Journal of Microbiological Methods 144 (2018) 91–98

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

VMG II transport medium stabilises oral microbiome samples for Next-Generation Sequencing

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ARTICLE INFO

Keywords:
Oral microbiome
Storage
Next-Generation Sequencing
VMG II

ABSTRACT

Next-Generation Sequencing is providing insights into the critical role of the oral microbiome in dental diseases. Application of this method can require the collection of dental plaque from large cohorts in field-type conditions, which necessitates a transport medium to preserve the microbiome composition. We evaluated the use of two transport media, VMG II and RNAprotect® Bacteria Reagent (Qiagen), for room temperature storage of dental plaque. VMG II has not previously been assessed for suitability to store microbiome samples intended for deep sequencing. We compared the microbiome composition of dental plaque (total n = 23) stored in either VMG II or RNAprotect Bacteria at room temperature with immediately-frozen plaque. 454 sequencing of 16S gene amplicons was used to assess the plaque microbial composition. While the bacterial diversity recovered was similar between storage conditions ($p > 0.1$), the abundance of bacteria was influenced by storage environment. Dental plaque stored in VMG II was most similar to immediately-frozen material, with only one of the 324 bacterial species being differentially abundant (*Neisseria*, $p < 0.001$). In comparison, dental plaque stored in RNAprotect Bacteria had 24 differentially abundant species compared with the immediately-frozen samples and a significantly different phylogenetic structure ($p < 0.01$). We have identified VMG II as a new transport medium for room temperature storage of dental plaque samples being subject to Next-Generation Sequencing that stabilises oral microbial DNA makeup.

1. Introduction

Understanding of the role of the oral microbiome in health and disease is being transformed by Next-Generation Sequencing (NGS). Initial deep sequencing studies of the oral microbiome present in health revealed for the first time the huge diversity of bacteria in the oral environment (Keijser et al., 2008), with up to 300 bacterial species found within an individual (Zaura et al., 2009). NGS studies examining the microbiome in common oral diseases caused by bacteria, such as caries (Belda-Ferre et al., 2012; Yang et al., 2012) and periodontal disease (Griffen et al., 2012), have revealed the highly polymicrobial nature of these conditions. Furthermore, NGS projects are starting to highlight the relationship between the oral microbiome and other body sites. For example, the composition of the oral microbiome has been found to be a predictor for gut microbiome makeup (Ding and Schloss, 2014). Aside from providing a greater understanding of the oral microbiome in health and disease, NGS is resulting in the production of a gene catalogue of the oral microbial community. This gene catalogue has the potential to direct the discovery of new therapeutics to tackle the huge problems of dental caries and periodontal disease.

NGS of the oral microbiome is moving from smaller case-based (Zaura et al., 2009) to larger cohort studies (Gomez et al., 2017; Human Microbiome Project, 2012) that can require remote sampling in field-type conditions. Cohort studies offer a population-level perspective, as demonstrated by the Human Microbiome Project, which has revealed how ethnic backgrounds have a strong influence on microbiome composition in health (Human Microbiome Project, 2012). However, large population studies can require sampling in participant's homes and in remotely-located communities. In these situations, the ideal treatment of microbiome samples for NGS, such as immediate DNA extraction or freezing (Goodrich et al., 2014), may be impossible. Hence, studies attempting to attain a population-wide understanding of the oral microbiome in health and disease may face sampling challenges, primarily associated with the need to transport microbiome material at room

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https://doi.org/10.1016/j.mimet.2017.11.012
Received 11 October 2017; Received in revised form 15 November 2017; Accepted 15 November 2017
Available online 22 November 2017
0167-7012/

temperature (RT).

Sample transport at RT requires storage conditions that preserve the original microbiome composition of samples, such as dental plaque. Extended transport of microbiome material neat (i.e. in no preserving agent) at RT has been found to alter the bacterial composition of samples when assessed by NGS (Cardona et al., 2012). Assessment of RT storage methods for microbiome analysis has primarily been undertaken on gut and not oral samples. RT storage methods tested on gut microbiome have included placing fecal samples on filtered paper that preserves nucleic acids, such as Flinders Technical Associates (FTA®) cards, and immersion of stool samples in ethanol, Tris-EDTA, OMNIgene.GUT (DNA GenoteK) and RNAlater® (Qiagen) (Choo et al., 2015; Dominianni et al., 2014; Hale et al., 2015; Nechvatal et al., 2008). The relevance of these findings on stool samples for oral biofilm samples is limited, due to the varied composition and physical properties of the two sample types. For oral microbiome samples, Aware Messenger™ (Calypte Life Sciences/Calypte Biomedical Corporation) fluid has been assessed for its ability to preserve microbial DNA in saliva at RT (Contreras et al., 2010). Hence, at present, there are few studies examining the storage conditions effects on oral biofilm samples. In addition, the majority of the above detailed RT storage methods examined on gut or oral samples are liquids and hence pose an issue for unsupervised sampling in the home or if the samples need to be posted. We are currently unaware of any non-liquid, RT storage method for dental plaque that has been assessed using NGS and found to accurately preserve microbial composition.

Our study assessed the use of RT storage media as an alternative to immediate freezing for oral microbial DNA preservation. Specifically, we investigated the use of semi-solid, non-liquid storage media that would be spill-proof. Whilst multiple DNA preservatives are available, the two we investigated for RT preservation of oral microbial DNA were the viability preserving microbiostatic medium (VMG II) because it is designed for oral samples, and RNAprotect® Bacteria Reagent (Qiagen) as RNA preservatives have been tested multiple times on gut microbiome samples (Choo et al., 2015; Dominianni et al., 2014; Hale et al., 2015; Nechvatal et al., 2008), and used in a large oral microbiome cohort study (Gomez et al., 2017), but not as yet tested for preservation of oral biofilm samples. VMG II was developed as a transport fluid to enable the storage of oral microbes at RT for up to 14 days (Moller, 1966, Syed and Loesche, 1972). Whilst originally intended for culturing, plaque samples stored in VMG II have been used for DNA isolation of periodontal pathogens (Doan et al., 2000). VMG II contains agar, making it semi-solid and a salt solution, which may provide the storage media with DNA preserving qualities (Caputo et al., 2011). However, it has yet to be examined if VMG II is a suitable storage media for dental plaque samples intended for NGS. RNAprotect Bacteria Reagent (RNAprotect Bacteria) enables stabilisation of RNA from both gram-positive and gram-negative bacteria at RT. RNAprotect Bacteria and other types of RNA preservatives contain tetradecyltrimethylammonium bromide, a cationic surfactant (detergent), which permeates cells and has been found to preserve bacterial DNA from mixed microbial community samples (Choo et al., 2015; Gray et al., 2013; Nechvatal et al., 2008). The mechanism of nucleic acid preservation is thought to be via hydrophobic interaction of this detergent with the DNA backbone (Cheng and Ran, 2014). RNAprotect Bacteria can be made into a semi-solid gel with the addition of agarose at 0.5%, although it is unknown what impact this may have on the ability of RNAprotect Bacteria to preserve nucleic acids.

To the best of our knowledge, the suitability of either VMG II or RNAprotect Bacteria with agar/agarose for RT stabilisation of DNA from oral microbiome samples has yet to be assessed using NGS. We used 454 sequencing of the 16S rRNA gene to compare the recovery of bacterial DNA from dental plaque frozen immediately (− 20 °C) with plaque stored at RT for two weeks in either VMG II with 0.5% agar or RNAprotect Bacteria with 0.5% agarose. Of the two RT storage media assessed, we found that only VMG II accurately stabilised the microbial composition of dental plaque when compared with immediately-frozen plaque. Our results provide a new method for RT storage of dental plaque samples intended for microbiome analysis.

2. Materials and methods

2.1. Population

We assessed the effect of storage conditions on dental plaque samples taken from eight Australian children. Written informed consent was obtained from the parents of all participants. Ethical approval for the study was obtained from the University of Adelaide HREC (H-78-2003). All methods in this study were performed in accordance with the guidelines and regulations stipulated by this ethical approval. Study participants were between the ages of 5.9 and 6.3 years. Among the study participants, we had a ratio of 3:1, males to females. Oral health examinations performed when sampling revealed the majority of study participants had good oral health, with the minority showing current or previous signs of caries. The oral and general health status of each study participant is detailed in Supplementary Table S1.

2.2. Dental plaque sampling and storage

Triplicate supra-gingival plaque samples were obtained from each individual at the University of Adelaide, Adelaide Dental School. Study participants were asked to refrain from brushing their teeth from 7 pm the evening before their appointment until after their clinical visit. Study participants were also asked not to eat or drink in the half an hour before the sample collection. All plaque samples were taken during the clinical visit by a registered dentist. Three sterile swabs (Tampone Swab, cultiplast®) were used to take the dental plaque samples. The three swabs were held together at the base, resulting in the cotton tips of the swabs to be pressed together in a line that were passed over the teeth and gums in unison for 1 min. Whilst this method would have resulted in some variability in the plaque picked up on each of the three swabs, this would have been random between individuals with respect to storage condition allocation of the swab.

Each of the triplicate swab samples taken per individual was stored under a different condition:

1. Sample 1/Swab 1 was frozen immediately at − 20 °C neat.
2. Sample 2/Swab 2 was stored in VMG II at RT for two weeks and then frozen at − 20 °C.
3. Sample 3/Swab 3 was stored in RNAprotect Bacteria at RT for two weeks and then frozen at − 20 °C.

An alternative method to using three swabs to collect the whole mouth plaque sample would be to take one swab, dispense the plaque into a liquid and aliquot the liquid to the different storage conditions. However, this approach was incompatible with the storage media tested. The aliquoted liquid would have been absorbed into the media during the storage time period. For DNA extraction, this would require the total volume of storage media to be extracted, which would have unknown effects on the DNA extraction.

The VMG II media was made up according to Moller (1966), and the protocol is detailed in the Supplementary Method. The RNAprotect Bacteria (Qiagen) was used in accordance with the manufacturer's protocol, with the addition of agarose (0.5%). The two methods while established as storage media for culture and Sanger sequencing, have yet to be examined for their suitability to preserve oral microbial DNA for NGS.

2.3. Genetic analysis

Genetic analysis of the dental plaque samples (n = 24) included DNA extraction, amplification of the 16S gene and pyrosequencing of