hours. All of the samples were fabricated on a per-experiment basis (6 at one time) in order to standardize the amount of time between completion of fabrication and surface microbial load sampling. The fabrication and sampling protocols were verified in the pilot study for feasibility and to establish a comprehensive list of materials. (Appendix L)

Disinfection Protocol and Control Definitions

In the first part of the experiment, 36 CSG were arranged into 6 different groups with 6 samples in each group (Table 1). The positive control for each group was randomly selected from the group of 6 and remained untreated before sampling. The 6 groups in the first part of the study were treated separately and conducted as 6 individual experiments. The time interval between the preparation of samples, media preparation, sampling, and analysis increased the overall time required per experiment resulting in a limited the number of samples per group (n=6). On average, each experiment required a total of three sessions; In the first session, the investigator prepared Brain Heart Infusion agar media (BHI) on petri plates according to manufacturer protocol at least 48 hours prior to sampling to ensure sufficient drying time and

rule out contamination prior to inoculation. Next, the 6 CSG were fabricated , documented for any surface irregularities, and placed into individually labeled sterile pouches for transport to be sampled. Each group was assigned a specific disinfectant method (Table 3); 1) a multi-purpose surface disinfectant spray (DS); 2) chlorhexidine gluconate (CHX) 0.12% immersion for 15 minutes; 3) an Isopropyl alcohol 70% (IPA) wipe for 3 minutes; 4) immediate use steam sterilization (“flash cycle”) for 4 minutes at 135°C (IUSS); 5) steam sterilization 30 min at 121°C (SL) and 6) a multi-purpose surface disinfectant wipe (DW) for 3 minutes. After disinfection the 5 samples were individually rinsed with 0.22 µm filtered deionized (DI) water for 2-3 seconds. The untreated CSG was sampled first, while the remaining 5 CSG were being treated with a disinfectant.

Specimen Surface Sampling and Quantification

The surface microbial load from each sample was determined by counting the CFU from surface samples plated on non-selective general purpose nutrient medium. The following protocol was strictly adhered to; The surgical guides were removed from the lab pouch, placed in a sterile 500mL beaker, and 10mL of 0.22 µm filtered sterile 0.9% Sodium Chloride solution (NaCl) was syringed directly onto the guide in the beaker. The investigator held the surgical guide in the beaker with sterile gloves and used an EO sterilized toothbrush to scrub the surface for a total of 2 minutes, 1 minute on each side. The gloves were changed between samples, however the same amount of pressure applied during scrubbing as well as the scrubbing time remained the same. After the 2 minutes of brushing, any viable surface cells were rinsed off of the guide into the beaker using 20 mL of filtered NaCl, and the guide was disposed. The NaCl was transferred from the beaker into a sterile 50mL polypropylene centrifuge tube, an additional 20mL of NaCl was used to wash the contents of the beaker into the tube. The remaining 6

bacterial pellets were resuspended into 1mL of fresh NaCl and transferred to a sterile 1.5mL microcentrifuge tube labeled 1:1. The 1:1 stock sample was used to prepare 1:4, and 1:10 dilutions. All samples were homogenized using a vortex mixer for 15 seconds and 0.1mL from each dilution was deposited on the surface of the BHI agar plate and spread evenly using the spread plate method. Each sample was plated in triplicate for each dilution (1:1, 1:4, 1:10) totaling in 9 plates per sample. The plates were labeled and kept undisturbed in an incubator for 48 hours at 37°C under aerobic conditions.

Negative Control Group

The purpose of this group was to verify the accuracy of tests and to determine the sterilization of NaCl and brushes used in sampling. Samples from the NaCl stock as well as the EO sterilized brush were plated in triplicate using the spread plate method.

Positive Control Group

The positive control was 1 surgical guide randomly selected from the 6 CSG that did not undergo any disinfection procedure. This surgical guide was sampled and plated in triplicate using the same techniques as the disinfected samples. This guide was anticipated to have the most CFU out of all the samples plated in the experimental group.

After 48 hours, the plates were removed from the incubator. The plates observed to have CFU within the target range of visual counting <300 CFU were subject to visual enumeration. . If a plate contained over 300 CFU, it was eliminated from the study and deemed non-usable data. The CFU on each plate was counted twice for verification. Each plate was photographed for documentation and future analysis of colony morphology. The CFU tallies were recorded into a data-set and the percent bacterial reduction was calculated per group using the median CFU/mL

from the positive control and individual samples. The percent bacterial reduction values were used to determine differences between disinfectant groups.

Comparison of Surgical Guide Type on Microbial Load

In the second part of the study, 7 untreated 3DG samples were fabricated and sampled identically. The CFU from the untreated 3DG samples were compared with CFU from untreated CSG obtained previously as the positive controls from each group. The purpose of this comparison was to determine if there were any differences between 3DG and CSG on microbial load. (Table 4) After completing the part 1 study, the CSG positive control samples consistently showed CFU formed across all samples and all replicates. (Table 6) However, after the initial sampling of 3D printed surgical guides (n=7) (3DG), little to no growth <10 total CFU was seen on across 63 plates and no further plating was done.

Sample Analysis

When multiple positive controls were available, the median was used to calculate percent of reduction for non-positive controls. Positive controls were removed from the disinfectant comparison for bacteria growth by the percent of reduction. The percent of reduction was descriptively summarized for each surgical guide sampled within disinfectant groups. (Table 3) Statistical analyses were applied to identify between-group differences, as well as differences between conventionally fabricated guides and 3D printed guides. When evaluating the data-set a non-parametric test, Kruskal-Wallis test, was applied to identify between-group differences, followed by Dunn tests for specific pairwise-group comparisons, where the p-values were adjusted for multiple testing. Considering the number of groups being compared was low, a Dunn's test was most appropriate post-hoc test to determine which of the sampling groups were significantly different. A p-value smaller than 5% was considered statistically significant. All the

statistical analyses were performed in R version 3.6.1. An overall percentage of reduction per group as well as a sample percent reduction derived from the median CFU of all replicates for that sample (Table 5). The standard deviation and coefficient of variation were calculated per sample. The primary objective of these analyses was to identify the disinfectant group which demonstrated the greatest amount of bacterial reduction. The secondary objective was to identify any differences in surface microbial load between CSG and 3DG prior to disinfection.

H. RESULTS

Statistically significant differences ($p < 0.0003$) were found between groups of disinfectant methods used to treat surgical guides. All disinfectant groups showed a percent reduction of CFU except for the disinfectant group with CHX. A list of p-values can be found in Table 5. When comparing various groups, the highest reduction of CFU was seen in groups treated with IUSS and SS. Out of all the chemical disinfectants, group 1 (DS) and group 6 (DW) worked the best ($p = 0.0003$). When evaluating untreated samples, the conventional surgical guides had significantly more bacterial growth than 3D printed guides ($p < 0.001$). Since the experiments in part 1 of the study were not performed at the same time, positive controls were obtained anew at each experiment. Thus, the percent reduction values were calculated for each surgical guide and each disinfectant group individually using specific positive controls from those groups. The percent reduction values for each group and sample were calculated using median CFU values from all the experimental replicates per surgical guide sample (Table 4) All list of percent reduction values are described in table 4. The group treated with disinfectant spray, Group 1 (DS) reduced a significant number of CFU compared to the positive control, demonstrating a 99.5% reduction with a standard deviation of 0.577. In contrast, Group 6 (DW) disinfectant wipe of the same chemical, showed a 100% reduction and no standard deviation. The difference between these two groups was not statistically significant. Group 1 (DS) was only statistically better when compared to Group 2 (CHX) which showed growth on treated plates ($p < 0.017$). The surgical guides treated with 70% IPA wipe showed a percent reduction of 47.16% with a standard deviation of 76.73. IPA was statistically better when compared to CHX ($p < 0.024$), however when comparing IPA with any other group it was not statistically significant. This result is likely due to degree of data variation and the limited sample size in the group. (Table 4)

The inclusion of 9 replicate plates per surgical guide sample was a way decrease the variation and increase the reliability of the sample, as a result median CFU values were used as opposed to the mean. Other than CHX, which showed more growth than the control across all sampled guides, this disinfectant was the least effective of the chemically based disinfectants tested in this study. (Table 3)

Both IUSS and SS, Groups 4 and 5, demonstrated the best reduction in bacteria showing a 100% reduction as compared to their positive control samples. There was no difference in CFU reduction when comparing the two steam disinfectant Groups 4(IUSS) and Group 5(SS). These results were expected as steam sterilization is routinely the gold standard for disinfection in the dental office. (Table 3)

The group treated with CHX demonstrated a (-1262.4%) reduction with a standard deviation 890.89 indicating that there was more bacterial growth in the treated groups as compared to the control group with no disinfectant. This was an unexpected finding in this study and is described further in the discussion. The bacterial growth in group 2 (CHX), was significantly more than those in other conventionally fabricated guide groups ($p < 0.0003$.) (Table 3)

A total of 18 surgical guide samples were excluded from the statistical analysis. There was completely no growth for the groups sterilized in groups 4 and 5, so one of the groups was entirely eliminated from the statistical analysis (Table 2 n=6)) Six samples were eliminated because there were too many CFU to count. (Group 1 (1), Group 2 (3), Group 3 (1), Group 6(1)).(n=6) To account for the reduced sample size, three additional trials were performed. (Group 2: trial 2 and 3, group 3: trial 2), however one of the trials (Group 2, trial 3) failed and the samples for that trial were not included. (n=6). This resulted in a total number 43 surgical guides subject to statistical analysis. (Table)

Finally, the conventionally fabricated acrylic resin surgical guides (CSG) had significantly more bacterial growth than surgical guides made from 3D printing technology (3DG) ($p < 0.001$).

(Table 6) When comparing the CFU values from the initial samples, it was determined that there was a significant difference between groups and no further plating was done. ($p < 0.0018$) The Wilcoxon rank sum test with continuity correction was used to show differences between the groups. ($W = 59.5$)

I. DISCUSSION

The results from this study rejected the null hypotheses and showed a statistically significant difference between disinfectant groups. This was also true between conventionally fabricated CSG and 3D printed surgical guides. Presently, there is limited published literature investigating the effects of steam, liquid, and chemical disinfection on auto-polymerized acrylic surgical guides as well as 3D printed surgical guides. The limitations observed in previous studies, who included surrogate acrylic blocks or surgical guide fragments as the treated samples, were mitigated in the present study by fabricating full size surgical guides, sampled in-vitro in true environmental conditions within the dental laboratory. Due to the lack of standardized decontamination protocols for these surgical (prosthetic) guides which come into contact with mucus membranes, alveolar bone, and elevated oral tissues, the results from this study will help dentists make an appropriate decision for the safety of their patients.

All of the instruments used in the surgical placement of dental implants, including surgical guides, should be considered critical items as described by Spaulding in 1968, as they come into contact with broken skin or bone during the time of surgery.^{12,37} In recent years, the development of new antimicrobial products has blurred the lines between cleaning, decontaminating, disinfecting, and sterilizing. Traditionally, a disinfectant was considered most effective if it was applied after cleaning. New disinfectant impregnated wipes claim to achieve disinfection and cleaning, however this is dependent on the wipe containing sufficient surfactant and wetness, and that the disinfectant is effective against the microbial population.³⁹ The present study evaluated the ability for several disinfectant groups to reduce the microbial load of surgical guides, as well as evaluating the mode of fabrication in the microbial load levels.

In the between-group comparison the steam sterilization (IUSS and SS) modalities showed the highest reduction in viable CFU, followed by multipurpose disinfectant spray (DS) and wipe (DW). The (IPA) (group 3) and (CHX) (group 2) showed the least amount of reduction potential ($p < 0.0003$). One unexpected finding observed in groups disinfected with CHX immersion for 15 minutes was an increase in the number of CFU as compared to the positive control. This finding is explained further.

In part 1 of this study, 36 CSG were randomly distributed into 6 groups containing 6 surgical guide samples. A total number of samples included in part 1 and part 2 of this study can be found in Table 1. A detailed breakdown of all samples removed from analysis due to error is seen in Table 2. Due to the fact that both of the sterilization groups, group 4 IUSS and group 5 SS, showed completely no growth, one of the groups (SS) was removed from the statistical analysis. (Table 2). Two additional trials were performed for CHX group in order to verify the unexpected high growth CFU values seen in the first CHX trial which were outside the range of visual countability range >300 . Trial 2 for CHX also produced higher levels of CFU in the disinfected samples than the untreated control and verified the results in trial 1. (Table 2). As a result of the unexpected findings the investigator conducted a third CHX trial. In the third trial, all of the CHX treated samples produced an elevated number of CFU as compared to other groups of disinfectants. Despite ample growth on the treated samples, the trial was not used in the statistical analysis because there was an absence of growth on the positive control suggesting sampling error. (Table 2) Similarly, a second trial was also performed for Group 3 (IPA) because some samples were eliminated due to high CFU >300 . The results from (IPA) trial 2 verified the results from trial 1 demonstrating similar percent reduction values. Due to the addition of a second trial to both the CHX and the IPA groups, the number of overall samples in the CHX

group increased by 3 surgical guides, and the total IPA samples increased by 4 surgical guides. (Table 2) However, the total number of samples included in the statistical analysis remained the same because 1 sample was removed from DS and 1 removed from DW group due to error. (Tables 1 and 2). In light of these changes, both of these groups showed the highest percent reduction of median CFU of all the chemical disinfectants tested and a non-parametric test still showed a statistically significant difference between groups ($p < 0.0003$). After completing the part 1 study, the positive control samples from each group were used in the part 2 analysis.

In the second part of the study, microbial load of 7 3D printed and 8 conventionally fabricated surgical guides (CSG) was studied. The data from the 8 CSG was taken from the previous part which included an untreated sample per experimental group. (Table 1) When comparing surgical guides prior to disinfection, out of the 3D printed guides and those fabricated using conventional laboratory methods, the 3D printed groups unanimously and consistently produced lesser colony forming units than conventional. (Table 5) The initial untreated 3D printed surgical guide samples ($n=7$) showed little to no growth < 10 CFU across 63 plates or 7 3DG. (Table 6). As a result of this finding, a Wilcoxon rank sum test with continuity correction determined that there was a significant difference between groups and no further plating was done. ($p < 0.0018$)

Chlorhexidine gluconate has widespread use in dentistry for its antimicrobial potential and thus many authors who conducted similar investigations evaluated CHX as one of the disinfecting agents.^{7,11,12,13,40} The common use and clinical acceptability of this agent can be attributed to the low systemic and local side effects reported from its use.^{41,42} The effectiveness of this agent relies on its ability to induce a cationic change in the microbial cell membrane, leading to greater permeability and osmotic imbalance in the bacterial cell.⁴³

In the present study, the unexpected microbial growth in the group disinfected with 0.12% chlorhexidine (-1262.42 CFU/mL percent reduction), led the investigator to repeat this experimental group on two more occasions, both of which demonstrated the same findings. Six studies indicate that low level chlorhexidine exposure quite often results in an antibiotic resistance, so far mainly described in biocide-sensitive strains from organic foods.

Some authors have reported 4% chlorhexidine as an effective means of reducing microbial growth.^{7,40} Conversely, an inability of 0.12% chlorhexidine to completely remove 100% of microbial populations has also been reported in similar investigations on surgical guides.^{7,12} Furthermore, one of these groups concluded, “disinfection of thermosensitive acrylic devices cannot be undertaken with CHX.”⁷ These observations warrant the investigation of an appropriate concentration of this antimicrobial agent. One published study evaluated the bactericidal activity of chlorhexidine digluconate tested at in-use concentrations (4%, 2%, 0.5%, 0.1%, 0.05% and 0.02%) for five minutes of contact time.⁴⁴ The results from their study indicated that 4% chlorhexidine was effective against susceptible bacteria, but at levels below 4% a decrease in bactericidal activity especially for *S. aureus* and *P. aeruginosa* was observed.⁴⁴ *S. aureus* has been reported to be a concern in postoperative wound biofilm infections. Additionally, a decreased susceptibility to chlorhexidine was identified in *s. epidermis* isolates associated with deep surgical site infections.⁴⁵ These findings along with the findings from the present study suggest although effective at higher concentrations, 0.12% chlorhexidine may not be sufficient for complete removal of potentially infectious pathogens.

The efficacy of alcohol has largely been underrated as a germicidal agent.³⁷ Alcohol towelettes or wipes have been used in hospitals for decades on multi-dose medication vials or vaccine bottles.²⁴ However due to the inability to demonstrate sporicidal effects, they are not

recommended for high level chemical disinfection.²⁴ In the present study the 70% alcohol wipe demonstrated a mean percent reduction of 47.13% CFU/mL. In a study evaluating 80% alcohol as a disinfectant against bacteria on acrylic resin specimen, they found a favorable long-lasting effect after only one minute of application and complete elimination of growth after a five minutes application. One difference between the present study and the reported literature, was that in the present study the alcohol was applied using a wipe and not via immersion. The ability of the disinfectant to completely saturate the surface and remain wet is an important aspect of its disinfecting capacity. Alcohol will evaporate very quickly making exposure time difficult to achieve unless the surgical guides are completely immersed.²⁴

The use of a quaternary ammonium compounds has been widely used as a multipurpose disinfectant on hospital blood pressure cuffs and other non-critical surfaces.²⁴ In the present study, the active ingredient in the disinfectant spray and wipe used for disinfectant groups I and VI was a quaternary ammonium compound. The mechanism of action is through inactivation energy-producing enzymes, denaturation of essential cell proteins, and disruption of the cell membranes in bacteria.⁴⁶ The mean percent reduction for the spray was 99.5% and 100% for the wipe, demonstrating a very effective agent for decontamination. Unlike alcohol, the wipe form of this agent performed well and can be attributed to the surfactant in the chemical which maintains the exposure time unlike the evaporating effects of alcohol. Despite being a very effective means of disinfection, the FDA strongly discourages the use of quaternary ammonium compounds on critical items such as endoscopes because of a lack of proven efficacy against all microorganisms and limits their use to cleaning surfaces. To the author's best knowledge, this is the first study to evaluate the efficacy of these agents on chemically activated conventionally fabricated and 3D printed surgical guides. Furthermore, only one study was found which evaluated heat

sterilization, a gold standard for sterilization in dentistry, in comparison to chemical disinfection of surgical guides and has limited data.^{10,39}

As described by the CDC, critical items such as surgical guides should be sterilized by steam sterilization or chemical sterilization. The availability of liquid chemical sterilants, ethylene oxide, and hydrogen peroxide gas plasma is limited to dental offices which warranted the investigation of the previously mentioned disinfectants. Steam sterilization is the gold standard for disinfection of critical items due to its high antimicrobial ability, and its capability to eliminate spores. The use of steam sterilization on acrylic resin surgical guides has become controversial due to the potential for chemically cured or photopolymerized resin to become unstable under the presence of high heat, rendering them clinically unacceptable.⁴⁸ Recent reports challenge the notion that surgical guides are in-fact heat-intolerable, and suggest no clinically significant changes can be observed.^{48,49,50} In the present study, our findings showed that both immediate use steam sterilization and routine steam sterilization demonstrated the highest mean percent reduction in microbial loads (100 percent reduction), with no growth seen.

When comparing conventionally fabricated surgical guides using chemically activated resin versus 3D printed guides, our study revealed significantly less bacterial growth on the 3D printed guides. One possibility for these findings can be the surface roughness of the samples. The 3D printed surgical showed consistent significantly smoother surfaces compared to the conventional surgical guides. Another reason can be because the post-processing steps of the 3D printed guides requires an alcohol wash > 95% alcohol and processing under UV light to remove the residual resin. Another important aspect which can account for the results is that the 3D printed guides did not come into contact with the pumice polish or the pressure pot, both high areas of contamination in the dental laboratory. (Figure 1)

The presence of microorganisms in the dental laboratories is an understanding in the dental community however their rapid reestablishment after disinfection and long survival times of viable and transmissible microbes is less considered.³⁹ To maintain validity in tests evaluating the surface bacterial population on surgical guides, the sampling methodology must accurately represent the environment in which they are cross-contaminated from.³⁹ The protocols for this experiment were and adapted from sampling and disinfecting protocols recommended by the FDA and those used in previous investigations on this topic.^{7,12,13,51} Some ways this study attempted to improve establishing a disinfection protocol was by using full size surgical guides and routine processing techniques to mimic the real-life environment.

One challenge discovered was insufficient bacterial recovery. There are several reasons which can account for this; insufficient surface bacterial load to begin with, user error in pipetting, or insufficient nutritional elements in the media. However, this aspect was unlikely due to the verification steps performed during the experiments showing ample growth in the positive controls and none on the samples. Also, the negative controls eliminated possibility of contamination. One aspect could be a non-homogenous stock sample leading to variability in the CFU. In the present study the investigator attempted to mitigate this issue by resuspending the stock sample using a vortex mixer >15 seconds at 2500, as described in literature.⁵² Another attempt at mitigating any variability in the CFU per sample was by study design. All fabrication steps were standardized to ensure consistency: three minutes for trimming under a lathe, three minutes for polishing in a dental laboratory pumice station, followed by steam spray to remove residual pumice, and lastly placing the sample in a sterile pouch for immediate sampling. (Figure 1) This is a limitation in the study and should be addressed in future investigations.

In order to ensure that each surgical guide was sampled directly after disinfecting, the samples were fabricated six at a time, disinfected individually and then sampled within six hours of fabrication. All the samples were fabricated, sampled and disinfected by one investigator so it was logistically prohibitive to conduct more than one experiment at a time. Therefore, each experiment evaluated 1 disinfectant group at a time, a total of 6 surgical guides per group. After fabrication, five of the six guides were disinfected while 1 was placed into the sterile pouch for transportation and served as a reflection of the number of bacteria that exist in the environmental sample, or a positive control. It was determined in the pilot study that the levels of microorganisms within the pumice area varied as a factor of time from when the station was last disinfected by the staff. (Figure 1) The quantity and diversity of species within common areas of the dental laboratory has been reported, but there are no definitive publications describing effective protocols for disinfection or the growth rates of these populations.^{8,36}

The Food and Drug Administration (FDA) standardized protocols defined for antimicrobial effectiveness testing and medical device bioburden describe four stages of bioburden estimation from the medical device as follows: 1. collection, 2. enumeration, 3. bioburden characterization and 4) application of the correction factor(s) determined during bioburden recovery studies in order to calculate the bioburden levels from the pre-sterilized control.⁵¹ This sampling methodology was adopted for use in the present study due to the validity and reliability.

Future Directions

In an era where infection control has taken front stage as new highly infectious pathogens, such as the 2019 novel coronavirus epidemic, viricidal agents will become a major area of investigation.⁵⁴ The emergence and rapid spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease or (COVID-19) is an example of how important infection

control protocols are in the healthcare setting.^{54,55} New targeted viricidal agents are rapidly emerging and the agents being used should be evaluated for their potential to reduce cross-contamination to and from the dental laboratory.⁵⁶ Selecting and implementing appropriate disinfectant protocols are paramount to prevent human to human transmission of such highly infectious pathogens.²⁴ The CDC recommends using products with EPA-approved claims against COVID-19, however if such a disinfectant with the emerging viral pathogen is not available then products with the label claim against human coronavirus should be used.⁵⁸ The disinfectant spray and wipe used in the present study, along with other chemicals listed in EPA List-N are labeled to contain a chemical with EPA-registered label claim against Human Coronavirus. (Metrex, Orange, CA).⁵⁹ As of May 13, 2020 the EPA has recognized a test protocol to evaluate the disinfection efficacy against this specific novel coronavirus strain, SARS-COV-2.⁶⁰ The company, Metrex, has performed third-party testing of this agent on SARS-CoV showing 3-log/99.9% reduction of the virus however the study result has not yet been reviewed or approved by the US EPA.⁶⁰

Several new chemical agents have been added to the CDC guidelines for disinfection and sterilization including hydrogen peroxide, peracetic acid and peracetic acid and hydrogen peroxide in combination.²⁴ These disinfectants should also be considered for future investigations on this topic. One consideration in the cross-contamination includes the reported contamination of the disinfecting agents themselves. Quaternary ammonium compounds have been linked to healthcare associated infections due to bacteria present in the disinfectant which were then used on cardiac catheters and other critical devices.⁵⁷ In the present study, only sealed previously unopened bottles of disinfectants were used, however future studies should consider sampling the disinfectants themselves on media to eliminate the possibility for growth.

J. STUDY LIMITATIONS

One obvious limitation in the present study is the low number of samples per group in this pilot study. Another limitation is the variability seen in the experimental replicates. This limitation is due to the following reasons; 1) The logistical limits such as time and resources to conduct more experiments by a single investigator. 2) The number of samples per group were further reduced because some samples were eliminated from analysis. Each sample took approximately 6 hours to prepare and sample. In addition, the materials preparation took approximately 4 hours to prepare for each sample on a separate day. Therefore, the total effort spent per sample was approximately 6 hours spanning for a total of 414 hours for all 61 samples in the main study, and 8 samples in the pilot study . Therefore, a power analysis to calculate the study sample was not performed. The samples obtained demonstrated within group similarity, and between group variability in CFU/mL counts, which was expected. However, in future studies the number of samples should be increased to increase the reliability of the data.

As the number of microorganisms in the pumice and pressure pot changes over time, this can account for the variability in CFU values observed. This was controlled by using a positive control for each experimental group, as well as using a dilution series for CFU to be in the visible countability range (75-300). However, despite these efforts a high level of CFU observed on some samples out of the target range and subsequently removed from analysis. To overcome the low number of samples, the investigator conducted additional trials which ultimately increased the total number of samples per group. The uneven group sizes were a limitation however were accounted for by using non-parametric statistical analyses and median values. The total number of samples per group can be seen in table 1. Despite the low number of

samples, the results from the statistical analyses demonstrated a statistical significance (p -value < 0.05).

The community of cultured bacteria may not necessarily reflect what is actually viable on the surface of our surgical guide samples, both in volume and diversity. A microorganism requires specific nutritional and environmental conditions in order to maintain viability and ability to culture. Some examples of this includes anaerobic pathogens which would not be identified in the aerobic bacterial cultures used in our study. Although it is possible that the total CFU/mL values obtained may not show all the organisms on the sample, the percent reduction observed in the visible organisms can represent the population as a whole.

To the author's best understanding, there is no comparison of actual full size surgical guide samples for their content of surface microbial load. The existing studies evaluate surrogate markers or samples as described in the introduction. Furthermore, to the author's best knowledge there are very few studies looking at only disinfectants commonly available to the average dentist which test the bacteria from the actual environment, existing studies use bacteria inoculated from a cultured sample. Future studies should incorporate true samples as well as test true environmental conditions to increase the clinical applicability of the laboratory study.

K. CONCLUSIONS

Surgical guides used in dental implant surgery come into contact with blood and bone and careful consideration should be used when selecting the appropriate protocol to reduce the microbial load of these lab fabricated samples. Based on the limitations of the study, the following conclusions were drawn:

1. All disinfectant groups, except for CHX group worked effectively by reducing the CFU/mL values as compared to the untreated controls. This indicates that chlorhexidine at the 0.12% concentration may not be the best suitable to remove microbial communities from surgical (prosthetic) guides prior to surgical procedures.
2. When comparing the modes of disinfection, steam sterilization was the optimal choice for eliminating microorganisms from surgical guides prior to use in a sterile field. However, if limited to using chemical disinfectants, a quaternary ammonium compound based disinfectant spray or wipe work equally well, but not as well as steam sterilization.
3. Due to the nature of their fabrication, 3D printed surgical guides had significantly less microbial contamination upon initial presentation prior to disinfection, as compared to conventional surgical guides.
4. Surgical guides with rough or porous surface such as those seen in conventionally fabricated guides presented with a higher microbial load. All other covariates studied such as sampling sites were insignificant.

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M. APPENDIX A

Pilot study

A pilot feasibility study was performed to identify the most appropriate sampling methods for the greatest recovery of viable surface bacteria. The main purpose of the pilot sampling was to identify a protocol designed to successfully sample the surface of surgical guides for viable bacteria. The second purpose of the protocol was to develop a method that would successfully to culture the organisms obtained in the surface sample within a range for visual countability (75-300 CFU/mL) A total of 8 surgical guides, of which 4 were fabricated using conventional methods and 4 were fabricated using 3D printing technology were prepared for surface microbial sampling. Two surgical guides (1 3D printed surgical guide and 1 conventional surgical guide) were included in each of the three sampling groups (A,B, and C). The remaining 2 guides were used to conduct a follow-up verification experiment of the best method and improving any identified errors. The groups are described as follows: A. A sterile cotton applicator was used to swab the surface of the guide and immediately placing into a sterile 1.5mL microcentrifuge tube containing 1.5 mL of 0.02µm filtered 0.9% sterile sodium chloride solution (0.9% saline). B. The surgical guide was sectioned into 3-4 segments which were then placed into a 50mL polypropylene tube with 40ml of 0.9% sterile saline. The tube was mixed vigorously using a vortex mixer at 2500rpm for <15s to dislodge viable cells into the saline solution. The saline solution was then dispensed onto a non-selective agar plate and allowed to incubate for 24 hours. C. The entire surgical guide was placed into a large 500mL sterile beaker and washed with 20mL of sterile saline. Then, a combination of surface agitation with a sterile brush along with a wash of sodium chloride was used to dislodge any viable cells. The remaining liquid samples from each group were transferred into a sterile microcentrifuge tube for further testing within 15

minutes. All liquid samples tubes were placed into a centrifuge at 5000 RPM for 12min, the supernatant was discarded, and the bacterial pellet was resuspended into a total 1 mL volume of 0.9% sterile saline.

The second half of the pilot test evaluated culturing methods and evaluated the following methods. The 1mL liquid sample containing viable cells in saline was resuspended and a 0.1mL aliquot was directly dispensed on non-selective media. The following media were tested: A. Tryptic Soy Agar (TSA) B. Brain heart infusion (BHI) Agar) using sterile microbiological methods. One sample from each method of surface sampling was for 24hr while the second 48hr at the incubation temperature of (37°C). The highest recovered CFU/mL were obtained from sampling method C, using BHI agar, for 48 hours at the temperature selected. This method was utilized in subsequent experimental analyses. A verification pilot assay was performed to standardized scrubbing times, dilution factors producing CFU/mL between 30-300 CFU, and sampling timepoint <6hr after fabrication to confirm this method. Variables that were understood from the pilot study include: The ideal plating dilutions, media requirements, sampling methods, and experimental timing.

Materials List

The following materials were used for microbiological and disinfection assay:

- 0.9% Sodium Chloride Solution (9 g/L Sodium Chloride)
- Deionized (DI) Water
- Autoclavable Petri Plates
- Tryptic Soy Agar (TSA)
- Blood Heart Infusion Agar (BHI)

- Cell Culture Incubator set to 37°C
- Immediate and Standard Steam Sterilization
- Magnetic Stirrer
- Sterile Cotton swab
- Benchtop Refrigerated Centrifuge capable of 5000rpm
- Ethylene Oxide Sterilization
- 50mL, 25ml Polypropylene Falcon tubes
- 1.5ml Micro Centrifuge tubes
- 10µL, 200µL, 1000 µL Sterile Aerosol Barrier Pipette tips
- Sterile PES Membrane Syringe 0.22 µm Filters
- 0.12% chlorhexidine gluconate
- 70% Ethanol wipes
- CaviCide™ (Diisobutylphenoxyethoxyethyl dimethylbenzyl ammonium chloride 0.28%, Ethylene Glycol Monobutyl Ether (2- Butoxyethanol) 1-5%, Isopropanol 17.2%, and Water 70-80%) multi-purpose decontaminant and disinfectant spray and wipes.

Materials list for Surgical Guide Preparation

- Jeltrate® Alginate Impression material
- Metal flask for auto-polymerizing acrylic resin.
- Self-Cure Orthodontic Resin, Dentsply Caulk Liquid (430ml)
- Self-Cure Orthodontic Resin, Dentsply Caulk Powder (605 g)
- Pressure Pot
- Acrylic burs

- Pumice and pumice wheel
- Steam pressure cleaner
- Ethylene Oxide
- Whip Mix VeriGuide™ generative resin
- Isopropanol >97%
- Ultrasonic bath
- Asiga MAX 3D Printer
- Asiga Composer Software
- STL File of Surgical Guide
- Asiga Flash Curing unit
- Acrylic burs

N. TABLES

Table 1: Summary of surgical guide samples analyzed in this laboratory investigation which studied the microbial load on surgical guide surfaces cross-contamination from the dental laboratory. The first part of the study investigated the effect of disinfectant type on cross-contaminated microbial load from the dental laboratory and included 28 disinfected samples with 8 untreated controls. In the second part of the study, a total of 7 3D printed and 8 of conventionally fabricated surgical guides were compared using their baseline surface microbial load CFU.

Total number of all surgical guide samples fabricated and sampled	61
Total number of all surgical guide samples included in the analysis	43
Total number of surgical guide samples excluded* from the analysis	18
Total number of all surgical guide samples in disinfectant comparison (part 1)	36
Total number of treated conventional surgical guide samples (part 1)	28
Total number of untreated conventional surgical guide samples (part 1)	8 [^]
Total number of all surgical guide samples in guide type comparison (part 2)	15
Total number of untreated conventional samples in (part 2)	8 [^]
Total number of untreated 3D Printed samples (part 2)	7
3DG =3D printed surgical guide	
CSG = Conventional surgical guide	
[^] = same dataset	
* = Refer to table 2	

Table 2: A total number of surgical guide samples excluded (n=18) from the statistical analysis.

The total number of surgical guide samples tested in this study was 61, resulting in 43 samples included in the statistical analysis.

Total number of surgical guide samples excluded from statistical analysis	18
CFU values outside of the range of countability (75-300)	6
non-usable data*x	6
redundant group data **	6
* = Defined as an error in the control reference value	
** = Group 5 (SS) and group 4 showed no CFU growth, only one of these groups was included in the final statistical analysis (group 4 (IUSS)).	

Table 3: The percent of CFU values for each group was used for comparisons between all disinfectant groups in part 1 of the study. The degree of variation within sample groups required the use of median CFU values for the calculation of percent reduction. The CFU data for group 4 (IUSS) and group 5 (SS) was identical, and was not included in statistical analysis. The total number of sampled (n=36) CSG, was comprised of 8 untreated control CSG used in the calculation for this data, and 28 disinfected samples listed below.

Percent of Reduction (%) (<i>n</i> =28)								
Group	Number	Minimum	25%	50%	75%	Maximum	Mean	Standard Deviation
Group 1 (DS)	4	99	99	99.5	100	100	99.5	0.577
Group 2 (CHX)	7	-2716	-1628.5	-1351	-694.5	-124	-1262.429	890.895
Group 3 (IPA)	8	-118	23.5	83.5	100	100	47.125	76.738
Group 4 (IUSS)	5	100	100	100	100	100	100	0
Group 6 (DW)	4	100	100	100	100	100	100	0

Table 4: Group comparison using Kruskal-Wallis rank sum test demonstrating an overall difference between groups. ($p < 0.0003$) The list of p-values shows a statistical difference between group 1 CHX and all other disinfectant groups. Group 5 (SS) was not included in group comparison because the results were identical to group 4 (IUSS) showing no growth.

Kruskal-Wallis rank sum test			
Kruskal-Wallis chi-squared = 21.091, df = 4, p-value = 0.0003038			
Comparison	Z	P value unadjusted	P value adjusted
Group 1 (DS)- Group 2 (IPA)	2.799	0.005	0.017
Group 1 (DS)- Group 3 (CHX)	0.676	0.499	0.554
Group 2(IPA) - Group 3 (CHX)	-2.589	0.010	0.024
Group 1 (DS)- Group 4 (IUSS)	-0.775	0.438	0.626
Group 2 (IPA)- Group 4 (IUSS)	-3.884	0.000	0.001
Group 3 (CHX)- Group 4 (IUSS)	-1.638	0.101	0.203
Group 1 (DS)- Group 6 (DW)	-0.735	0.462	0.578
Group 2 (IPA)- Group 6 (DW)	-3.628	0.000	0.001
Group 3 (CHX)- Group 6 (DW)	-1.525	0.127	0.212
Group 4 (IUSS)- Group 6 (DW)	0.000	1.000	1.000

Table 5: Part 2 of the study Conventionally fabricated and 3D Printed surgical guides. The conventional surgical guides (CSG) had significantly more bacterial growth than 3D printed guides (3DG) ($p < 0.001$) A total number of 15 samples, 8 CSG ($n=8, \bar{x} = 14715, \sigma = 19814$)* and 7 (3DG) ($n=9, \bar{x} =0, \sigma =0$)* were determined to be sufficient in showing a statistical difference between the two guide types.

Bacteria Growth (CFU/ML)								
Group	n	Minimum	25%	50%	75%	maximum	\bar{x}	SD
Cohort 1: CSG	8	0	680	4100	13820	49200	14715	19814.11
Cohort 2: 3DG	7	0	0	0	0	0	0	0

* n= number of samples, \bar{x} = mean, σ = standard deviation

O. FIGURES

FIGURE 1: Image of a BHI agar plate showing the CFU observed from a sample of pumice (left) and pressure pot water (right) from the dental laboratory where samples were fabricated for this study. These plates are plated at 1:1 and contain a significant number of CFU and colony morphologies which are difficult to differentiate due to the number of CFU.

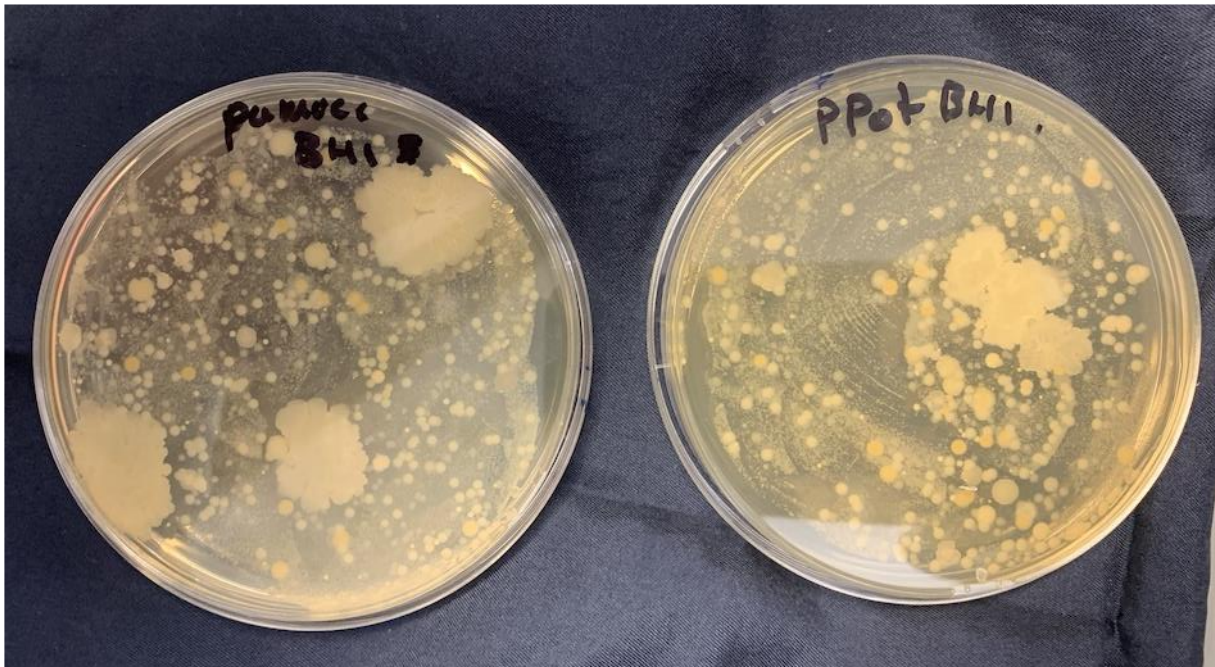


FIGURE 2: A picture of a BHI agar plate showing the CFU from a conventionally fabricated surgical guide disinfected with disinfectant spray used in group 1. The sample was plated at 1:4 dilution and contains several different types of colony morphologies; 1. Large round yellow, 2. Large white round.

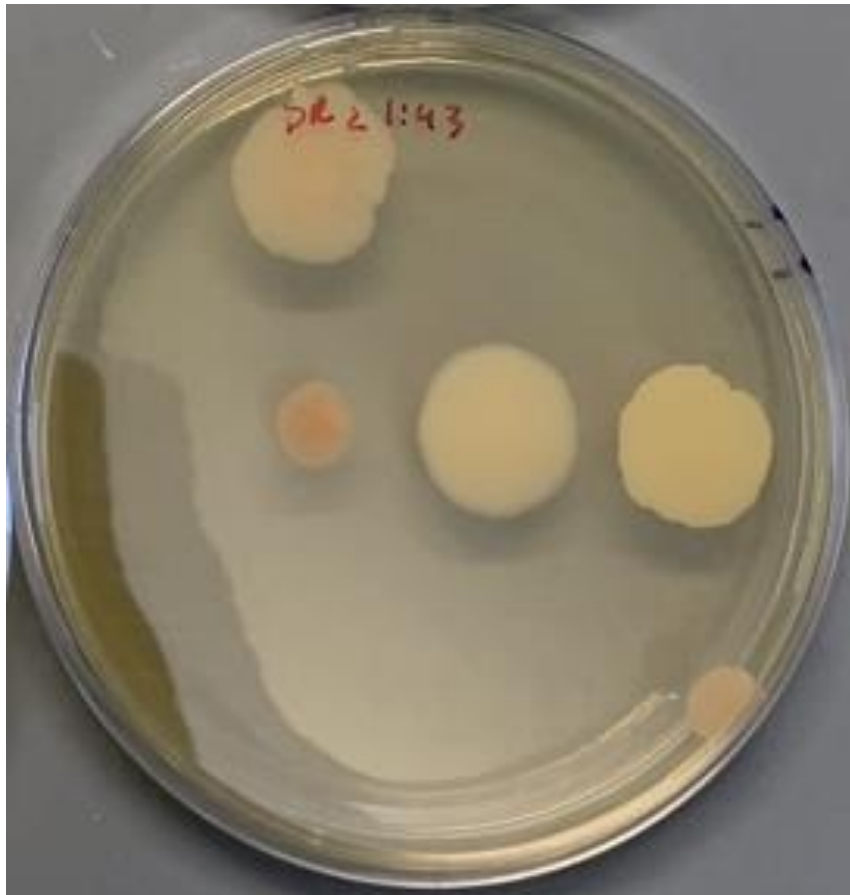


FIGURE 3: A picture of a BHI agar plate showing the CFU from a conventionally fabricated surgical guide disinfected with 0.12% chlorhexidine used in group 2. The sample was plated at 1:4 dilution and contains several different types of colony morphologies; 1. Large round yellow, 2. Large transparent round, 3. Small white round, 4. Small transparent round.



FIGURE 4: A picture of a BHI agar plate showing the CFU from a conventionally fabricated surgical guide disinfected with 70% Isopropyl alcohol used in group 3. The sample was plated at 1:1 dilution and contains several different types of colony morphologies; 1. Large round yellow, 2. Large transparent round, 3. Small white round, 4. Small transparent round.

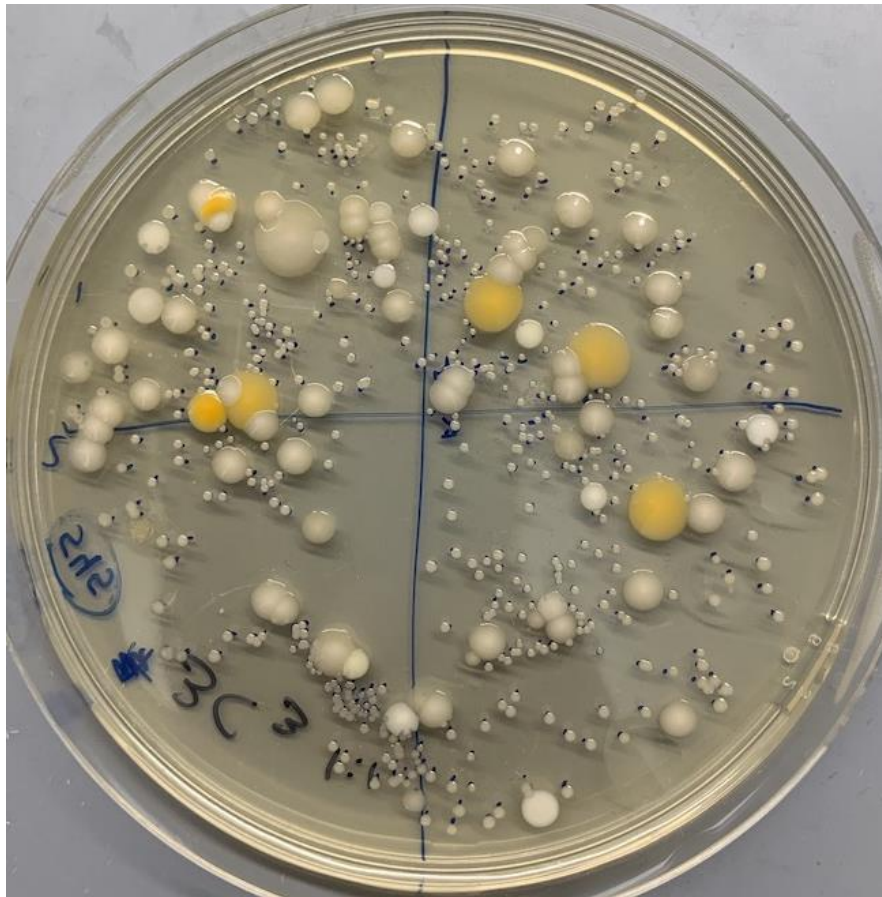


FIGURE 5: A picture of a BHI agar plate showing no growth a conventionally fabricated surgical guide sample sterilized by immediate use steam sterilization (“flash cycle”) for 4 minutes at 135°C (IUSS) in group 4. Group 5 samples are identical in findings. The sample was plated at 1:1 dilution and contains no CFU.

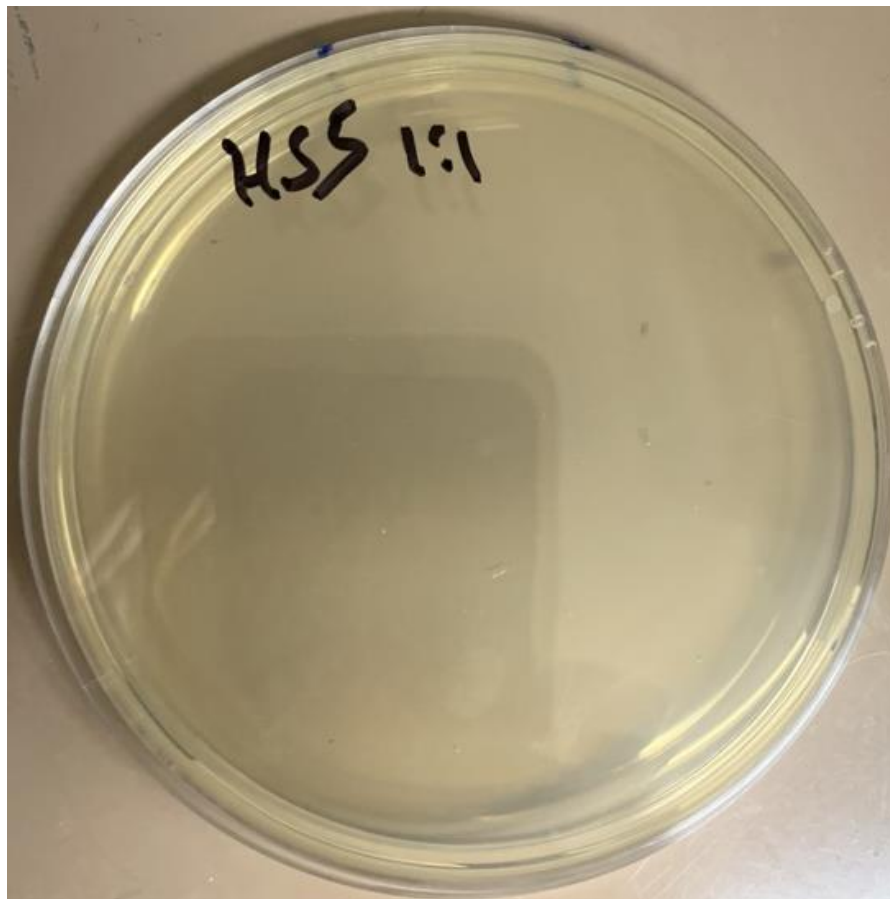


FIGURE 5: A picture of a BHI agar plate showing no growth a conventionally fabricated surgical guide sample sterilized by steam sterilization for 30 minutes at 121°C (SS) in group 5. The sample was plated at 1:1 dilution and contains no CFU.

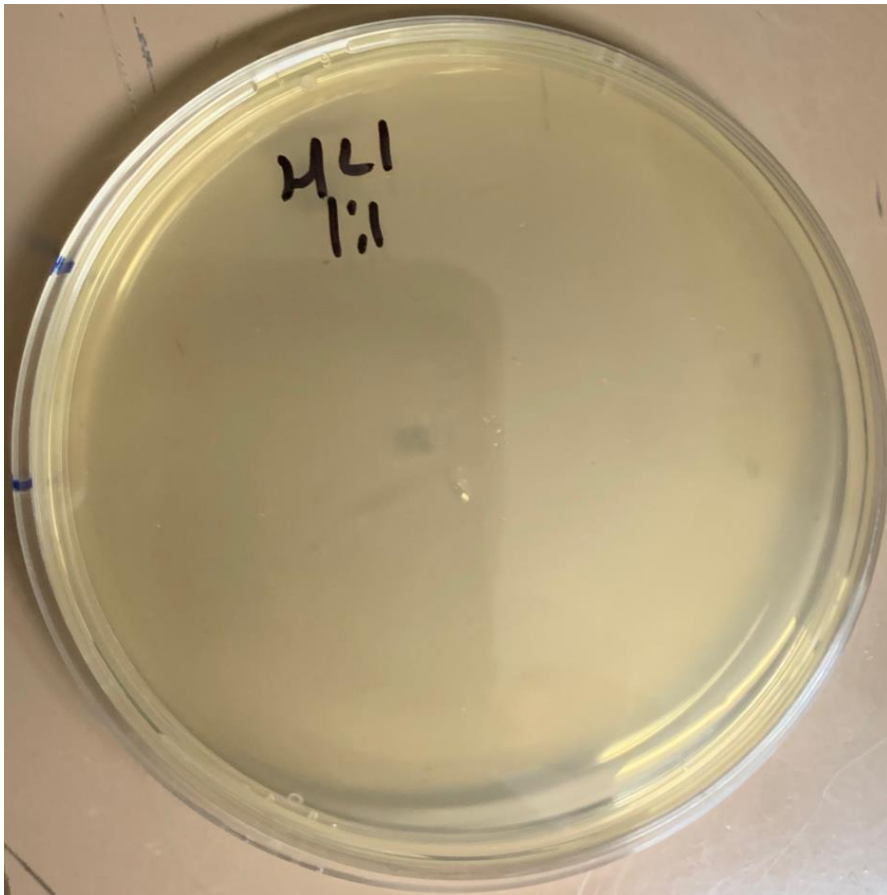


FIGURE 6: A picture of a BHI agar plate showing the CFU from a conventionally fabricated surgical guide disinfected with disinfectant spray used in group 6. The sample was plated at 1:4 dilution and contains several different types of colony morphologies; 1. Large round yellow, 2. Medium white round, 3. Small white round, 4. Small transparent round, 5. Medium transparent round.



FIGURE 7: A frontal photo of a maxillary conventionally designed acrylic resin surgical guide before immediate use steam sterilization (“flash cycle”) for 4 minutes at 135°C (IUSS). The surface of this sample shows surface irregularities and is rough.



FIGURE 8: A frontal photo of a maxillary conventionally designed acrylic resin surgical guide after immediate use steam sterilization (“flash cycle”) for 4 minutes at 135°C (IUSS).



FIGURE 9: A photo of a maxillary surgical guide fabricated using 3D Printed technology. The surface of this guide is smooth and has no roughened texture.

