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Dental caries shallow-layer microbe harvest and data display method V.1

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We are still developing and optimizing this protocol

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Abstract

We report a new harvest method and data display of taxa in material dissected from within dental caries lesions for culture or non-culture sequencing and taxa identification. This method is significant because it preserves the microbe spatial positions relative to lesion depth and uses micro-surgical aseptic techniques that allow exact dissection of precisely selected portions of the lesion while preventing contamination. Samples are harvested in shallow layers and each layer harvested becomes a separate sample for sequencing and taxa identification. The layers are coded in a way that allows sequential reassembling of the layers after taxa identification. Reassembling the harvested layers is analogous to reassembling pieces in a puzzle that rebuild a picture. In this case, the reassembled layers build a "lesion diagram" showing where within the pre-harvest lesion the identified taxa resided relative to lesion depth in the pulpal direction. After reassembling lesions, taxa detail not possible previously showed taxa location, abundance and diversity relative to lesion pulpal depth, plus absolute abundance per milligram and taxa transitions and fluctuations from superficial through deepest dissected layers.

Before start

This site is reviewed and updated as the authors continue with this work. Please check back for updates and additions.

Dental caries shallow-layer harvest and data display method shows taxa pre-harvest spatial positions, absolute and relative abundance and diversity relative to lesion pulpal depth

1 Introduction

This method was originally developed and used for dissection of dental caries lesions in situ but is applicable broadly to other tissues, particularly hard tissues. Readers will find this description references teeth and the oral cavity of humans and will need to apply the principles and procedures to their own applications.

This description of our method includes helpful hints and notes from the developers' experiences. As new ideas and information are developed, they will be added. **Please check back for additional edits frequently.**

Note

We found contamination problems with supplies and instruments promoted and sold as "sterile", and learned not to trust this designation. Instead, we individually repackage all items needed to be sterile, and sterilize them in small batches that are biologically monitored. Contaminants adversely affect both diversity and abundance results.

Note

Operating glove preparation - Problems with gloves have been infrequent (about 1-2% of the time) but persistent and easy to miss. Problems noted have been inclusions within the glove materials, tears between the fingers at their base, and random culturing that showed bacterial and fungal organisms present. Some glove brands could not be used after steam sterilization. If sterilized too long before use, internal surfaces of some brands adhered to each other and would not separate to allow a hand to be inserted. We purchase multiple brands and processed them through the autoclave to identify brands free of these problems, that we could rely on. For sterilization we learned to individually layer each glove within sterilization wrap about 2-4 weeks before use and then steam sterilize them about 1 week before use. It is important to test your preferred gloves *before the day of use* to assure they can be sterilized and then donned easily.

2 MATERIALS & METHODS

The following method was developed to combine surgical microscopy, micro-dissection, layered sampling, and a new data display design that presents a layered diagram of the

pre-harvest location of a lesion's internal taxa relative to its pulpal depth. This section describes the design of the aseptic operating area, procedural steps for shallow-layer dissection and sample collection, and the protocol used for microbial processing and data visualization. Each component of the method was developed to provide sample integrity, minimize contamination, and allow reconstruction of the pre-harvest microbial distribution relative to pulpal depth of the lesion.

In our application we used a 9-member team to expedite sample progressing and to maintain detailed care and attention to accuracy and asepsis in the process. Below is a listing of these 9 positions and their assigned responsibilities and invite your review and use or adaption to your situation.

A nine-member team performed the lesion dissection as follows:

- **Team Leader.** Wearing full-body sterilized protective covering, performed resin dam isolation, disinfection, dissection, and Micro-Brush collection of tooth material under operating microscope magnification (10-18x) while recording observations verbally into a microphone taped to cheek under a face mask (Isolator Plus N95 Disposable Respirator, Crosstex).
- **Lead-Assistant.** Provided and assisted with all items requested by the Team Leader using aseptic techniques, confirmation of codes, and passing the brush-filled vials to Hand Recorder.
- **Associate-Assistant.** Aseptically replaced sterile operating gloves after each layer dissection, provided sterile-wrapped new bur and/or number-coded pre-weighed sterile micro-brush (Multi-Brush, Magic-Brush). At completion of each dissection layer, confirmed all codes with Lead Assistant and Hand Recorder.
- **Hand Recorder.** Received from Lead Assistant each letter-coded brush-filled vial, confirmed codes, then hand recorded tube and brush codes and dissection layer number.
- **Runner.** Transported vial containing brushes to Anaerobe Chamber #1 Operator for weighing, then to Anaerobe Chamber #2 Operator for vortexing and division into three aliquots, leaving the original vial in chamber #2 to become the anaerobic cultured aliquot, then deposited the other two aliquots into the portable incubator to be labeled for non-culture NGS sequencing or ultra-low temperature storage.
- **Anaerobe Chamber #1 Operator.** Received vial containing micro-brushes from Runner, confirmed codes, then weighed, calculated, recorded and communicated sample weight to Lead Assistant, Hand Recorder and Team Leader.
- **Anaerobe Chamber #2 Operator.** Received weighed vial containing micro-brushes from Runner, vortexed and prepared three aliquots, retaining the vial containing the micro-brushes as the anaerobic aliquot within chamber #2, and passed to Runner the other two aliquots coded for non-culture NGS sequencing or ultra-low temperature storage into the portable incubator for temporary short-term holding until processed for NGS and ultra-low temperature storage.

- **Handpiece/Attachment/Bur-Assistant.** At completion of each layer dissection, received back used handpiece/attachment/bur from Team Leader, and with Lead-Assistant help aseptically replaced all with sterile components, and discarded used bur.
- **Microbiology Assistant.** Labeled the two aliquots—one for non-culture NGS sequencing and the other for ultra-low temperature storage, then placed all tubes into 4°C refrigerator until further processing after completion of the procedure on the subject

3 Aseptic Operating Area and Contents

A vacuum evacuated microbiology laboratory was floor-to-ceiling curtain-partitioned to form an isolated space 14×6×8 feet that included a 2×4 foot downdraft HEPA air filtration unit (Fantom, Gordon Cleanroom Products) ceiling mounted over the oral cavity operating area and confirmed at 1.2 air exchanges per minute at high setting (Hot Wire Thermoanemometer WD20250-16, Digi-Sense). Items within the operating area included: dental subject chair with attached light; clinician chair; ceiling mounted surgical microscope (PROergo, Zeiss); surgical electric control unit with 20 handpieces and 20 attachments (Surgic XT Plus, NSK) to aseptically power micro-size cutting burs at low rpm using no compressed air or water; hand-held SLR camera with close-up lens (S-1 Pro, Fuji with 105mm Lens, Nikon) to produce high quality close-up digital photographs of lesion appearance during dissection; audio recording system (Tascam DR-44WL, Teac) to record real-time observations during dissection. See Figure 1.

Note

Air Purification - Proper air purification is critical in the sample dissection/collection area to avoid introduction of contaminants into the dissected samples. In prior work, testing common air purification products (i.e.: blowers, filters, suction, ultraviolet light, ion generators, foggers, certified negative pressure) to clear the area around a patient's head, we identified the ECM Phantom (Gordon's Cleanroom Products) as most effective at keeping this area free of particles. The 2x4 foot ceiling mounted forced fan unit is located in the ceiling directly over the patient's oral cavity operating area. It emits a string downdraft of HEPA filtered air (~700cfm) and filters the air in the 14x8x8 foot curtained area ~1.2 times per minute. To help keep the air clean, all personnel present in the curtained treatment area must wear a high filtration face mask with snug peripheral fil, clean/sterile PPE, minimize talking, and keep movements minimal and deliberate.

4 Equipment and instruments outside the isolated area

Two certified anaerobe chambers designated chamber #1 (AS-580, Anaerobe Systems) containing a certified microbalance (MSA6.6SOTRDM, Sartorius) and deionizer (STAT-FAN 0-dc/YIB01-OUR, Sartorius) for weighing samples; chamber #2 (Whitley MG500,

Microbiology International) for dividing each layer sample into two aliquots used for culture and Sanger sequencing and ultra-low temperature storage; portable incubator (Incufridge RS-IF-202, Revolutionary Science) for warming sterile resin dam material; steam sterilizer (PSS11-AA-MSSD, Primus); HEPA air filtered cleanroom with certified biologic safety cabinet (NU-543-600, Nuaire); incubators; refrigerators; and ultra-low temperature freezers.

5 **Aseptic Shallow-Layer Dissection Procedure**

Steps below are ordered as performed by a team wearing protective covering appropriate for assigned functions.

- 5.1 **Subject pre-dissection preparation:** In an adjacent outpatient dental clinic, the subject received local anesthetic, surface biofilm removal using sterile gauze and sterile gloves, and placement of an elastic dam to provide retraction of lips, cheeks, and tongue and isolate the tooth containing the test lesion.

Note

The elastic dam leaks and saliva ingress is constant around the edges adjacent to the isolated tooth, so steps outlined below are performed to prevent this leakage.

- 5.2 **Dissection site disinfection:** Within the isolated area, the elastic isolation dam and its metal retaining clamps were disinfected with 2 ml 95% ethyl alcohol (Everclear Grain Alcohol, Luxco) pipetted aseptically separately onto 5 sterile gauze sponges used separately and allowed surfaces to dry after each application in the purified ambient air (total wet contact time ≥ 5 minutes).
- 5.3 **Sterile isolation of dissection site:** Sterilized warmed (37°C) resin dam material (Dental Dam, Den-Mat) was syringe applied and immediately light-polymerized over small contiguous sections of the thoroughly dry elastic dam, metal clamps and intact tooth surfaces where it formed a fragile seal that ultimately surrounded the dissection site to provide a sterile zone surrounding the operating site and prevent saliva ingress.

Note

The resin dam application is a critical and technically delicate procedure that involves 2 people -- one applying the resin material from a syringe and the second person operating the special light that initiates polymerization of the resin dam material.

All surfaces must be thoroughly dry to obtain the seal to the elastic dam and the tooth. Once the seal is established care must be taken to use very light touch for fulcrums and the subject cannot leave the treatment area because the seal will be broken due to large movements.

Note

Foregoing steps were used when culture and Sanger sequencing were performed. If culture-free NGS is used, disinfection described by Widmer, et al should be considered. (J Endod 2018; 44:1132-1139) DOI:10.1016/j.joen.2018.03.009

- 5.4 **Sterility monitoring:** Samples of all sterilized items were monitored for sterility before and throughout the procedure to check maintenance of sterile conditions.
- 5.5 **Electric motor aseptic assembling:** Immediately before tooth dissection, the surgical electric control unit connected to the barrier-covered electric motor received, sequentially and aseptically, a sterile handpiece, sterile attachment, and unused sterilized $\frac{1}{8}$ or $\frac{1}{4}$ round carbide bur, long or short shank (Brasseler).
- 5.6 **Shallow-layer dissection procedure:** Lesion dissection began when the surgical electric control unit, set at 1,000 rpm (2,000 for enamel), was foot activated and the bur applied in intermittent, light-touch slow stroking motions on the lesion or restorative material surface with gentle minimal surface contact, allowing the miniature sharp new blades to dislodge fine fragments in a controlled manner. The dissection was restricted to the lesion core, excluding edges. Dissection of the resin-based composite treated lesions in posterior teeth was started at the central fissure of the restoration and moved in shallow layers to the buccal resin-tooth interface, then moved in shallow layers apically to the pulpal floor of the tooth preparation (the lingual margins remained undisturbed and were covered with sterile resin dam material). Bur use was stopped repeatedly after short intervals and the fragments were collected to maintain visual access, prevent fragment scattering, and prevent heat buildup. Average fragment weight per dissected layer was 6mg (SD 2.65; range 0.6–9.7).

Note

Smaller samples (less weight) were collected as the lesion deepens and proximity to the pulp chamber increases.

Steps outlined below detail preparation required before use of collection brushes and NUNC tubes on dissection day. Attached Excel "Brush and tube worksheet.xlsx" has worksheets to record weights of each brush and tube, calculate the average weight of each brush and tube, and calculate the weight of the sample collected from each layer's dissection. The information from these worksheets is used to generate the lesion diagrams outlined in Step 6 below.

A - BRUSH PREPARATION

Brushes are prepared as follows:

- To remove loose bristles from the micro brushes, thoroughly blow bristles on brush heads using compressed air.
- Cut brush handle to a length that allows about ¼ inch clearance when placed in the collection tube and the cap is securely placed.
- Heat the cut end of the brush handle to seal exposed cavity in brush handle and remove sharp/pointed edges on handle.
- Bend the brush handle about ½ inch back from the bristles to about a 45° angle.
- Wrap brushes in groups of 25 in a double layer of sterilization wrap.
- Autoclave wrapped brushes 20 minutes at 121°C.
- After sterilization, place packs of brushes in anaerobe chamber to "dry" 2+ days.
- We chose to do all of our weighing in an anaerobe chamber to facilitate processing of samples at the time of dissection/collection. Place microbalance system in anaerobe chamber #1 at least 1 day before use to allow system to equilibrate to chamber conditions. In the anaerobe chamber open a sterilized pack of brushes and position brushes on blue wrap under the deionizer output for 1 hour before weighing each brush.
- Weigh each brush on the micro balance a minimum of 2 times. Handling brushes with non-latex covered hands carefully, touching only their handles using forceps to avoid contaminants being deposited onto brushes handles and to avoid static electricity issues. Repeat weights for each brush must be plus/minus 1µg (.000001g). If greater than this, weigh the brush a 3rd time. Record each weight on the "Weight Sheet" (see attached Excel workbook file) line which number correlates with the number on the sterilization pouch the brush will be placed in. This worksheet automatically calculates the average weight of each brush. The average brush weight is used in final calculation of the lesion sample weight collected.
- Carefully place the weighed brush into its numbered sterilization bag.

- Autoclave weighed and bagged brushes 20 minutes at 121°C. This is the second sterilization of each brush.
- Dry brush/bag a minimum of overnight before use.

B - NUNC TUBE PREPARATION

NUNC tubes are prepared as follows (NOTE: non-latex gloves are worn to reduce static electricity issues):

- Label 3 sets of tubes with the letters A to Z, 2 sets using Black ink and the third using Red ink.
- Mark the tube caps thoroughly with Black for one black labeled set of tubes and Red for the red labeled set of tubes. This additional color coding facilitates quick and easy identification of the correct tubes for use for the different purposes. Place both black labeled sets of tubes in anaerobe chamber #2.
Add to each red labeled tube with the red cap 1.5ml sterile Brain Heart Infusion broth. Place tubes in anaerobe chamber #1 for at least 72 hours before proceeding.
- After 48 hours visually check medium in each tube for absolute clarity. If turbid, discard and replace. Prior to using on dissection day, weigh each tube containing sterile BHI broth. Record weights on record sheet. (See Step 6 below.)

- 5.7 **Sample collection:** Dislodged fragments were collected onto filaments of pre-weighed sterilized brushes (Magic-Brush Extra Small and/or Multi-Brush Extra Small, Denbur), dampened in sterile Brain Heart Infusion (BHI) broth immediately before use to facilitate pick-up. Once filled, each brush was deposited immediately and aseptically into a previously letter-coded pre-weighed sterile screw top vial (NuncTM Cryotube Vial, Thermo Fisher Scientific) containing 1.5 ml sterile reduced BHI broth. Consecutive letters were used to identify the dissected layer order. The vial was brought adjacent to the dissection site, uncapped just before entry of each brush, recapped immediately after receiving each brush, and moved away until needed again for loading of the next brush. Each vial was allowed to contain four brushes maximum. Dissection of each new layer received new sterile items including gloves, unused bur, handpiece and attachment, pre-weighed collection brushes, and pre-weighed vial containing BHI medium.

Note

We have not yet found a way to recover the microbes on the used burs because a component of the bur itself kills the microbes during culture. Instead, each bur is discarded after use for dissection of one layer only for two reasons: 1- the blades become chipped and dulled during use and 2- complete cleaning of the bur before sterilization is very difficult and theoretically impossible.

- 5.8 **Collected sample processing:** Each letter-coded dissected layer corresponded to one sample for culture, molecular sequencing and taxa identification, and was processed as follows: Immediately after each vial was loaded, it was transported by runner to

anaerobe chamber #1 and weighed, then to anaerobe chamber #2 where it was vortexed and separated into two aliquots. One aliquot received glycerol to 20% (v/v) and was frozen at -20°C for 10-12 hours before transfer to an ultra-low temperature freezer (-80°C) for archival storage. The aliquot remaining in the NUNC™ tube containing the micro-brushes was retained in anaerobe chamber #2 for serial 1:10 dilution using Phosphate Buffered Saline (PBS, Thermo Fisher Scientific) and plating of 100 µL onto duplicate pre-reduced blood agar plates (Remel Anaerobic (CDC) Blood Agar (RO1036), Thermo Fisher Scientific), then incubated anaerobically (80% N₂, 10% CO₂, 10% H₂) at 37°C until colonies matured (7-21 days). Mature colonies were digitally photographed, lysed, and the DNA extract checked for quantity and quality using the Nanodrop. Samples with adequate DNA (>20ug/ul) of acceptable quality (A260/A280 >1w.8) as sent to a commercial laboratory (Charles River Laboratories, Newark, DE) for Sanger sequencing and taxa identification where molecular identity of the isolates was determined by their proprietary 500 bp 16S rDNA sequencing and analysis methods, AccuGENX-JD, and the identification reference library at Charles River. The DNA was isolated with standard alkaline lysis methods and the extract used in PCR amplification with standard M13-tailed 16S primers, 0005F (TGGAGAGTTTGATCCTGGCTCAG) and 0527R (GTATTACCGCGGCTGCTGGCAC) with the primer numbering based on the *Escherichia coli* 16S rRNA gene.¹³ Sequencing reactions were carried out using manufacturer's recommendations and BigDye™ Terminator v1.1 cycle sequencing kits (Thermo Fisher Scientific, Carlsbad, CA) and run on the ABI 3130XL or ABI 3500XL Genetic Analyzer capillary sequencer (Thermo Fisher Scientific, Carlsbad, CA). Data meeting a Phred score of ≥20 on the internal developed sequencing assessment software were assembled and analyzed by a semi-automated reference method and sequences compared to the Accugenix proprietary, validated, reference database for an identification. The 16S rRNA gene sequences of the samples were aligned together with other closely related reference species and analyzed phylogenetically using proprietary software. Evolutionary distance was calculated on the basis of the Jukes and Cantor¹⁴ model and phylogenetic trees were inferred on the basis of the neighbor-joining¹⁵ models. Identifications were made upon consideration of the sequence alignment and the neighbor-joining tree for each isolate.

- 5.9 **Photographic documentation:** When tooth fragment collection was completed for a layer, the dissection site was prepared for digital photographing by dampening a new sterilized Multi-Brush or Magic-Brush with sterile BHI broth used to moisten the dissected area to accentuate surface color and detail in close-up images produced with the hand-held SLR digital camera. The brush used for moistening was discarded. In preparation for dissection of the next layer, all used sterilized items were replaced (see step 7 above for listing of items).
- 5.10 **Dissection termination:** The above steps were repeated until two clinicians agreed on the dissection termination point based on tissue hardness, appearance and/or pulp chamber proximity.

5.11 **Post-dissection site restoration:** After dissection completion, the subject was returned to the adjacent dental clinic to receive a restoration placed by the clinic's dentist to restore the tooth's form, function and esthetics.

6

Layered lesion diagram creation



Master - Map Generation Workboo... 79.6KB

The last step in the method was creation of the layered lesion diagram to communicate taxa identification results, visually and numerically, displaying the taxa identified in each consecutive layer dissected.

When identification reports were received back from the sequencing lab, lesion diagrams were generated to display the microbial growth by layer. Attached workbook contains example worksheets we used to generate the information used to build each lesion diagram.

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