Tracing of Microbes in Prepared Cavity Following Different Minimally Invasive Caries Removal Protocols

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ABSTRACT
Aims: The conventional caries removal technique has been replaced with minimally invasive (MI) techniques to preserve healthy natural teeth and to provide durable dental restorations. Each of these MI caries removal protocols is reported to be favorable in dealing with different caries conditions. The current study aimed to trace the residual bacteria that may remain in a prepared cavity following a visual-tactile (VT), caries detection dye (CDD), and chemo-mechanical caries removal (CMCR) protocol.

Materials and methods: A total of 29 extracted human molar teeth with visible caries lesions were randomly divided into three groups. The cavity preparation and caries removal of each group was accomplished following one of the MI caries removal protocols. Swab samples (one from each specimen) were taken and inoculated onto a blood agar plate and incubated for 48 hours. The growth of the bacterial colony was observed under a microscope and the specific genome of the bacteria was identified by polymerase chain reaction (PCR) test.

Results: The maximum number of traceable bacteria was observed following the chemo-mechanical caries removal group followed by the caries detection dye group and the least in the visual-tactile group. The PCR test revealed the presence of Streptococcus sobrinus in all the observed colonies; however, Streptococcus sobrinus was absent completely. The Chi-square test reveals a statistically insignificant ($\chi^2 = 0.646$) difference among the tested groups.

Conclusion: All of the MI caries removal protocols used in this study showed a trace of microbes in certain teeth. The cavity prepared following a visual-tactile protocol showed the least amount of traceable bacteria in the prepared cavity.

Clinical significance: Cavity that is prepared following individual MI protocol has a risk of leaving microbes in it.

Keywords: Caries detection dye, Chemo-mechanical caries removal, Minimally invasive caries removal, Residual microbes, Visual tactile.

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INTRODUCTION
The term dental caries refers to a disease caused by microbial invasion on a susceptible tooth structure that ultimately results in the decay of the tooth. It begins and progresses when demineralization outgains remineralization. It has been found that bacteria are a part of the resident flora in the oral cavity. However, when a low pH environment is achieved, these bacteria express their pathogenicity by secreting acids. Streptococcus mutans and Lactobacilli have been linked to this process. S. mutans is involved in the initiation of caries, while lactobacilli contribute to its progression.1,2

The concepts of caries removal techniques have been changing over time. GV Black’s caries removal technique, also known as the “extension for prevention” technique, revolutionized the field of dentistry when it was introduced in the early 20th century. Black believed that caries should be removed completely, leaving behind only sound tooth structure, to prevent further decay. This technique involved creating a cavity that extended beyond the visible decayed area and into the surrounding sound tooth structure. This allowed for the placement of restorative materials that would provide a barrier against further decay.3 Black’s technique remains a cornerstone of modern dentistry, and his principles have been refined and adapted over the years to improve patient outcomes.

The conventional caries removal technique is a widely used method in dentistry to remove decayed or infected tooth structures. It involves the use of rotary instruments such as high-speed handpieces, low-speed handpieces, and burs to remove the carious lesions from the tooth. The technique aims to completely remove the decayed tissue, leaving behind a sound tooth structure.4 Studies have shown that this technique is effective in removing caries and restoring the tooth’s structural integrity. However, the conventional caries removal technique has some disadvantages...
such as the risk of damaging healthy tooth structure, the generation of heat during the procedure which can lead to tooth sensitivity, and the potential for the spread of bacteria during the removal process. Therefore, dentists are increasingly adopting minimally invasive (MI) techniques such as selective caries removal to preserve healthy tooth structure while removing the diseased portion. Nowadays, researchers advocate a more conservative method, as it is now limited to the removal of carious tooth structure only while preserving sound structure.

Minimally invasive caries removal techniques are gaining popularity in modern dentistry as they offer several advantages over traditional methods. These techniques involve removing only the diseased portion of the tooth, leaving the healthy structure intact. This approach minimizes the amount of healthy tooth structure lost, reduces the risk of postoperative sensitivity, and preserves the natural anatomy of the tooth. Furthermore, MI techniques reduce the need for anesthesia and can often be performed without the need for a dental drill. Several studies have shown that MI caries removal techniques can be as effective as traditional methods in treating carious lesions, with similar rates of success and patient satisfaction. Overall, these techniques provide a more conservative and patient-friendly approach to treating dental caries.

The visual-tactile (VT) method is the most common method of caries removal following the MI concept. This method involves using a dental explorer and mirror to visually examine the tooth surface for any soft or sticky areas, which may indicate the presence of caries. Additionally, a tactile examination is performed using a dental probe to determine the consistency of the tooth surface. However, it is considered subjective, less sensitive, and of poor specificity.

Caries detection dye (CDD) is a commonly used tool in dentistry to identify areas of tooth decay. This dye works by highlighting areas of demineralization on the tooth surface, making it easier for dentists to identify and treat cavities. Comparing conventional excavation and excavation with CDD, it was found that the addition of dye is beneficial in the detection and removal of infected dentin without increasing the size of the cavity.

Chemo-mechanical caries removal (CMCR) is a non-invasive method that eliminates infected dentin with the use of a chemical agent. This method requires excavation instead of drilling, which helps overcome dental anxiety due to the use of local anesthesia, the preservation of tooth structure, and to limit the damage to the pulp by the use of heat.

Despite having several advantages, all of these three MI techniques have some limitations. The purpose of this study is to identify a technique that results in the least amount of microbes residing within cavity preparations to determine the best possible caries removal technique, for restorations that last longer with a lower risk of failure.

**Materials and Methods**

**Specimens Preparation**

This study was conducted under the ethical approval number RAKMHU-REC-028-2020/21-UG-D which was obtained prior to the experiment. This study was conducted in 2021 over a period of 6 months. A total of 30 extracted human teeth were used in this study. The specimens were collected using the convenience non-probability sampling method. The inclusion criteria for the specimens were permanent teeth, teeth with sufficient tooth structure, and teeth with carious lesions. Teeth with pulp therapy, and exposed pulp were excluded from this study. The teeth were preserved in natural saliva (collected from the volunteers) until the experiment was conducted.

**Caries Removal Procedures**

Three trained operators performed the caries removal as per the instruction provided by the manufacturer. The caries lesions were removed using either the VT method, CDD method, or CMCR method. Ten specimens were allocated per groups following previous articles published using similar protocol. For the VT method, the caries was removed using a spoon excavator and low-speed round carbide bur (as per the nature and size of the caries lesion) by evaluating the color, consistency, and texture of the prepared cavity. For the CDD method, CDD (Caries Finder™ G, ZEST DENTAL SOLUTIONS, CA, USA) was applied on the caries lesion and was excavated using a spoon excavator and low-speed carbide bur following the color guidance and manufacturer instruction. For the CMCR method, the gel (Papacarie® Duo gel, Fórmula & Ação, São Paulo, SP, Brazil) was applied to the caries lesion following the manufacturer’s instructions and were gently excavated using a spoon excavator and rinse with water spray.

**Isolation and Identification of Bacterial Stains**

The prepared surfaces were swabbed with saline-soaked swab sticks. These swabs were plated onto blood agar plates (BioWorld Inc. USA) and incubated at 37°C under aerobic conditions for 48 hours. The inoculum/suspension was serially diluted by adding 1x of suspension to 2x of diluent. The surfaces of the plates were sufficiently dry to allow a 20 μL drop to be absorbed in 15–20 minutes. Plates were divided into equal sectors. The sectors were labeled with dilutions. In each sector, 1 × 20 μL of the appropriate dilution was dropped onto the surface of the agar and the drop allowed to spread naturally. The plates were left upright on the bench to dry before inversion and incubation at 37°C for 48 hours. Each sector was observed for growth, high concentrations gave a confluent growth over the area of the drop, or a large number of small/merged colonies. Colonies were counted in the sector where the highest number of full-size discrete colonies can be seen. Identification of bacterial isolates was carried out using standard microbiological techniques.

**Amplification of DNA Strand Using Polymerase Chain Reaction (PCR)**

Polymerase chain reaction was used to amplify target sequence of DNA using polymerase enzyme (HiPurA™ 96 Bacterial Genomic DNA Purification Kit, HIMedia Laboratories Pvt. Ltd. India). The specimens were heated for 5 minutes and then centrifuged. Two microliters of the supernatant fluid were the source of genomic DNA in PCR. The PCR was carried out for 30 cycles.

**Preparation of Samples for PCR**

Each reaction mixture (total volume = 25 μL) contained 12 μL of PCR Master Mix (HOT FIREPol® EvaGreen® qPCR Mix Plus, Solis BioDyne, Estonia), 2 μL of each primer, 2 μL of genomic DNA as template, and 7 μL of nuclease free water. The reaction was conducted as follows: 95°C for 5 min; followed by 30 cycles of 95°C for 15 sec, 56°C for 30 sec, and 72°C for 1 min, and finally, 7 min at
72°C for extension using Applied Biosystems 2720 Thermal Cycler. PCR products were evaluated by 1.5% agarose gel electrophoresis in tris-borate ethylenediaminetetraacetic acid (EDTA) buffer, stained with ethidium bromide, and then visualized using BioRad Gel Doc Ez Imaging System. To evaluate the effectiveness of the experimental group, *S. mutans* (ATCC 25175) and *Lactobacillus casei* (ATCC 393) were used as the positive and negative controls, respectively. The methodology is shown in *Flowchart 1*.

**Statistical Analysis**

The data were analyzed using statistical software (SPSS 24.0, IBM, USA). The trace of microbes was analyzed using descriptive statistics and significance among the tested groups was determined by the Chi-square test.

**Results**

Inoculation of swab samples onto blood agar media obtained from prepared cavity showed growth of bacterial colony after 48 hours of incubation. A well-defined colony was observed with naked eye and under a microscope. The representative image of the bacterial colony showed in *Figure 1*. All three groups showed a different number of colonies within the groups. Three teeth specimens out of 10 that prepared following VT method showed a visible growth of bacterial colony. In case of CDD, 4 of 9 and for CMCR 5 of 10 specimens showed a visible growth of bacterial colony. The PCR test reveals the specific genome of bacteria that grew in blood agar media. The agarose gel electrophoresis showed a positive band of specific microbes. *Figure 2* shows the result of PCR to detect the presence of genomic DNA of *S. mutans* in the samples tested. All 12 specimens that showed visible bacterial colony expressed a positive band for *S. mutans*. *Figure 3* shows the result of PCR to detect the presence of genomic DNA of *S. sobrinus* in the samples tested. None of the tested specimen expressed a positive band for *S. sobrinus* using agarose gel electrophoresis except the positive control. Chi-square analysis with the frequency of traceable bacteria did not show any statistically significant difference among the tested groups. *Table 1* show the frequency of traceable bacteria for each group.

**Discussion**

An efficient MI method of caries removal should identify and remove the caries-infected dentin and preserve caries-affected dentin which is free of microbes and has the potential of remineralization. Currently, the VT, CDD, and CMCR are the most common and clinically practiced MI caries removal technique. This study was intended to compare the accuracy in microbe-free cavity preparation following the VT, CDD, and CMCR gel.

The VT method is a traditional technique for removing microbes from prepared cavities in dentistry. It involves the use of hand
Microbes in Minimally Invasive Prepared Cavity

Table 1: The frequency of traceable bacteria in each experimental group

<table>
<thead>
<tr>
<th>Microbes</th>
<th>VT Count</th>
<th>VT % within group</th>
<th>CDD Count</th>
<th>CDD % within group</th>
<th>CMCR Count</th>
<th>CMCR % within group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans</td>
<td>Absent</td>
<td></td>
<td>Present</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Count</td>
<td>7</td>
<td>41.2</td>
<td>29.4</td>
<td>5</td>
<td>56.6</td>
<td>70.0</td>
</tr>
<tr>
<td></td>
<td>% within group</td>
<td>5</td>
<td>29.4</td>
<td>56.6</td>
<td>70.0</td>
<td>0.646</td>
<td></td>
</tr>
<tr>
<td>S. sobrinus</td>
<td>Absent</td>
<td></td>
<td>Present</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Count</td>
<td>10</td>
<td>34.5</td>
<td>31.0</td>
<td>10</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>% within group</td>
<td>10</td>
<td>34.5</td>
<td>100.0</td>
<td>100.0</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Count</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td></td>
<td>% within group</td>
<td>0</td>
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</tr>
</tbody>
</table>

Instruments such as a dental explorer and mirrors to evaluate the caries removal by examining the color, consistency, and texture of the prepared cavity after caries removal. Although this method is effective, it has several limitations that may compromise the quality of the restoration and increase the risk of postoperative complications. One limitation of the VT method is its reliance on the operator’s subjective judgment and manual dexterity. The operator must rely on their sense of touch and vision to detect and remove all infected tissue and debris from the cavity. This can be challenging, especially in deep or complex cavities, and can lead to incomplete removal of microbes and subsequent failure of the restoration. Another limitation of the VT method is its potential to cause damage to healthy tooth structures. Hand instruments can cause micro-fractures and micro-cracks in the surrounding dentin, which can weaken the tooth and increase the risk of fracture or postoperative sensitivity. In our study, the trace of S. mutans was detected in 30% of the total cavity prepared using the VT method. The result of our study reflects and supports the above-mentioned limitation of the VT method in caries removal. A study conducted by Ntovas et al. reported that experienced professionals showed statistically significant higher specificity compared with young dentists and students in the detection of residual cavities using VT method. Another study indicates that the use of fluorescence-aided caries excavation can improve the accuracy of the VT caries removal technique.

Several protein dyes have also been marketed as caries-detection agents. Implemented to achieve complete removal of infected carious dentin and was advocated for a pain-free caries removal technique without the need to use local anesthesia. The technique is based on staining, which involved multiple dye applications and repeated removal using spoon excavators and low-speed handpieces. However, stained surfaces are claimed to be less mineralized rather than bacteria-free, as the CDD use was intended to only stain-less mineralized and denatured collagen dentin surfaces, with the risk of overpreparation. This method can also be subjective in the interpretation of the color of the dye, which might lead to unknowingly leaving caries in the preparation due to the misjudgment of dentists. In our study, of 45% specimens that were prepared following color guidance of the CDD showed a trace of S. mutans in the prepared cavity. As discussed earlier, the major drawback of CDD is the color-based evaluation that may vary among individual operators. Another concern is the sensitivity and specificity of CDD. Research on CDD is still ongoing and debatable, as many clinicians believe it has resulted in sound tooth structure removal, and on the other hand, other clinicians found it crucial in removing residual S. mutans. A previous study also reported similar results in accordance with our result.

Chemo-mechanical caries removal is a MI method for removing dental caries without the use of traditional dental drills. Chemo-mechanical caries removal involves the application of a chemical agent that selectively dissolves the carious tissue, followed by the mechanical removal of the softened caries with hand instruments. Moreover, it is considered a patient-friendly approach as it is painless and overcomes the fear of anesthesia and the noise of rotational instruments. Papacarie, which has been showing promising results as a CMCR agent, consists of papain, chloramines, and toluidine blue. Papain interacts with exposed collagen by the dissolution of dentine minerals through bacteria, and thus softens the infected dentine and allows for painless removal. This method has been proven to be effective with pediatric patients as it is painless, providing relief and comfort while encouraging a positive dental attitude. However, in our study cavities that were prepared using CMCR showed the highest amount (50%) of traceable S. mutans in the prepared cavities. Most of the previous studies reported the efficiency of CMCR using a primary tooth that contains less minerals than the permanent tooth. In our study, the use of permanent molar teeth might contribute to achieving lower efficiency. Another limitation of CMCR noticed in our study is the poor dissolving/softening capability of CMCR that requires a repeated application and a comparatively long time to prepare the cavity. This fact was also highlighted in a previously published article.

In view of the result of this study, none of the caries removal techniques used in this study could achieve a microbe-free cavity. The most commonly used VT showed the highest efficiency, however, not enough to achieve 100% accuracy. Several previous studies recommended using a combination of multiple techniques to enhance caries removal efficiency. Final disinfection of the cavity with an antibacterial solution such as a low-concentration sodium hypochlorite might be used for a cavity with complex anatomy.

**Conclusion**

Within the limitation of the in vitro study model, this study concludes that achieving microbe-free cavities is difficult following individual MI methods. The VT method showed the least amount of residual bacteria followed by CDD method. The CMCR showed the maximum number of residual bacteria. There is scope for developing new technique and improving the accuracy of currently available cavity preparation techniques. The VT method in conjunction with other methods might be the best choice to achieve a microbe-free cavity.
Clinical Significance
Cavity that is prepared following individual MI protocol has a risk of leaving microbes in it. The clinician should use collective MI protocol to assure microbe-free cavity for restoration.

REFERENCES