Effect of Saliva Storage Conditions on Bacterial DNA Quantification by Real-time Polymerase Chain Reaction

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Abstract

Objective: This research aimed to study the effects of temperature and duration of saliva samples storage on quantification of bacterial DNA by Real-time polymerase chain reaction (Real-time PCR)

Materials and Methods: Human saliva samples were collected from 3 healthy volunteers, aliquoted into microcentrifuge tubes and stored in the following conditions; centrifuged immediately before storing at -80^{o}C, kept at room temperature, on ice, or dry ice for 6 hours, 24 hours or 120 hours. Genomic DNA was extracted for quantitative analysis of total bacteria by Real-time PCR.

Results: Absolute quantitative real-time PCR showed that concentrations of bacterial DNA from saliva samples stored at almost every condition which were stored at room temperature for 120 hours, on ice for every duration, or on dry ice for 6 or 24 hours were similar to that of the control, saliva centrifuged and frozen immediately after collection. However, bacterial DNA concentration from saliva stored at room temperature for 6 or 24 hours was significantly higher than that of the control. In contrast, the concentration of DNA from saliva stored on dry ice for 120 hours was significantly lower than that of the control.

Conclusion: Temperature and duration had effects on bacterial DNA quantity. Saliva storage at room temperature within 24 hours had increasing bacterial DNA as quantify by Real-time PCR. Saliva storage on ice and dry ice could maintain bacterial DNA similar to sample centrifugation immediately and then frozen at -80^{o}C. But long duration of storage, 120 hours, would decrease bacterial quantity.

Keywords: saliva sample storage, bacterial DNA, Real-time Polymerase Chain Reaction

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Introduction

Salivary bacterial DNA has shown potential in many applications. It can be used to identify saliva stain on subject for forensic investigation (1). It has potential to be diagnosis tools for many diseases (2). For examples, in pediatric ciliac disease, there was significant increase in the Bacteroidetes phylum, while the Actinobacteria phylum, *Rothia* genus and *R.aeria* showed a significant decrease (3). In oral squamous cell carcinoma (OSCC), *Actinobacteria, Fusobacterium, Moraxella, Bacillus,* and *Veillonella* species exhibited strong correlations with OSCC (4). Different in microbial DNA profiles provided information for further development of diagnosis tools.

In most studies, salivary DNA was extracted immediately (1) or froze for a period of time before extraction (4,5). But in research studies required to collect saliva specimens in the fields or remote areas, the specimen storage in the freezer may not be available.

There are not many studies of effect of temperature and time used for saliva storage on DNA analysis and the data showed some controversial results. In human gene genotype analysis study, storage of saliva at room temperature up to 5 days before DNA extraction and genotypes analysis had no effect on the analysis (6).

In the study of impact of temperature and time storage on the microbial detection of saliva mixed with supragingival biofilm samples by Checkerboard DNA-DNA hybridization method, total bacterial count of sample stored at -20^{o}C for 2 weeks was higher than that of the sample processed immediately (7). While the samples kept at room temperature or 4^{o}C or -80^{o}C show similar count to the control. The author showed that storage for 12 months reduced the bacterial counts detected by this method.

Storage of saliva samples at -20^{o}C for 24 hours or 36 months before DNA extraction, followed by quantitative real-time PCR analysis showed no significant different of bacterial DNA quantity when the DNA was extracted using QIAamp DNA Blood Mini Kit (8). However, when using phenol-chloroform extraction, DNA quantity of the 36 months sample was lower.

In bacterial DNA semi-quantitation by conventional PCR, saliva sample that was pelleted and kept for 1 week at -70^{o}C before DNA extraction provided glutamine-fructose-6-phosphate transaminase 1 (GFPT1) PCR band quantity more than that of sample that was stored at 4^{o}C or -70^{o}C for 1 week before pellet formation and DNA extraction (9).

Freezing of saliva samples maintain the sample integrity, but in some studies, such as in remote areas, freezer may not be available. Storage of saliva samples on ice and dry ice, materials provide low temperature that are easy to obtain from markets, have not been studied. We designed to examine the effect on bacterial DNA quantity of saliva samples storage at room temperature or on ice or on dry ice. Time of storage was 6 hours or 24 hours or 120 hours (5 days) which is the time used for delivery of samples to laboratory in different situations. Microbial DNA quantities in the samples were analyzed by real-time quantitative PCR as it is one of the reliable DNA quantification techniques.

Materials and Methods

Saliva samples collection and storage conditions

This project was approved by Naresuan University Institutional Review Board, IRB No. 183/57. Three healthy volunteers with no dental cavities, no oral mucosal lesions, and no antibiotic treatments within 3 months were included for saliva samples collections. DMFT index, plaque index and gingival index values of all volunteers were examined. The volunteers were instructed not to drink or eat for 2 hours before saliva collections. The volunteers chewed paraffin for 5 minutes and then 5 ml of stimulated saliva samples were collected from right buccal vestibule using sterile syringe. One hundred and fifty microliters of saliva sample were aliquoted into 1.5 ml microcentrifuge tubes and stored in the following conditions; 1) centrifuged immediately at 14100 x g for 10 min at 4^{o}C and the pellet was stored at -80^{o}C for 5 days before DNA extraction, 2) the saliva samples were stored at room temperature (25^{o}C) for 6, 24, or 120 hours before DNA extraction, 3) the saliva samples were stored on ice for 6, 24, or 120 hours before DNA extraction, and 4) the saliva samples were stored on dry ice for 6, 24, or 120 hours before DNA extraction. For storage on ice or dry ice, the samples were placed in a box containing ice or dry ice and the box was sealed with sealing tape. Melted ice or evaporated dry ice was replaced with new ice or dry ice every 6 hours.

Bacterial genomic DNA extraction

Bacterial genomic DNA from saliva samples were extracted using PrestoTM Mini gDNA Bacteria Kit (Geneaid Biotech Ltd., Taiwan). The saliva pellet was mixed with 200 µl of Gram+ buffer containing 4 mg/ml of Lysozyme and incubated at 37^{o}C for 30 minutes. The mixture was added with 20 µl of Proteinase K and incubated at 60oC for 10 minutes. Bacteria were further lysed by addition of 200 µl of GB buffer containing 50 mg/ml of RNase A and incubation at 70^{o}C for 10 minutes. The bacterial lysate was added with 200 µl of absolute ethanol and the mixture was placed in the GD column. The column was washed with 400 µl of W1 buffer and further washed with 600 µl of Wash buffer. Bacterial DNA was eluted with 100 µl of Elution buffer. Bacterial DNA concentration was determined using NanoDrop^{TM} 200c UV-Vis spectrophotometer (Thermo ScientificTM, Thermo Fisher Scientific, USA).

Real-time PCR for Quantitation of bacterial DNA in saliva samples

Real-time PCR was performed with Prbac1 (5' ACTACGTGCCAGCAGCC 3') and Prbac2 (5' GGACTACCAGGGTATCTAATCC 3') primers for 16s rRNA gene of all bacteria (10). The PCR product sizes were ranged from 296 to 300 bp. *S. mutans* DNA, 0.0005–50 ng/ml, was used to create a standard graph of DNA concentration. Ten nanograms of salivary bacterial DNA was mixed with LightCycler® 480 II SYBR Green I Master mix (Roche Applied Science, Germany), primers, and water. The PCR mixture was amplified and analyzed using LightCycler® 480 II Instrument (Roche Applied Science, Germany) with Absolute Quantification program (LightCycler® 480 II Software, Version 1.5).

Statistical analysis

Concentrations of the extracted DNA or the DNA concentrations quantified by real-time PCR of each storage condition were tested for normality of data distribution by Shapiro-Wilk test. Concentration of extracted DNA data from

every storage condition passed normality test. Difference between groups was analyzed by one-way ANOVA and found that there was no statistically difference ($p = 0.44$).

Data of DNA concentration quantified by real-time PCR from every storage condition passed normality test, except data of storage on ice for 120 hours group. Difference between groups was analyzed by Kruskal-Wallis test and found that there was statistically difference ($p = 0.0036$). Difference between 2 normal distribution data groups was further analyzed by unpaired t test, while difference between normal distribution data group and non-normal distribution data group was analyzed by Mann-Whitney test. All statistical analysis was performed using GraphPad Prism software version 10.3.1.

Results

DMFT index mean value of three healthy volunteers, aged between 22 to 24 years old, was 8.67 +/- 2.08 (Table 1). Plaque index and gingival index mean values were 73.51 +/- 7.44 and 1.28 +/- 0.23, respectively.

Table 1. DMFT, plaque, and gingival index values of three volunteers.

	DMFT index	plaque index	Gingival index
Volunteer 1	11	65.17	1.29
Volunteer 2	8	75.89	1.05
Volunteer 3	8	79.46	1.5
Mean +/- SD	8.67 +/- 2.08	73.51+/- 7.44	1.28 +/- 0.23

Genomic DNA from saliva samples stored at different conditions were extracted and concentration of the DNA samples were measured using NanoDrop 200c UV-Vis spectrophotometer. Concentration of bacterial DNA extracted from saliva stored at different conditions was similar ($p = 0.44$) as shown in figure 1.

The extracted genomic DNA was subjected to analyze by absolute quantitative real-time PCR using primers for 16s rRNA gene of all bacteria (10). The quantification analysis showed that concentration of bacterial DNA from saliva stored at room temperature for 6 or 24 hours was significantly higher than that of DNA extracted from the control, the saliva sample that was centrifuged and frozen immediately after collection (figure 2). In contrast, the concentration of bacterial DNA from saliva stored on dry ice for 120 hours was significantly lower than that of the control. In other storage conditions, storage at room temperature for 120 hours, on ice for every duration, or on dry ice for 6 or 24 hours, the concentration of extracted DNA was similar to that of the control. Even though the storage of saliva sample on ice for 120 hours seemed to show lower concentration of bacterial DNA but it was not statistically significant.

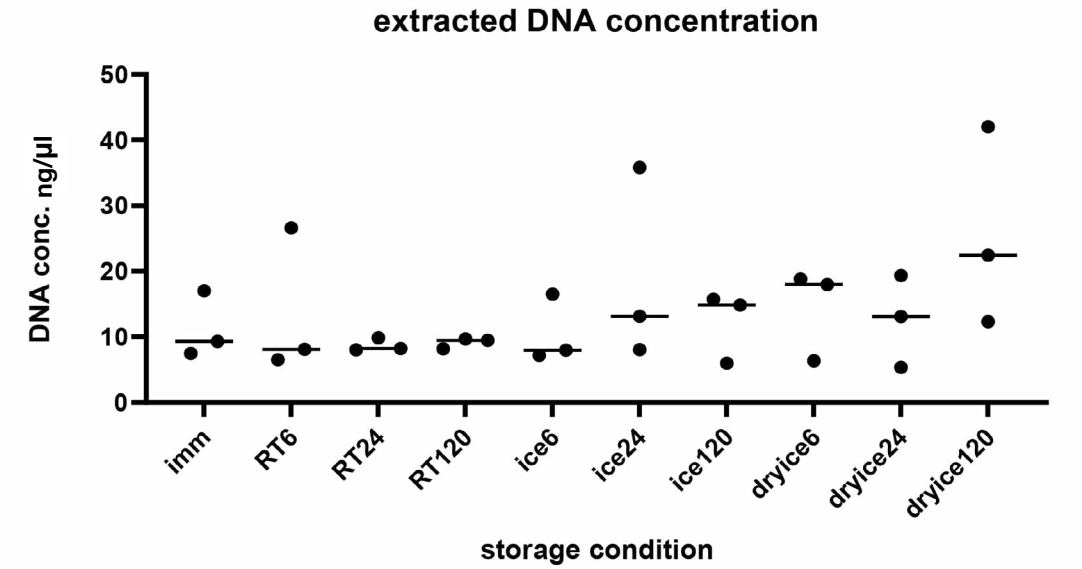

Fig.1 Concentration of genomic DNA extracted from saliva stored at different temperature and duration, Bacterial genomic DNA was extracted from saliva sample stored at -80°C after immediately centrifuged (imm), stored at room temperature for 6, 24, or 120 hours (RT6, RT24, or RT120), stored on ice for 6, 24, or 120 hours (ice6, ice24, or ice120), or store on dry ice for 6, 24, or 120 hours (dryice6, dryice24, or dryice120). Each dot represents extracted DNA concentration in ng/µl of each volunteer.

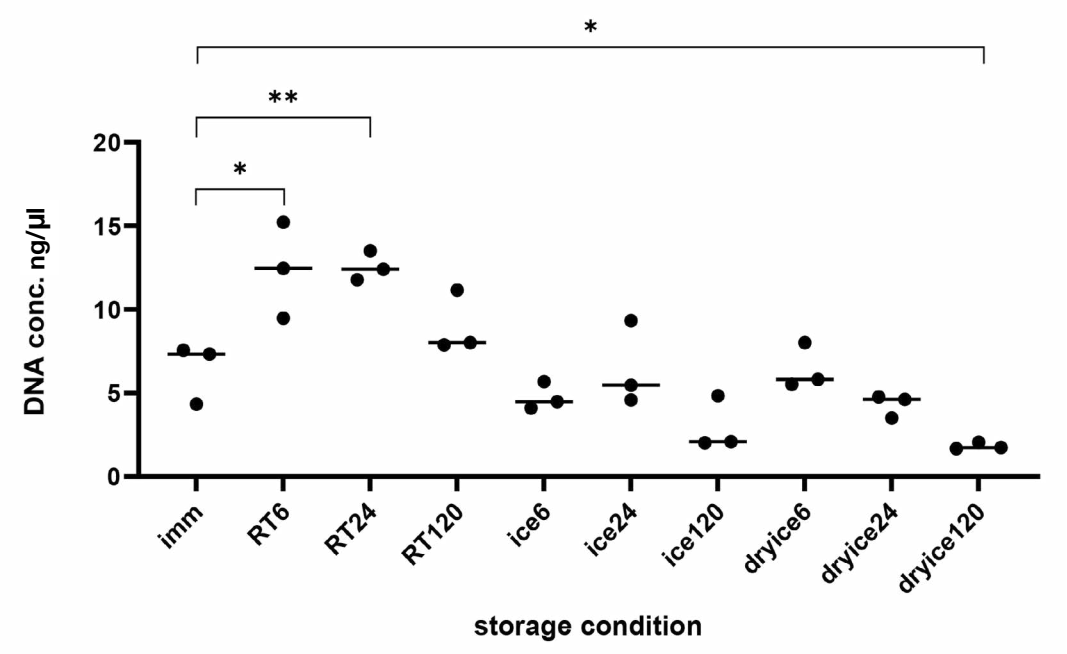

Fig. 2 Concentration of bacterial DNA quantified by real-time PCR, Bacterial genomic DNA extracted from saliva sample stored -80oC after immediately centrifuged (imm), stored at room temperature for 6, 24, or 120 hours (RT6, RT24, or RT120), stored on ice for 6, 24, or 120 hours (ice6, ice24, or ice120), or store on dry ice for 6, 24, or 120 hours (dryice6, dryice24, or dryice120) was quantified by absolute quantitative real-time PCR using 16s rRNA gene primers for total bacteria. Each dot represents DNA concentration in ng/µl from each volunteer. *indicates that p-value is lower than 0.05; **indicates that p-value is lower than 0.01.

Discussion

Saliva samples stored at room temperature for 6 or 24 hours appeared to contain more bacterial DNA than that of the sample pelleted and frozen immediately at -80°C. This might be resulted from bacterial growth using nutrients present in saliva (11-13). The increased bacteria would die as the nutrient was exhausted and bacterial waste was produced. Since bacterial DNA would be destroyed by bacterial and salivary DNase (14,15), bacterial DNA quantity at 120 hours was reduced to the level similar to the control.

Storage of saliva on ice up to 120 hours or on dry ice, about -80°C, for 6 or 24 hours had no effect on bacterial DNA quantity. These results were similar to the study that stored saliva at -20°C for up to 36 months before DNA extraction and followed by quantitative real-time PCR (8). However, storage on dry ice for 120 hours the DNA quantity was reduced significantly. This result was similar to the study that analyzed salivary DNA with conventional PCR (9). But it was different to the study that quantify bacterial count by Checkerboard DNA-DNA hybridization method (7). This might be a result of different quantitation techniques used in each study.

From this study, storage of saliva on ice for 120 hours seemed to reduce bacterial DNA concentration but it was not statistically significant. Study with more saliva sample number is needed to confirm this result.

Certainly, storage on ice or dry ice for up to 24 hours could maintain quantity of bacterial genomic DNA. Therefore, saliva samples collected for bacterial DNA quantitative analysis could be kept on ice or dry ice for transportation from a research field to a laboratory within 24 hours. Storage on ice or dry ice for a longer period of time might affect DNA quantity. The effect of saliva storage on ice or dry ice for 2 to 4 days should be studied. If storage within 4 days doesn't affect DNA quantity, transportation of saliva samples from the study field that is far away from the laboratory is possible.

Conclusion

As quantify by Real-time PCR, saliva storage at room temperature at 6 or 24 hours had increasing bacterial DNA. Saliva storage on ice and dry ice could maintain bacterial DNA similar to centrifugation immediately and then frozen at -80°C. However, storage on dry ice for 120 hours reduced bacterial DNA quantity.

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