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“The Effectiveness of the removal of DNA from the surfaces of EVA-based Mouthguards using Standard Cleaning Protocols”

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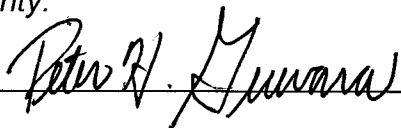
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The Effectiveness of the removal of DNA from the surfaces of EVA-based Mouthguards using Standard Cleaning Protocols.

Dean Chuenklung, DDS; Michael A. Washington, PhD; Peter H. Guevara DMD

Background: The American Dental Association has recognized the value of mouthguards for all those who participate in contact sports and recreational activities in the reduction of sport-related dental injuries.^{1,2} It has been well documented that participants of all ages, genders and skill level are at risk of sustaining dental injuries in sporting activities, including organized and unorganized sports at both recreational and competitive levels.³ Since mouthguards are repeatedly transferred between the oral cavity and the exterior environment, they are potential sources of infection.^{4,5,6} This is particularly true due to the fact that mouth guards are often used by athletes and military personnel in outdoor settings and given the fact that soil microbes are known reservoirs of antibiotic resistance genes.

Currently, only acrylic resin denture cleaning regimens have been developed to reduce the potential for bacterial and fungal contamination.^{7,8,9} Although many studies call for daily sanitizing compared to studies of dentures showing marked reductions in microbial loads, no studies on Ethylene-Vinyl-Acetate mouthguards have suggested which method of sanitizing is most effective.

However, the presence of residual deoxyribonucleic acid (DNA) on the surface of mouthguards can lead to the formation of biofilms and increase the potential for horizontal gene transfer (HGT). Horizontal gene transfer occurs through mechanisms of transformation, conjugation and transduction. In transduction, bacteriophages will introduce their own or foreign DNA in the host's genome. Proteins often protect bacteriophage double-stranded DNA from destruction by host endonucleases.¹⁰ Conjugation is like "bacterial sex," it is the transfer of genetic material between bacteria through cell to cell contact, DNA is transferred from plasmids or transposons. The genetic material transferred is often beneficial to the recipient with possible benefits of antibiotic resistance i.e. tetM located on Tn916 of *Streptococcus* from mother to child through saliva.¹⁰⁻¹² Transformation is the process that allows bacteria to take up DNA from its environment/surroundings. This takes place through "quorum-sensing" which allows competent cells to bind free double-stranded DNA from the environment and transfer across cell surfaces. Examples of competent bacterial genera at all times are *Haemophilus*, *Campylobacter*, and *Neisseria*, while others such as *Streptococcus* are competent in certain physiological states.^{11,13} HGT between residual bacterial DNA on the surface of mouthguards acquired from the environment and bacteria present in the oral cavity may transform normally innocuous bacteria into virulent and even antibiotic-resistant organisms.¹¹

Hypothesis: The capability of current cleaning protocols varies with respect to the removal of DNA from ethylene vinyl acetate (EVA) sheets.

Study Type: Randomized control study

Study Design and Methods: Ten EVA sheets were vacuumed formed with six individual stainless steel maxillary first molars (#14). Inoculated with vertebrate genomic DNA, then applied to five different disinfection methods and analyzed quantitatively for residual DNA bound to EVA wells using spectrophotometer.

Results: “Alcohol Free” Listerine mouthwash (which is not truly alcohol free since it contains high concentrations of the sugar alcohols and propylene glycol) did much worse at removing remaining DNA than all the other treatments. The lowest value for the mouthwash, 15.5 ng/μl, was higher than the maximum value for all the other treatments. Overall p-value=0.008 based on logged analysis. Air dry and Commercial mouthguard cleaning tablet performed better than the antibacterial hand soap (p=0.009 and p=0.026)

Conclusion: We concluded that alcohol free Listerine by itself is not sufficient to remove DNA contamination from EVA sheets. In order to prevent possible HGT events, we suggest the use of water wash, air dry, or commercial cleaning tablets. However, we recognize that Listerine was not designed to remove DNA. It was designed as an antiseptic and although this capability was not tested in this study, the use of a primary Listerine rinse to kill bacteria, followed by a water wash, air dry, or tablet treatment to remove DNA may be recommended. Further studies will focus on determining the best method for removing both bacteria and residual DNA.

Materials and methods

Specimen fabrication and sterilization

Sixty vacuumed tooth formed EVA specimens were obtained by means of 6 individual identical stainless steel crowns of Maxillary left first molar #14. Dental stone was poured into the intaglio surface to remove undercuts of mold. Six molds were then equally placed onto the MiniSTAR S® (Scheu Dental) along with each individual 3mm EVA sheet (Bioplast®Clear material) heated to Manufacture recommended time of 90seconds @3 bars of pressure or 45psi. After vacuum form of each sheet, stainless steel molds were removed and sheets were placed in individual containers with 100% Listerine mouthwash.

All specimens were bench cooled for one hour and immersed in Listerine mouthwash at room temperature 37°C for 24 hours for initial disinfection as simulated from laboratory practices.

Specimens received a distilled water rinse to remove all residue of mouthwash solution. The disinfection methods’ efficacies were evaluated against standard Sheared Salmon Sperm DNA (Ambion, AM9680).

Source of DNA irrelevant due to the fact that DNA is chemically identical whether, human, bacteria, or salmon.

Innoculation of the specimens

Each of the EVA sheets containing six well depressions was inoculated with 750uL of 1:50 concentration of Salmon Sperm DNA for 2 hours at room temperature. Pipet DNA solution from all six well depressions of 10 sheets and discard. Experimental designed around the instructions of Commercial Mouthguard cleaner (FreshGuard™).

1. Freshguard™ method: Soak for 5 minutes in warm water mixed with packet contents of Freshguard. Solution will foam up and turn blue. The solution will start to turn clear when it is ready. Remove your device, and rinse thoroughly.
2. Mouthwash (Listerine Zero™) method: 750uL of 50:50 concentration into designated contaminated well. Solution allowed to sit for 5 minutes and then pipet removal and discarded.
3. Antibacterial Hand Soap (Soft Soap) method: 750uL of 1:50 concentration placed into designated well using Micropipet. Allowed to sit for 5 minutes and removed via pipet and discarded.
4. Tap water (City Water) method: 750uL of tap water at room temperature was pipet into designated well. Allowed to sit for 5 minutes and removed via pipet and discarded.
5. Air Dry (Countertop) method: no solution was placed into designated well. After 5 minutes of countertop drying,

All 5 procedures were performed independently, wash was performed by pipetting 350uL of Phosphate buffered Saline 10 times and discarding.

Controls

Negative control: To confirm sterilization of the specimens, previous inoculation of genomic DNA were placed into 5 wells, except for the absence of inoculum in the 6th well. This control well was given a wash of 350uL PBS and immediately measured for Nucleic Acid absorbance.

DNA Quantification

For all specimens, 350uL of PBS was used to remove unbound DNA via pipetting up and down several times. Absorbance at 260nm for nucleic acid was read using Nanodrop spectrophotometer manufactured by Thermo Scientific.

Data Analysis

When comparing the four treatments other than Listerine (SoftSoap-S, City water-W, FreshGuard- F, and Air Dry-A), the overall treatment effect from the ANOVA was significant ($p=0.008$), and follow-up pairwise comparisons found that treatments F (geometric mean=2.1 ng/ μ l) and A (geometric mean=1.8 ng/ μ l) were significantly lower on average than S (geometric mean=5.7 ng/ μ l), $p=0.026$ for F vs. S, and

p=0.009 for A vs. S. None of the treatments differed significantly from W (geometric mean=3.9 ng/μl). P-values were adjusted for multiple comparisons based on Tukey-Kramer adjustment.

Results:

Air dry and Commercial mouthguard cleaning solutions were the best methods of DNA contamination removal. Air dry performed better than the antibacterial hand soap (p=0.009 and p=0.026) Listerine mouthwash did much worse at removing remaining DNA than all the other treatments. The lowest value for the mouthwash, 15.5 ng/μl, was higher than the maximum value for all the other treatments. Overall p-value=0.008 based on logged analysis.

Discussion:

In this study, the data support the hypothesis that cleaning methods vary in their effectiveness at removing DNA from EVA sheets. It was shown that the alcohol free Listerine method did not reduce DNA bound to the EVA intaglio surface while two other methods proved to be significant reducers of DNA contamination bound to EVA surface.

On comparison, results showed that the Commercial Mouthguard cleaner and Countertop Air dry significantly lowered the DNA contaminant than Antibacterial Hand soap, none of the treatments differed significantly from tap water rinse.

We hypothesize, that the Alcohol Free Listerine cleaning method is not suitable for the removal of DNA from EVA sheets. This is most likely due to the fact that although it does not contain ethyl alcohol, Alcohol Free Listerine contains sorbitol which is a sugar alcohol and propylene glycol which is an organic compound containing several hydroxyl (alcohol functional) groups. These compounds may have served to precipitate the DNA on the EVA membrane and prevented its removal by subsequent washes. In fact, glycol containing compounds are often included in DNA precipitation protocols.

Conclusion:

We conclude that Alcohol Free Listerine by itself is not sufficient to remove DNA contamination from EVA sheets. In order to prevent possible HGT events, we suggest the use of water wash, air dry, or commercial cleaning tablets. However, we recognize that Listerine was not designed to remove DNA, it was designed as an antiseptic and although this capability was not tested in this study, the use of a primary Listerine rinse to kill bacteria, followed by a water wash, air dry, or tablet treatment to remove DNA may be recommended. Further studies will focus on determining the best method for removing both bacteria and residual DNA.

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