Journal of Microbiological Methods 118 (2015) 85–92

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

In vitro method for prediction of plaque reduction by dentifrice

Bruce Tepper [a,*,1], Brian Howard [b,*], Daniel Schnell [c], Lisa Mills [a], Jian Xu [d]

[a] Microbiology Capability Organization, The Procter & Gamble Company, 8700 Mason-Montgomery Road, Mason, OH 45040, USA
[b] Life Sciences Innovation, The Procter & Gamble Company, 8700 Mason-Montgomery Road, Mason, OH 45040, USA
[c] Statistics and Data Management, The Procter & Gamble Company, 8700 Mason-Montgomery Road, Mason, OH 45040, USA
[d] Single-Cell Center and Shandong Key Laboratory of Energy Genetics, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, Shandong 266101, China

ARTICLE INFO

Article history:
Received 14 May 2015
Received in revised form 26 June 2015
Accepted 27 June 2015
Available online 4 July 2015

Keywords:
Oral biofilm
Biofilm cleaning
454 pyrosequencing

ABSTRACT

An in vitro Particle Based Biofilm (PBB) model was developed to enable high throughput screening tests to predict clinical plaque reduction. Multi-species oral biofilms were cultured from pooled stimulated human saliva on continuously-colliding hydroxyapatite particles. After three days PBBs were saline washed prior to use in screening tests. Testing involved dosing PBBs for 1 min followed by neutralization of test materials and rinsing. PBBs were then assayed for intact biofilm activity measured as ATP. The ranking of commercial dentifrices from most to least reduction of intact biofilm activity was Crest ProHealth Clinical Gum Protection, Crest ProHealth, Colgate Total and Crest Cavity Protection. We demonstrated five advantages of the PBB model: 1) the ATP metric had a linear response over ≥1000-fold dynamic range, 2) potential interference with the ATP assay by treatments was easily eliminated by rinsing PBBs with saline, 3) discriminating power was statistically excellent between all treatment comparisons with the negative controls, 4) screening test results were reproducible across four tests, and 5) the screening test produced the same rank order for dentifrices as clinical studies that measured plaque reduction. In addition, 454 pyrosequencing of the PBBs indicated an oral microbial consortium was present. The most prevalent genera were Neisseria, Rothia, Streptococcus, Porphyromonas, Prevotella, Actinomyces, Fusobacterium, Veillonella and Haemophilus. We conclude these in vitro methods offer an efficient, effective and relevant screening tool for reduction of intact biofilm activity by dentifrices. Moreover, dentifrice rankings by the in vitro test method are expected to predict clinical results for plaque reduction.

1. Introduction

Caries and gingivitis continue to be prevalent oral health issues globally for children and adults in developed and developing countries (WHO, 2012, 2014). The best means to prevent and control caries and gingivitis is generally recognized as removal and control of bacterial plaque by daily brushing (Loe, 2000). Demonstrating new dentifrices have improved plaque reduction requires clinical studies. Predicting which new dentifrice formulations should progress to clinical studies has become problematic because in vitro methods to evaluate oral biofilm cleaning efficacy have not kept pace with dentifrice improvements.

The challenge was to find a simple rapid method to culture adequate amounts of oral biofilm that resisted cleaning and could be used for weekly high throughput screening. The biofilms would have to be representative of dental plaque which resists complete physical and chemical cleaning during routine oral hygiene. We reasoned that, just as routine brushing of teeth selects for residual plaque that must be removed by dental prophylaxis, high shear during biofilm culturing would be needed to select for similar biofilm with a combination of high adhesion to physical substrate and high cohesion within the biofilm. Ideally a test method using such biofilms to discriminate cleaning efficacy of new dentifrice formulations would predict their clinical reduction of plaque.

Existing in vitro biofilm culturing and testing models did not meet our needs (see reviews: Sissons, 1997; Coenye and Nelis, 2010; Lebeaux et al., 2013; Salli and Ouwehand, 2015). Batch microtiter plate biofilm models such as Calgary Biofilm Device and Biofilm Ring Test are simple and capable of high throughput screening at relatively low cost, but the biofilms do not resist realistic in vitro cleaning due to minimal shear force during culturing. Lower throughput flow displacement models that culture with some shear such as the Centers for Disease Control Reactor, Modified Robbins Device, flow cells of various types and sizes, and the Constant Depth Film Fermentor along with other rotating disc reactors produce biofilms with limited resistance to cleaning and are not amenable to high throughput testing. Bench-scale chemostats modified to continuously culture PBBs under high shear have appeal. Like their industrial-scale counterparts they can be adapted to study kinetics, mass-transfer and biodegradation (Nicolella et al., 2000). Chemostats, however, are challenging to maintain,

* Corresponding authors.
E-mail addresses: brucetepper@yahoo.com (B. Tepper), howard.bw@pg.com (B. Howard).
[1] Retired.

http://dx.doi.org/10.1016/j.mimet.2015.06.017
0167-7012/

expensive to purchase and operate, and require expertise to produce consistent biofilms over time.

We chose to pursue a combination of PBBs, batch culturing and 96-well plate testing. We reasoned that biofilms that resist cleaning can be created by culturing biofilms on particles with various levels of non-laminar or discontinuous mixing to create shear force via high-frequency particle collisions. Using batch systems requires the least effort and expense while offering flexibility of culturing conditions. Once produced, PBBs can be distributed into 96-well plates and quickly separated from liquids by settling for various procedures and high throughput testing. In addition, shear conditions via particle collisions can be created during a test such that chemical and/or physical cleaning can be evaluated. Moreover, multiple endpoints can be measured in the same test on the same treatment replicate because each replicate treatment well contains hundreds of PBBs that can be parsed across multiple assay plates. In addition, fundamental research involving kinetics and biofilm community responses to stress would be possible.

One key issue to resolve was which particles to use. Oral PBBs were used in the 1970s to evaluate anti-plaque agents (Sudo et al., 1976), effects of culture conditions on oral biofilm species composition (Sudo, 1977), and adhesion and aggregation of oral bacteria to saliva-coated and uncoated surfaces (Clark et al., 1978; Wheeler et al., 1979). These early PBBs were cultured on either glass beads or glass beads coated with hydroxyapatite to improve colonization. Bacterial cells were recovered from these biofilms for viable cell counts to compare treatment effects. This was possible primarily because these biofilms had only modest adhesion and cohesion. Our initial trials with different sizes of glass beads with high shear culturing conditions demonstrated the expected; biofilm formation was minimal. Moreover, any hydroxyapatite coating would likely shear off the glass. The solution was to use hydroxyapatite particles of an appropriate size range as a way to manage shear forces due to collisions.

The other key issue to resolve was an appropriate rapid and applicable measure of treatment effects. Biofilms with high adhesion to substrate and cohesion within the biofilm resist cleaning and, therefore, would not easily release bacterial cells for viable cell counts. In addition, a more rapid and less expensive endpoint than cell counts was desired. Adenosine triphosphate (ATP) was chosen as the primary metric for cleaning efficacy. In our new model treated PBBs are compared for reduction of intact biofilm activity after treatment. Less ATP associated with treated intact biofilm than a negative control represents a combination of cell death, inhibition and/or dispersion. To distinguish among these causal factors requires additional metrics.

After developing our PBB culturing methods and a manual dentifrice cleaning test we migrated the test method to a robotic fluid handler. This reports on a single-lab validation of the automated method to demonstrate test results are sufficiently precise to distinguish treatment effects over a wide dynamic range, robust to potential interferences, repeatable over time and relevant to clinical plaque reduction by dentifrices.

2. Materials & methods

2.1. Saliva

For each test, first morning stimulated human saliva (prior to oral hygiene) was self-collected daily by five to seven donors for three days. Donors were 21 to 60 years old in good health with no prophylaxis or treatment for any oral disease within 30 days. Other donor criteria for at least 48 h prior to and during collection included: no use of mouthrinse, floss, toothpicks or dentifrice other than Crest Cavity Protection; no fever >38 C, communicable disease or oral infection; no use of tobacco products, oral antibiotics or steroids; no use of antihistamines, decongestants or other cold/flu/allergy medicines; and females could not be pregnant or lactating. All donors were required to brush their teeth unsupervised twice daily during a minimum two-day washout period and during the collection period using supplied Crest Cavity Protection dentifrice containing 0.243% sodium fluoride and a standard manual toothbrush. Donors chewed on supplied sterile pieces of paraffin with beeswax or polypropylene tubing each morning to collect 25 to 30 mL saliva in 100 mL sterile wide-mouth screw-cap containers. After collection the containers were sealed and chilled for transport to the lab. Daily for three days equal volumes of saliva, 20 to 25 mL, from each donor were pooled in a sterile 500 mL Erlenmeyer flask with 10 to 12 sterile borosilicate glass beads, 5 to 6 mm diameter (CG-1101-04, Chemglass Life Sciences, Vineland, NJ, USA). Pooled saliva was sheared by vortex at ca. 100 rpm for 60 to 120 s. Sheared pooled saliva sans settled solids was diluted 50% with sterile 0.9% saline (NDC0338-0048-04, Baxter Healthcare Corp., Deerfield, IL, USA) to form sheared pooled saliva diluted (SPSD). SPSD was amended only on the first culturing day to ca. 1% sucrose using a filter-sterilized 10% sucrose stock (OmniPur® Sucrose, EMD Chemicals Inc., Gibbstown, NJ, USA).

2.2. Biofilms

For each test PBBs were cultured in eight 50 mL centrifuge tubes, each tube containing 725 to 775 mg UV-sterilized hydroxyapatite powder (HAP), 53 to 124 μm mean diameter (Clarkson Chromatography Products Inc., South Williamsport, PA, USA). Culture tubes containing HAP plus 20 mL SPSD with sucrose were sealed with caps, transferred to a 31 to 33 C aerobic incubator, laid on their sides on a rocking platform (Model 200, VWR Scientific Products, Radnor, PA, USA) and held in place with 15 cm bungee cords. Rocking speed was set between 7 and 8 to keep particles continually moving and colliding. After ca. 22 and 46 h culture tubes were removed from incubation and stood upright for at least 1 min to settle PBBs to tube bottoms. From each tube 18.5 to 19 mL supernatant was aspirated and replaced with 20 mL fresh SPSD before continuing incubation. After 68 to 72 h culture tubes were removed from incubation and supernatant was again removed. PBBs were rinsed twice, each time with 20 mL sterile saline, including inverting tubes ca. 10 times to effect thorough mixing. The bulk of PBBs settled to tube bottoms after 60 s leaving only the smallest particles suspended to cloud rinse supernatants. Suspended PBBs and planktonic bacteria were removed in rinse supernatants and discarded. Little to no cloudiness was observed in 10 mL sterile saline added to each tube to enable transfer of PBBs into a reservoir. Note that when the test method (2.4) is performed manually PBBs are transferred into a 50 mL disposable pipetting reservoir. When the method is performed on an epMotion 5075 automated pipetting system (Eppendorf, Hamburg, Germany) PBBs are transferred into a 100 mL epMotion reservoir.

2.3. Treatments

Sterile 0.9% saline was the negative control and used to prepare all treatments. Sodium lauryl sulfate (Stepanol, Stepan Co., Winder, GA, USA) at 0.1% in saline was the positive control, which approximated SLS present in 1:10 diluted dentifrice treatments. Crest ProHealth Clinical Gum Protection, Crest ProHealth, Crest Cavity Protection and Colgate Total Clean Mint dentifrices were purchased from a supermarket in Mason, OH, USA. Dentifrice slurries were prepared within 30 min of use by diluting ca. 2 g dentifrice with saline 10× (weight to volume) and homogenizing for 10 to 12 s using an alcohol-sterilized immersible BioHomogenizer (Model M133/1281-0, Biospec Products, Inc., Bartlesville, OK, USA). Supernatant (14 mL) of each diluted dentifrice treatment was transferred into its own well in a 12-deepwell reservoir (82007-294, VWR). Treatments were assigned the same plate columns in 96-well plates for each test. Since plate location bias can occur in 96-well plate assays, replicated treatments were used to check for bias across the plate, as follows: Saline #1 and #2 were in columns 1 and 12, respectively; SLS #1 and #2 in 2 and 11; Cavity Protection #1 and #2 in 3 and 10; and Colgate Total #1 and #2 in 6 and 9. Plate bias would be indicated