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Effect of preparation method and storage period on the stability of saliva DNA

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ABSTRACT

Saliva is an attractive source for oral microbial detection and quantification since sampling is non-invasive and rapid.

Objectives: To determine whether different saliva preparation methods or preservation time periods affect DNA stability.

Methods: Saliva samples from 4 healthy adult volunteers were processed to obtain 3 different preparations: whole saliva, and after centrifugation pellet and supernatant. Purified DNA (MasterPure™) from each sample was divided into 4 aliquots, one for immediate analysis and 3 (stored at −80 °C) for later analyses after 1 week and 2 and 6 months. DNA concentrations and qPCR based quantities of *Porphyromonas gingivalis, Prevotella intermedia, Parvimonas micra, Fusobacterium nucleatum, Filifactor alocis* and *Streptococcus mutans* were determined.

Results: DNA concentration did not decrease ($P > 0.05$) during the 6-month period in any sample. Mean (SE) DNA concentrations (ng/μl) in whole saliva were 152.2 (51.2) and 147.8 (50) at day 0 and 6 months, respectively. Similarly, the values for pellet were 134.9 (42.5) and 133.6 (42.9), and for supernatant, 11 (1.9) and 8.9 (2.3), the difference being significant ($P < 0.001$) between supernatant and whole saliva or pellet. The quantities of most bacterial species found at day 0 remained stable over the 6-month period in all saliva preparations. In supernatant, species quantities were lower ($P < 0.05$) than in whole saliva or pellet.

Conclusions: DNA concentrations were comparable between whole saliva and pellet, suggesting that either of them can be used for DNA-based analyses. Our results also demonstrated that DNA extracted from saliva can be preserved at −80 °C for at least 6 months without decrease in DNA concentration.

1. Introduction

Saliva contains a variety of indigenous bacteria released from biofilms growing on soft and hard oral surfaces. The procedure of sampling whole saliva is noninvasive and rapid and does not require specialist skills or equipment. The easy access has led to a widespread research effort on exploring possibilities of using saliva for various diagnostic purposes including detection and quantification of salivary bacteria (Sakamoto, Takeuchi, Umeda, Ishikawa, & Benno, 2001; von Troil-Linden, Torkko, Alaluusua, Jousimies-Somer, & Asikainen, 1995; Zhou et al., 2015). Both culture and DNA-based methods have revealed presence of periodontitis- and caries-associated bacteria in saliva (Asikainen, Alaluusua, & Saxen, 1991; Darout, Albandar, Skaug, & Ali, 2002; Kononen, Jousimies-Somer, & Asikainen, 1994; Umeda, Contreras, Chen, Bakker, & Slots, 1998). In addition, salivary levels of these species have been linked to increased risk of disease severity (Slots & Slots, 2011; Umeda et al., 1998; von Troil-Linden et al., 1995). Little is known of the prognostic value of the presence or quantity of these bacteria in saliva.

DNA-based methods have gained popularity among researchers due to their straight-forward laboratory protocols that are simpler, faster and more discriminative than culture for bacterial identification. Preparation of the saliva sample for DNA extraction may affect the DNA yield and detection of target molecules. Whole saliva or, after centrifugation, the pellet have been used for DNA extraction (Durdiakova, Kamodyova, Ostatnikova, Vlkova, & Celec, 2012; Ng, Koh, Choo, Ng, & Fu, 2004) more often than the respective supernatant (Gu et al., 2009; Li, Zhou, St John, & Wong, 2004; Vandewoestyne, Van Hoofstat, Franssen, Van Nieuwerburgh, & Deforce, 2013). Since free DNA is released from living (Draghi & Turner, 2006) and lysed bacteria into the extracellular milieu, saliva supernatant may also serve as material for the analysis of oral bacteria by DNA-based techniques. No

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comparisons of DNA yield from various salivary biolayers were found.

In clinical studies, the extracted DNA samples are commonly preserved frozen until the intended study material is complete and can concurrently be analyzed. The preservation time obviously depends on the study design, but short-term preservation for a couple of months seems reasonable for many current studies. However, possible DNA loss during preservation period may bring a source of error to the results. In this study, we wanted to know firstly, whether different preparations of saliva samples i.e., whole saliva as such and, after centrifugation, the pellet and the supernatant give different DNA yields and secondly, whether preservation time affects the quantity of DNA samples or that of select oral bacteria.

2. Methods

2.1. Reference bacteria and culture conditions

Reference strains *Filifactor alocis* CCUG 47790, *Porphyromonas gingivalis* ATCC 33277 *Parvimonas micra* CCUG 46357, *Prevotella intermedia* ATCC 25611, and *Fusobacterium nucleatum* ssp. *polymorphum* NCTC 10562 were grown on brucella blood agar plates for 3 days. All strains were incubated in anaerobic conditions (10% H_2, 5% CO_2, 85% N_2) at 37 °C using Anoxomat™ Mark II anaerobic gas filling system (Mart Microbiology, The Netherlands). *Streptococcus mutans* CCUG 11877 was also cultured on brucella blood agar plates, but it was incubated at 37 °C in 5% CO_2 in air for 2 days.

2.2. Saliva samples

A schematic illustration of the study design is presented in Fig. 1. Four healthy volunteers (age from 34 to 41 years) were chosen for this study since all target species could be found in their saliva samples in a prior pilot study. Paraffin-stimulated whole saliva samples (each 4 ml) were collected into sterile 15-ml tubes (Falcon, BD). Each subject's saliva sample was divided into 3 equal 1.3-ml aliquots, which were transferred into 3 sterile microcentrifuge tubes (Eppendorf). One tube with whole saliva was left on ice until the 2 remaining tubes with whole saliva were centrifuged at 14000 × *g* (4 °C, 15 min). After centrifugation, salivary pellet in one tube and supernatant in the other tube were separated. Thus, for each subject 1.3-ml aliquots of whole saliva and supernatant, and the separated pellet were subjected to DNA purification and subsequent preservation at −80 °C.

2.3. DNA purification

Total DNA from the above saliva samples was purified using MasterPure™ DNA purification kit according to manufacturer's instructions (Epicenter Biotechnologies). Briefly, 1.3 ml of whole saliva or supernatant was mixed with equal volume of 2 × Tissue and Cell lysis solution containing Proteinase K. The saliva pellet was resuspended in 2.6 ml of the above lysis solution and mixed thoroughly. The mixture was incubated at 65 °C for 15 min vortexing every 5 min. After placing the lysed samples on ice for 5 min, 175 µl of protein precipitation reagent was added and vortexed vigorously. Followed by centrifugation at 10,000 × *g* (4 °C, 10 min), supernatant was transferred to a clean sterile tube and 400 µl isopropanol added. The tube was inverted 30–40 times and DNA was pelleted by centrifuging as above. After carefully pouring off isopropanol the DNA pellet was rinsed twice with 70% ethanol. Residual ethanol was completely removed and the pellet air-dried before resuspending in 150 µl TE buffer. The purified DNA from each of whole saliva, pellet, and supernatant samples was aliquoted into 4 parts to test DNA stability upon preservation. Aliquots for 1 week, and 2 and 6 months were preserved at −80 °C, while those for 0 day were immediately subjected to DNA concentration measurement using NanoDrop™ presented below and bacterial quantification by qPCR as described below. Genomic DNA from reference bacterial strains was purified as described earlier (Karched, Bhardwaj, Inbamani, & Asikainen, 2015).

2.4. Measurement of DNA concentration

DNA concentration was measured by UV spectrometry method using NanoDrop™ 1000. Purity of the eluted DNA was assessed by A260/280 ratio. Duplicate readings were taken for each sample.

2.5. qPCR

For qPCR quantification of the 6 oral species, previously validated species-specific primers (Table 1) were chosen and their specificity revalidated using *in silico* analysis tools as described (Karched et al., 2015). The reactions were performed on ABI 7500 Fast Real-Time PCR machine. The reaction mixture was as follows: 12.5 µl SYBR Green master mix (Power SYBR Green® Kit, Applied Biosystems), 1 µl each of forward and reverse primer (0.2 µM), 5.5 µl H_2O and 5 µl DNA template. Thermal cycling included a 10-min initial denaturation at 95 °C followed by 40 cycles of 95 °C for 15 s, 50–63 °C (depending on the primer pair) for 30 s and 72 °C for 30 s. Data from fluorescent signal was acquired at the elongation step. Data was analyzed using the software SDS v2.0.6. Serial dilutions of DNA from the above species were used in the reaction and the Ct values were plotted against deduced bacterial cell concentration (cells/ml) for each species to construct standard curves using the above software. Prior to qPCR experiments, bacterial cell concentration at OD_{600} = 1 was determined as follows: Each species was grown on respective culture media and conditions as above. The colonies were harvested from agar plates, suspended in 1 ml sterile PBS, washed by centrifugation (5000 × *g*, 5 min) and adjusted to OD_{600} = 1 and a 10-fold dilution was prepared for cell counting. Cell numbers per ml as enumerated by using Neubauer counting chamber (Celeromics, Cambridge, UK) were as follows: *P. gingivalis* 8×10^7, *P. intermedia* 2.5×10^8, *F. nucleatum* 1×10^8, *P. micra* 1.2×10^8, *F. alocis* 1.7×10^8, *S. mutans* 2.7×10^8.

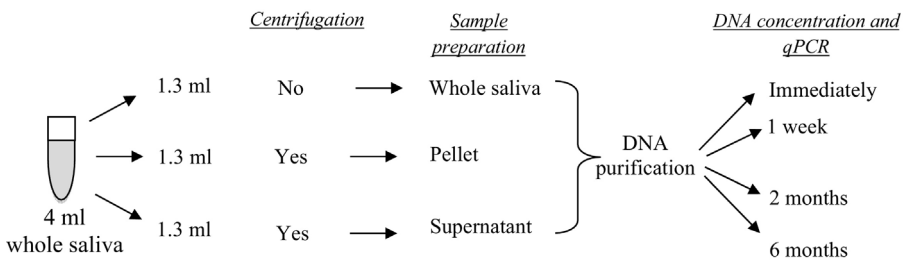

Fig. 1. A schematic illustration of the study design. Paraffin-stimulated whole saliva samples from 4 healthy volunteers were divided into 3 equal 1.3-ml aliquots. After sample preparation with or without centrifugation, the samples were subjected to DNA purification. Purified DNA from each sample preparation was either immediately analyzed or preserved at −80 °C for different periods before analyses.