EVALUATION OF ALTERNATIVE METHODS FOR DISINFECTION OF ORAL APPLIANCES CONTAMINATED WITH POTENTIALLY INFECTIOUS PATHOGENS

A Thesis
Submitted to
the Temple University Graduate Board

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in Partial Fulfillment
of the Requirements for the Degree
MASTER OF SCIENCE

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August, 2014

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ABSTRACT

Objectives: The oral cavity may be an important and overlooked reservoir of systemic infection, and athletic appliances may constitute a potentially important and under-recognized risk factor for infection.

Athletic mouthguards are difficult to clean and provide retentive sites for microorganisms. These microrganisms are organized as biofilms and can adhere to the acrylic, which has porosities on its outer and inner surfaces that create favorable conditions for bacterial colonization. Athletic appliances are not disinfected routinely at home and there is no specific clinical protocol for the control of bacterial biofilm on these appliances. Although adequate brushing or scrubbing with a dentrifice is an effective means of controlling the biofilm, inappropriate home-care quality and frequency are factors that compromise the efficacy of the mechanical control of biofilm. In addition, many athletic mouthguards are often left in the locker-room, and while in use at the athletic site, are often transferred from the mouth to hands, and temporarily stored or carried at distant sites (eg helmuts, shorts, sleeves,socks etc).

The two most frequently gram-positive cocci isolated from athletic mouthguards were *Staphylocccus aureus* and *Staphylococcus epidermidis*, prominent skin pathogens that are also associated with endocarditis, pericarditis, pneumonia, osteomyelitis, food intoxication, and athletic equipment contamination. Methicillin-resistant *S. aureus* is an important nosocomial pathogen, which is often transmitted from the colonized site to the site of subsequent infection, and is responsible for considerable morbidity and mortality.

Athletic mouthguards may be a reservoir for these bacteria to hibernate and thus be a mechanism of transmittal from one site to another. An increase in community associated MRSA (aka close-quarter MRSA) outbreaks has been noted in basketball, football, rugby, volleyball, and wrestling athletes. and some strains of *S. aureus* are the most common cause of cultured skin abscesses in athletes.

Good personal hygiene is the key to prevention and control of community associated MRSA outbreaks. Such practices among athletes include frequent hand washing, covering abrasions or seeping wounds, disallowing athletes with open wounds in whirlpools or saunas, discouraging shared personal items, requiring showers after all practices and games, wearing sandals in showers, isolating athletes who have infections, and washing protective gear after each use. Recommended infection control measures include regular and thorough cleaning of equipment, however when antimicrobial treatment is recommended, specific products are not identified. Therefore , the purpose of this study was to assess alternative methods of disinfecting samples of acrylic from surface and subsurface inoculates of specific bacteria, having in mind methods that maybe used at the athletic site and in the locker-room.

Methods: In this study, we tested the efficacy of the Nature/Zone^{Tm} ozone sanitizing chamber and Purell^{Tm} hand sanitiazer in disinfecting surfaces of acrylic specimens prepared from mouthguards. *Staphylococcus epidermidis* (aka *S. aureus*), *Lactobacillus casei,* and *Escherichia coli* were purchased from the American Type Culture Collection, Manasas, Va. All experiments were conducted in a laminar flow hood and all equipment was autoclaved prior to use. Growing cells were placed on the surface of sterile acrylic discs and then either Purell was added to the disc for three

minutes, or the inoculated discs were subjected to ozone in the chamber for three minutes. Alternatively, growing cells were placed in Eppendorf tubes to simulate porosities in the acrylic. All specimens (discs or inoculates) were then transferred to 10 ml of culture broth, grown overnight, and compared to positive and negative controls.

After overnight incubation, each working solution was diluted in ten-fold steps for the purpose of estimating bacterial cell numbers. From the dilutions , 0.1 ml was spread evenly across the surface of their respective agars. All agar plates were incubated for 24 hrs. at 37 C, after which all visible colonies were counted. The number of colony forming units (CFU) were determined by counting segments of the plates. All experiments were performed in triplicate, and experimental samples were compared to positive and negative controls. One way analysis of variance was used to compare colony counts between positive control samples , experimental ozone chamber samples, and experimental Purell™ samples.

Results: Three trials indicated that there was no inhibition of growth of the organisms when sterile discs or eppendorf tubes were incubated in the ozone chamber for 3 minutes. As compared to positive control and the ozone chamber experimental samples, surface application of hand sanitizer (Purell™) for 3 minutes significantly inhibited growth of all three bacteria ($p = 0.05$, one way ANOVA).

Conclusions: These findings indicate that ozone produced by the Nature/Zone UV sanitizer is ineffective in reducing bacterial counts of the three organisms used in these in vitro experiments. On the other hand, Purell hand sanitizer, significantly reduced the number of bacterial counts of all three organisms. Thus, whereas the hand sanitizer is an effective disinfectant, the ozone chamber is not an effective disinfectant.

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CHAPTER 1

INTRODUCTION

Oral devices, eg toothbrushes, mouthguards and dentures, have the ability to transmit organisms responsible for both local and systemic disease (Glass 1992). Fitted mouth guards provide protection from injury for athletes (news of dentistry, J Am Dent Assoc, 1972), but also may be harmful by causing bruises, lacerations and sores to soft tissues allowing portals of entry for microorgaisnms (Glass et al. 2009). Home-based sterilization procedures that are effective for cleaning toothbrushes, eg rinsing with soap and water, mouthrinses, and microwave irradiation, may not be effective for sanitizing mouthguards, because microorganisms exist in the porosities of the appliance , as revealed by scanning electron micrographs. (Glass et al. 2011).

Oral appliances become colonized by microorganisms after a certain time in the mouth (Sreenivasan et al. 1995). Gram-positive cocci, gram-positive bacilli, gram-negative cocci, gram-negative bacilli, yeasts, and molds were isolated from 81 athletic mouthguards submitted for microbial analysis (Glass et al. 2011). The two most frequently gram-positive cocci isolated from athletic mouthguards were *Staphylocccus aureus* and *Staphylococcus epidermidis*, prominent skin pathogens that are also associated with endocarditis, pericarditis, pneumonia, osteomyelitis, food intoxication, and athletic equipment contamination (Glass et al. 2004, and Glass et al. 2001).

Athletic mouthguards are difficult to clean and provide retentive sites for microorganisms. These microrganisms are organized as biofilms and can adhere to the

acrylic, which has porosities on its outer and inner surfaces that create favorable conditions for bacterial colonization (Morgan and Wilson 2000). Athletic appliances are not disinfected routinely at home and there is no specific clinical protocol for the control of bacterial biofilm on these appliances. Although adequate brushing or scrubbing with a dentrifice is an effective means of controlling the biofilm, inappropriate home-care quality and frequency are factors that compromise the efficacy of the mechanical control of biofilm (Dills et al 1988). In addition, many athletic mouthguards are often left in the locker-room, and while in use at the athletic site, are often transferred from the mouth to hands, and temporarily stored or carried at distant sites (eg helmuts, shorts, sleeves, socks etc).

The oral cavity may be an important and overlooked reservoir of systemic infection, and athletic appliances may constitute a potentially important and under-recognized risk factor for infection (Passariello et al. 2012). The possible disease transmission by contaminated athletic mouthguards, one to a leg abscess and another to lung infection, has been documented in two young football players (Glass et al. 2007). The oral cavity is commonly colonized with *S. aureus* (4-46%) which may be associated with a wide range of serious infections, especially methicillin-resistant *S. aureus* (MRSA) (Smith 2001), Athletes have been the subject of an increasing number of reported MRSA cases (Patel et al. 2007).

Methicillin-resistant *S. aureus* is an important nosocomial pathogen, which is often transmitted from the colonized site to the site of subsequent infection, and is

responsible for considerable morbidity and mortality. Two types of MRSA are identified in the literature: hospital-associated MRSA and community associated MRSA. An increase in community associated MRSA (aka close-quarter MRSA) outbreaks has been noted in basketball, football, rugby, volleyball, and wrestling athletes. and some strains of *S. aureus* are the most common cause of cultured skin abscesses in athletes (Weber, 2009). Among athletes, MRSA may be spread from skin to skin contact with open abrasions and contaminated persons, from surface to skin contact with contaminated sports equipment (eg mouth-guards), or by sharing personal items (Weber 2009).

Guidelines are lacking on what treatment strategies might lead to eradication of MRSA from the mouth, although it is thought that this is difficult to achieve. A recent systematic review of clinical studies concluded that the clinical evidence was lacking and that, although many chemical agents contained in oral hygiene products have proven in vitro activity against *S. aureus*, their clinical efficacy remains to be confirmed by further high-quality randomized controlled trials (Lam et al. 2012). However, a recent study showed that commercial mouthrinses are ineffective against oral MRSA biofilm (Smith et al. 2013).

Good personal hygiene is the key to prevention and control of community associated MRSA outbreaks. Such practices among athletes include frequent hand washing, covering abrasions or seeping wounds, disallowing athletes with open wounds in whirlpools or saunas, discouraging shared personal items, requiring showers after all practices and games, wearing sandals in showers, isolating athletes who have infections, and washing protective gear after each use (Johnson 2009). Recommended infection

control measures include regular and thorough cleaning of equipment, however when antimicrobial treatment is recommended, specific products are not identified. Therefore, the purpose of this study was to assess alternative methods of disinfecting samples of acrylic from surface and subsurface inoculates of specific bacteria.

CHAPTER 2

MATERIALS AND METHODS

Bacterial cultivation and preparation of cells. *Staphylococcus epidermidis* (aka *S. aureus*), *Lactobacillus casei*, and *Escherichia coli* were purchased from the American Type Culture Collection, Manasas, Va. *S. epidermidis* ATCC 12228 was grown on/in brain heart infusion agar and broth. *L. casei* (aka *L. rhamnosus*) ATCC 7469 was grown on/in Lactobacillus agar and broth. *E. coli* ATCC 10798 was grown on/in trypticase soy agar/broth.

All tubes, broths, agars and pipet tips were sterilized by autoclaving at 121 C for 20 minutes. The purchased freeze dried cells were reconstituted in their respective broths and then streaked on agar plates. Colonies were examined for purity and single colonies were transferred to broth and grown to log phase at which time they were used experimentally.

Devices and Materials to be Tested: The NatureZone ^{Tm} UV/ozone sanitizer was purchased from Henry Schein Co. Purell ^{Tm} and Chlorox were purchased from local pharmacies.

Preparation of acrylic spicimens. A standard hole puncher was used to make circular discs from a hard-soft acrylic mouth guard (Shock Doctor). Disc sizes were approximately 6 mm wide in diameter and 2.5 mm thick. Eppendorf tubes (50 µl) and the cut discs were sterilized by autoclaving at 121 C for 20 min.

Experimental Procedure Using Surface Application of S. epidermidi., E. coli, and L. casei. All experiments were conducted in a laminar control hood (Environmental Air

Control, Inc., Hagerstown, Md). The surface of the hood was wiped with Chlorox wipes prior to conducting the experiments. Aliquots (20 µl) of broth or cells were placed on the surface of five sterile discs and treated as follows: (negative control sample) 20 µl of sterile broth was placed on a disc, and after 3 minutes the disc was transferred to 10 ml of broth; (positive control sample) 20 µl of log phase cells was placed on the surface of the disc, 10 µl of sterile broth was also added, and after 3 minutes the disc was transferred to 10 ml broth; (experimental ozone chamber sample) 20 µl of log phase cells was placed on the surface of the disc, 10 µl of sterile broth was added, the disc was placed into the sterilization chamber, the lid was closed, the the uv-light was activated by pushing on the start button, and after 3 minutes of exposure to ozone, the disc was transferred to 10 ml of broth; (experimental Chlorox sample) 20 µl of log phase cells was placed on the surface of the disc, 10 µl of chlorox was added, and after three minutes, the disc was transferred to 10 ml of broth; (experimental Purell sample) 20 µl of log phase cells was placed on the surface of the disc, 10 µl of Purell was added, and after 3 minutes the disc was transferred to 10 ml of broth. All five tubes of broth containing the discs (designated as working solutions) were then incubated at 37 C for 12 hours (overnight).

After overnight incubation, each working solution was diluted in ten-fold steps for the purpose of estimating bacterial cell numbers. From the dilutions , 0.1 ml was spread evenly across the surface of their respective agars. All agar plates were incubated for 24 hrs. at 37 C, after which all visible colonies were counted. The number of colony

forming units (CFU) were determined by counting segments of the plates similar to the procedure described Loeshe et al. 1979. All experiments were performed in triplicate.

Experimental Procedures Using Subsurface Application of S. epidermidis and L. casei. Bacteria were applied to sealed eppendorf tubes to mimic bacteria existing in porosities of acrylic appliances. Aliquots (20 µl) were placed into sterile eppendorf tubes and treated as follows: (negative control) 20 µl of broth left at room temperature for 3 min.; (positive control) 20 µl of log phase cells left at room temperature for 3 min; (experimental ozone chamber sample) 20 µl of log phase cells, placed into sterile eppendorf tubes, after which the tubes were placed into the ozone sanitizing chamber, the lid was closed and the start button was pushed to activate the uv light and generate ozone; (experimental boiling sample) 20 µl of log phase cells, placed into sterile eppendorf tubes, after which the tubes were placed into boiling water for 1 min. The contents of all eppendorf tubes (20 µl aliquots of negative control sample, positive control sample, experimental ozone chamber sample, experimental boiling sample) were placed into 10 ml of broth (designated as working solutions) and incubated for 12 hr at 37 C.

After overnight incubation, each working solution was diluted in ten-fold steps for the purpose of estimating bacterial cell numbers. From the dilutions , 0.1 ml was spread evenly across the surface of their respective agars. All agar plates were incubated for 24 hrs., at 37 C, after which all visible colonies were counted. The number of colony forming units (CFU) were determined by counting segments of the plates similar to the procedure described Loeshe et al. 1979. All experiments were performed in triplicate.

Statistical analysis. One way analysis of variance was used to compare colony counts between positive control samples , experimental ozone chamber samples, and experimental Purell™ samples.

CHAPTER 3

RESULTS

Three trials indicated that surface application of 2%sodium hypochlorite (Chlorox) to the bacterial inoculum for 3 minutes and boiling the bacterial inoculum in eppendorf tubes for 1 minute completely inhibited growth of all three organisms (data not shown). In contrast, there was no inhibition of growth of the organisms when sterile discs or eppendorf tubes were incubated in the ozone chamber for 3 minutes (Table 1, Figures 1). As compared to positive control and the ozone chamber experimental samples, surface application of hand sanitizer (Purell^{Tm}) for 3 minutes significantly inhibited growth of all three bacteria (Table 1; $p = 0.05$, one way ANOVA).

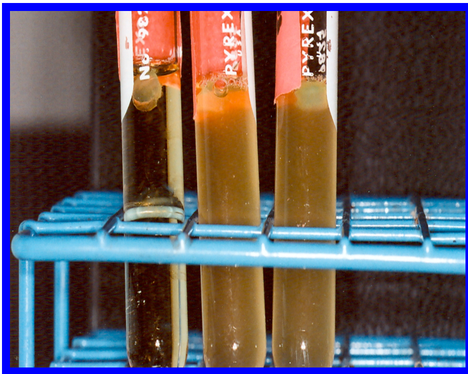

Fig 1. Overnight incubation of discs; from left to right, negative control, positive control, experimental ozone chamber sample.

Table 1: Summary of Experiments Comparing Positive Control,Experimental Ozone Chamber, and Hand Sanitizer Treatment of Acrylic Discs Inoculated with Bacteria		
Organism	Treatment	CFU 10^{-6} dilution (singles or clusters)*
S. epdermidis	none (+control)	41.7 ± 9
	ozone	46.0 ± 7.2
	hand sanitizer	10.7 ± 3.5
L. casei	none (+ control)	25 .5 ± 5.6
	ozone	28.2 ± 6.9
	hand sanitizer	5.4 ± 1.3
E. coli	none (+ control)	58.4 ± 8.8
	ozone	50.6 ± 9.4
	hand sanitizer	14.4 ± 4.6
• Data is presented as the median ± standard deviation of three trials.		

CHAPTER 4

DISCUSSION

Athletic mouth-guards become colonized by microorganisms after a certain period of time in the mouth. Previous studies have reported that these appliances become contaminated by several oral microorganism that may be involved in oral and systemic diseases (Lessa et al. 2007). Although dentists usually recommend at-home disinfection of dentures by immersion in antimicrobial agents for a certain length of time (Pavaina et al. 2003) data is lacking concerning the effectiveness of antimicrobials on bacteria lodged in the porosities of these appliances. In addition, in contrast to dentures, there is no emphasis on disinfection of mouthguards either at home or at the arena.

Oral bacteria with pathogenic potential, *L. casei* , *S. aureus*, and *E. coli*, were selected for this study. *L. casei* is characteristically known to lead to the progression of carious lesions, and bacteremia, meningitis, and endocarditis have been reported, particularly in immunocompromised patients (Salvana and Frank 2006). *S. aureus* causes several diseases, such as pneumonia, sepsis, abscesses, infective endocarditis, and osteomyelitis and MRSA(Smith 2001). *E. coli* is an important cause of diarrhea, urinary tract infections, and septicemia (Kaper et al. 2004).

Sodium hypochlorite 2% (Chlorox,bleach,NaOCl) and heating bacteria to 100 C for 3min (boiling) were considered the gold standard for disinfection from bacteria. NaOCl is widely used as the main root canal irrigant because of its broad antimicrobial activity in endodontics. It is a also a low-cost method to disinfect an appliance (Glass et al. 2001). Da Silva et al. (2008) investigated the effectiveness of different disinfectant

solutions for disinfecting acrylic resin specimens contaminated with *Streptococcus mutans*, *S. aureus*, and *E. coli* . They found 2% NaOCl to be the best antimicrobial agent against the tested microorganisms. This result is supported by Salvia et al. (2013). However, although boiling and the use of NaOCl, are effective and low cost methods of sterilization, they are not applicable to use in the locker-room or on the playing arena.

In this study, we evaluated the efficacy of 2 alternative protocols – application of hand sanitizer and use of an ozone sterilization chamber – for disinfection of mouthguard specimens compared with the gold standards of chlorox application and boiling. Disinfection means to eliminate most harmful microorganisms from surfaces or objects. Purell reduced the levels of all bacteria but did not eliminate the microroganisms completeluy. The efficacy of Purell in this instance, ie it disinfects but does not sterilize, may be explained by the fact that the active ingrediant, alcohol, is dispersed in a viscous carrier, glycerol, which may not allow contact with all of the organisms on the specimen. The ozone chamber, on the other hand, had no effect on any of the bacteria, and did not reduce CFU in any of these in vitro experiments.

Ozone has well-documented bactericidal properties due to multiple oxidation effects that are optimal at high humidity (Fan et al. 2002). Ozone is known as an oxidizing agent both in aqueous solutions and in the gas phase (Berrington and Pedler 1998). It has been shown that an ozone generator producing a concentration of 25ppm is bactericidal to both wet and dry samples across a range of gram-positive and gram-negative bacteria placed onto plastic and soft surfaces (Sharma and Hudson 2008). Ozone is not effective at cleaning the air except at extremely high, unsafe levels, and then

it is only partially effective. Devices that emit ozone at or below health standards set by the US Food & Drug Administration (FDA) do not effectively remove particles such as dust and pollen from the air, nor do they kill bacteria, viruses, mold, or other biological contaminants, despite claims made by advertisers. Further, if bacteria and/or mold are imbedded inside of porous materials such as carpet fibers or furniture cushions, ozone at or below health standards will have no effect on these biological contaminants (Berrington and Pedler 1998).

The failure of the ozone generator used in this study to disinfect the mouth-guard samples may be related to insufficient energy to generate ozone concentrations that are bactericidal, and there is no humidity in the chamber. The optimal requirement for high humidity suggest that additional radicals such as hydroxyl and peroxides may be necessary in addition to ozone radicals. A similar result was recently found by Astorga et al. (2014) using a similar new commercial ozone generator (Curozone X^4). Alternatively, it is feasible that proteins in the growth media may be oxidized by the ozone, and that such oxidation blocks ozone interaction with bacteria. However, this is unlikely because no reduction in bacterial counts was noted in any of the twelve experiments that were conducted in this study.

CHAPTER 5

CONCLUSIONS

This in vitro study demonstrates the susceptibiliuty of *S. aureus*, *E. coli*, and *L. casei* to hand sanitizer (Purell^{Tm}) and suggests that it disinfects the surfaces of acrylic specimens of mouth-guards. The study also indicates that the Naturezone^{Tm} ozone generator is not capable of disinfecting the same samples under the conditions used in these experiements.

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