The Effects of Storage of Human Saliva on DNA Isolation and Stability

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The most important progress in diagnostic sciences is the increased sensitivity and specificity in diagnostic procedures due to the development of micromethodologies and increasing availability of immunological and molecular biological reagents. The technological advances led to consider the diagnostic use of saliva for an array of analytes and DNA source.

The purpose of the present study was to compare DNA from saliva with those from blood and buccal swab, to evaluate diagnostic and forensic application of saliva, to investigate the changes of genomic DNA in saliva according to the storage temperature and period of saliva samples, and to evaluate the integrity of the DNA from saliva stored under various storage conditions by PCR analysis. Peripheral venous blood, unstimulated whole saliva, stimulated whole saliva, and buccal swab were obtained from healthy 10 subjects (mean age: 29.9 ± 9.8 years) and genomic DNA was extracted using commercial kit. For the study of effects of various storage conditions on genomic DNA from saliva, stimulated whole saliva were obtained from healthy 20 subjects (mean age: 32.3 ± 6.6 years). After making aliquots from fresh saliva, they were stored at room temperature, 4℃, -20℃, and -70℃. Saliva samples after lyophilization and dry-out procedure were stored at room temperature. After 1, 3, and 5 months, the same experiment was performed to investigate the changes in genomic DNA in saliva samples. In case of saliva aliquots stored at room temperature and dry-out samples, the results in 2 weeks were also included. Integrity of DNA from saliva stored under various storage conditions was also evaluated by PCR amplification analysis of ß-globin gene fragments (989-bp).

The results were as follows:

1. Concentration of genomic DNA extracted from saliva was lower than that from blood ($p<0.05$), but there were no significant differences among various types of saliva samples. Purities of genomic DNA extracted from stimulated whole saliva and lyophilized one were significantly higher than that from blood ($p<0.05$). Purity of genomic DNA extracted from buccal swab was lower than those from various types of saliva samples ($p<0.05$).
2. Concentration of genomic DNA from saliva stored at room temperature showed gradual reduction after 1 month, and decreased significantly in 3 and 5 months ($p<0.05$, $p<0.01$, respectively). Purities of DNA from saliva stored for 3 and 5 months showed significant differences with those of fresh saliva and stored saliva for 1 month ($p<0.05$).
3. In the case of saliva stored at 4℃ and -20℃, there were no significant changes of concentration of genomic DNA in 3 months. Concentration of DNA decreased significantly in 5 months ($p<0.05$).
4. There were no significant differences of concentration of genomic DNA from saliva stored at -70℃ and from lyophilized one according to storage period. Concentration of DNA showed decreasing tendency in 5 months.
5. Concentration of genomic DNA immediately extracted from saliva dried on Petri dish were 60% compared with that of fresh saliva. Concentration of DNA from saliva stored at room temperature after dry-out showed rapid reduction within 2 weeks ($p<0.05$).
6. Amplification of ß-globin gene using PCR was successful in all lyophilized saliva stored for 5 months. At the time of 1 month, ß-globin gene was successfully amplified in all saliva samples stored at -20℃ and -70℃, and in some saliva samples stored at 4℃. ß-globin gene was failed to amplify in saliva stored at room temperature and dry-out saliva.

Key words : Saliva, DNA, Storage, PCR, Stability

Ⅰ. INTRODUCTION

Over the past decade, scientists have increasingly discovered a large number of genes for human diseases and many scientists now appreciate that with the possible exception of diseases caused by trauma, essentially all human diseases have genetic components. These diseases include not only inherited diseases, but also viral, bacterial, and fungal infectious diseases, neoplastic diseases, cardiovascular and cerebrovascular diseases, and many craniofacial diseases[1]. With developed methodologies, the diagnostic use of DNA has been used for several infectious diseases[2-4] and forensic purpose[5], and there have been reports that genetic examination may be useful screening test for breast cancer[6] and cystic fibrosis[7] from general population.

A dominant theme in dental field becomes the application of concepts and techniques developed in molecular genetics, clinical genetics, immuno-genetics, and developmental biology to the study of craniofacial-oral-dental disorders. This approach will continue to provide DNA probes and antibodies that can be used as clinical diagnostic screening reagents for disorders, provide novel molecular approaches to gene-based therapeutics, create biomaterials for dental application, and provide a molecular and developmental rationale for gene therapy studies[8].

In the field of dentistry, there were also several reports about application of DNA for specific diseases. Kornman *et al.*[9] studied a genetic contribution to the pathogenesis of periodontitis, and demonstrated that specific genetic markers, which have been associated with increased IL-1 production, were a strong indicator of susceptibility to severe periodontitis in adults. Boyle *et al.*[10] demonstrated the detection of cancer cells in the saliva of patients with head and neck cancer by using genetic marker, and suggested a potential application of a genetic based test of saliva for clinical carcinoma research.

Many scientists have used various specimens such as blood, hair roots, semen, tissue, and bone for sources of DNA[11-15]. In forensic field, most conventional uses of saliva or saliva-stained materials recovered from crime scenes rely primarily on the identification of blood group antigens[16], rarely on isoenzyme[17], and polymorphic proteins[18]. However discriminating methods using these markers have several limitations compared with using DNA as markers because of their limited detectability and less sensitivity[16]. Therefore it has been paid attention to DNA in saliva for further discrimination.

Amount of analyte or concentration of molecule is no longer limitation of diagnostics because the sensitivity and specificity in diagnostic procedure were increased due to the development of micromethodologies and increasing availability of immunological and molecular biological reagents. Therefore, it is becoming increasingly apparent that saliva is useful diagnostic medium because the collection of saliva is very simple and safe[19].

Recently, there were some reports concerning the diagnostic and forensic application of saliva using genomic DNA extracted from saliva. van Schie *et al.*[20] analyzed Fc receptor allelic polymorphism of IgG using genomic DNA extracted from saliva, reported that saliva appeared to be an excellent source of DNA as a substitute of blood. Walsh *et al.*[21] examined the extraction procedure of DNA from saliva and saliva-stained materials for the purpose of forensic use of saliva, demonstrated that intact DNA was readily isolated and the DNA banding patterns obtained from saliva or saliva-stained material were indistinguishable from the patterns obtained from blood or hair from the same individual. In spite of several studies, there is a little information on the condition of DNA

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extracted from saliva stored in varied conditions.

The purpose of the present study was to compare genomic DNA extracted from saliva with those from blood and buccal swab, to investigate the changes of genomic DNA in saliva according to the storage temperature and period of saliva samples, and to evaluate the integrity of the DNA from saliva stored under various storage conditions by PCR analysis.

Ⅱ. MATERIALS AND METHODS

1. Comparative study of genomic DNA from blood, saliva, and buccal swab

1) Collection of blood, saliva, and buccal swab

Peripheral venous blood was obtained with standard venepuncture technique and collected into glass tube containing 250 μL of 0.5 M EDTA (pH 8.0).

Unstimulated and stimulated whole saliva were collected from 10 healthy adults (mean age: 29.9 ± 9.8 years). Collection of saliva was carried out between 9:00 am - 12:00 am to minimize effects of the diurnal variability in salivary compositions. Sample from the subjects was collected into the chilled sterile 15 mL polypropylene tube before meals or at least two hs after meals. All subjects were abstained from smoking, eating or drinking for 2 hs before examination. Unstimulated whole saliva was collected for about 5 min by spitting method (*i.e.,* after swallowing, saliva is collected with closed lips, and then all saliva is expectorated into a vessel one or two times per min) and in case of stimulated whole saliva, the gum base was used as stimulus.

In order to investigate the effect of lyophilization on genomic DNA, lyophilized stimulated whole saliva was prepared. Aliquots of stimulated whole saliva, 600 μL drawn into 1.5 mL sterile microcentrifuge tube, were frozen at -70℃, and lyophilized in a FD5505 freeze dryer system (Il-Shin Lab Co, Ltd, Seoul, Korea). These lyophilized stimulated whole saliva were resuspended with 600 μL distilled, deionized water (DDW), and used immediately for extraction of DNA.

To obtain buccal swab, metal spatula was scraped lightly 3 times on the buccal cheek of each individual. Buccal swab sample was collected in microcentrifuge tube containing 600 μL DDW by swing the metal spatula. Obtained blood, saliva, and buccal swab samples were used immediately for extraction of DNA.

2) Extraction of genomic DNA

Genomic DNA was extracted from blood using a commercial DNA purification kit (Wizard® Genomic DNA Purification kit, Promega Co, WI, USA). Each 300 μL of prepared blood sample was added into the 1.5 mL microcentrifuge tube containing 900 μL of cell lysis solution and invert the tube 5-6 times to mix. Samples were incubated for 10 min to lysis the cells, centrifuged at 13,000-16,000 xg for 20 sec at room temperature. Supernatant was carefully removed as possible without disturbing the visible white pellet. Nuclei lysis solution (100 μL) was added into the tube, the solution was pipetted 5-6 times, and incubated at 37℃ until the clumps are disrupted. If the clumps were still visible after 1 h, nuclei lysis solution was added, and repeated the incubation. Into the tube, 1.5 μL of RNase solution was added, and the mixture was incubated at 37℃ for 15 min, and then cooled to room temperature, and 100 μL of protein precipitation solution were added to the nuclear lysate. The sample was mixed thoroughly and centrifuged at 13,000-16,000 xg for 3 min at room temperature. The supernatant was transferred to a clean 1.5 mL microcentrifuge tube containing 300 μL of room temperature isopropanol and the sample was gently mixed by inversion, centrifuged at 13,000-16,000 xg for 1 min at room temperature. The supernatant was removed and one sample volume of room temperature 70% ethanol was added. The tube was gently inverted several times to wash the DNA pellet and centrifuged at 13,000-16,000 xg for 1 min at room temperature. After careful removal of the ethanol, the tube was inverted on clean absorbent paper and dried by air

for 10-15 min. Finally 65 μL of DNA rehydration solution was added, and the tube was incubated at 65℃ for 1 h. Extracted DNA yield was determined spectrophotometrically at 260 nm (Ultrospec 2000, Pharmacia Biotech Ltd, Cambridge, UK). The purity of DNA was determined as rate of optical density at 260 nm and 280 nm. The extracted DNA was stored at -20℃ until examination.

DNA was extracted from the unstimulated whole saliva and stimulated whole saliva with same method with exception of sample volume to 600 μL as previously described in blood using a commercial DNA purification kit (Wizard® Genomic DNA Purification kit, Promega Co, WI, USA). DNA was extracted from obtained buccal swab sample with same method in saliva.

3) DNA gel electrophoresis

High molecular mass genomic DNA from blood, saliva, and buccal swab were examined by electrophoresis. Seven μL of extracted DNA sample from blood, 15 μL from saliva, and 20 μL from buccal swab were loaded on the gel. Electrophoresis were performed on 1% agarose submarine gel at constant 100 V in TBE buffer (pH 8.8) (134 mM Tris, 75 mM boric acid, 25 mM EDTA) for 1 h. Ethidium bromide was incorporated in the gel matrix to enable fluorescent visualization of the DNA under UV light.

2. Effects of various storage conditions on genomic DNA

1) Collection and storage of saliva

For the examination of extraction rate of genomic DNA from saliva stored in various conditions, 20 mL of stimulated whole saliva were collected from 20 healthy adults (mean age: 32.3 ± 6.6 years). Salivary aliquots were made by distribution of each 600 μL saliva into sterile 1.5 mL microcentrifuge tube and were stored at room temperature, 4℃, -20 ℃ and -70℃ until examination for 5 months.

To examine change of DNA after drying, dried-out sample and lyophilized sample were prepared. 600 μL of stimulated whole saliva was inoculated into the Petri dish and dried out naturally at room temperature and stored for 5 months at room temperature. In order to determine yield of DNA recovery from Petri dish, genomic DNA was extracted immediately after completion of drying. At the time of extraction of DNA, the dried sample was rehydrated with 10 mL of DDW and centrifuged. The pellet was resuspended in 600 μL DDW and subsequently the final salivary analyte was obtained. Salivary aliquots were quickly frozen in -70℃ and water of frozen saliva was vaporized. These lyophilized samples were stored at room temperature under atmospheric condition for 5 months until examination.

2) Extraction of genomic DNA

DNA was extracted from the saliva stored various conditions by the same method as previously described in fresh saliva using a commercial DNA purification kit (Wizard® Genomic DNA Purification kit, Promega Co, WI, USA).

3) PCR-based assays of extracted genomic DNA

Integrity of DNA extracts was also evaluated by PCR analysis. The 989-bp sized fragments of ß-globin gene from the extracted DNA were amplified using a commercial PCR kit (Premix Taq(Takara Ex Taq™ version), Takara Bio Inc, Shiga, Japan). The primers RS80 (5′-TGGTAGCTGGATTGTAGCTG-3′) and RS40 (5′-ATTTTCCCACCCTTAGGCTG-3′) were used to generate a 989-bp fragments. Each 1 ng of extracted DNA was added into the 0.2 mL sterile PCR tube containing 25 μL of Premix Taq solution and each 5 μL of primers under cooling condition, sterilized distilled water was added up to 50 μL of total volume. Samples were underwent 40 amplification cycles (1 min at 95℃, 1 min at 55℃, and 2 min at 72℃) after predenaturation of 10 min at 95℃, followed by a final extension of 5 min at 72 ℃ (Master Cycler Gradient, Eppendorf Inc, Hamburg, Germany).

4) DNA gel electrophoresis

High molecular mass genomic DNA extracted from stored saliva and PCR amplicons of extracted genomic DNA were also examined by the same electrophoretic procedure as previously described. Fifteen μL of extracted DNA from saliva, and 10 μL of amplicons were loaded on the gel.

Ⅲ. RESULTS

1. Comparative study of genomic DNA from blood, saliva, and buccal swab

Genomic DNA extracted from blood, saliva, and buccal swab immediately after sample collection were examined and compared each other. Mean concentration of genomic DNA extracted from blood was 20.23 ± 8.68 ㎍/mL, and those from stimulated whole saliva and unstimulated whole saliva were 6.16 ± 2.78 ㎍/mL and 3.57 ± 2.53 ㎍ /mL. There was significant difference of concentration of DNA between blood and saliva ($p<0.05$). Although concentration of genomic DNA from stimulated whole saliva was higher than that from unstimulated whole saliva, there was no significant difference. Concentration of DNA from lyophilized stimulated whole saliva was 5.38 ± 3.45 ㎍/mL, slightly lower than that from fresh saliva, but there was no significant difference. Total amount of DNA yields scraped 3 times by metal spatula was 0.22 ± 0.08 ㎍. Purity of DNA from buccal swab was 1.53 ± 0.19, there were significant differences with various types of saliva samples ($p<0.05$) (Table 1). High molecular mass genomic DNA from saliva and buccal swab were located at same position of that from blood in electrophoretic pattern (Fig. 1).

2. Effects of various storage conditions on genomic DNA

Stimulated whole saliva was used in the study of effects of various storage conditions on genomic DNA, because stimulated whole saliva is easily gained than unstimulated whole saliva and concentration of DNA from stimulated whole saliva was higher than that of unstimulated one. Changes of DNA from saliva stored at room temperature for different period were examined (Table 2). Concentration of DNA in 2 weeks was not differ from initial concentration, but after 1 month,

Table 1. Concentration and purity of DNA from blood, saliva, and buccal swab

Sample (n = 10)		Concentration (㎍/mL)	Purity
Blood		20.23 ± 8.68	1.70 ± 0.13
Saliva	UWS	3.57 ± 2.53	1.75 ± 0.19
	SWS	6.16 ± 2.78	1.82 ± 0.15
	LSWS	5.38 ± 3.45	1.93 ± 0.05
BS		0.22 ± 0.08†	1.53 ± 0.19
Significance		*(Blood, UWS), *(Blood, SWS), *(Blood, LSWS), *(Blood, BS), *(UWS, BS), *(SWS, BS), *(LSWS, BS)	*(Blood, SWS), *(Blood, LSWS), *(UWS, LSWS), *(UWS, BS), *(SWS, LSWS), *(SWS, BS), *(LSWS, BS)

*: $p<0.05$; †: total amount of DNA yield scraped 3 times, unit is ㎍
SWS: stimulated whole saliva; UWS: unstimulated whole saliva
LSWS: lyophilized stimulated whole saliva; BS: buccal swab

Table 2. Differences of concentration and purity of DNA from stimulated whole saliva according to period of storage at room temperature

Sample (n = 20)	Time of DNA extraction					Significance
	Imm	2 W	1 M	3 M	5 M	
Concentration (μg/mL)	4.73 ± 4.67	4.86 ± 4.77	3.02 ± 2.23	1.50 ± 1.51	0.79 ± 0.50	*(Imm, 3 M), **(Imm, 5 M), **(2 W, 5 M), *(2 W, 3 M), *(1M, 3M), **(1M, 5M)
Purity	1.87 ± 0.12	1.86 ± 0.16	1.76 ± 0.19	1.55 ± 0.17	1.54 ± 0.18	*(Imm, 3 M), *(Imm, 5 M), *(2 W, 3 M), *(2 W, 5 M), *(1 M, 3 M), *(1 M, 5 M)

**: $p<0.01$; *: $p<0.05$

Imm: immediately; W: Week after collection time; M: Month after collection time

Fig. 1. Electrophoretic pattern of high molecular mass genomic DNA. Lane 1: blood, lane 2: stimulated whole saliva, lane 3: unstimulated whole saliva, lane 4: lyophilized stimulated whole saliva, lane 5: buccal swab. Electrophoresis were performed on 1% agarose submarine gel at constant 100 V in TBE buffer for 1 h, with ethidium bromide.

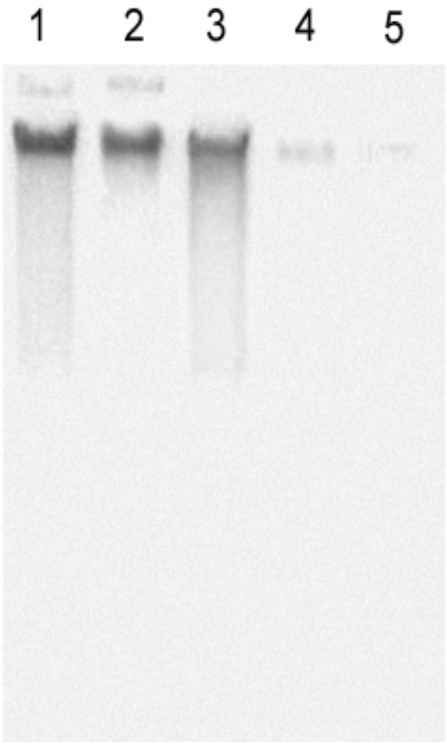

Fig. 2. Electrophoretic pattern of high molecular mass genomic DNA from stimulated whole saliva stored at room temperature. Lane 1: 0 day, lane 2: 2 weeks, lane 3: 1 month, lane 4: 3 months, lane 5: 5 months. Electrophoresis were performed on 1% agarose submarine gel at constant 100 V in TBE buffer for 1 h, with ethidium bromide.

Table 3. Differences of concentration and purity of DNA from stimulated whole saliva according to period of storage at 4℃

Sample (n = 20)	Time of DNA extraction				Significance
	Imm	1 M	3 M	5 M	
Concentration (μg/mL)	4.73 ± 4.67	4.49 ± 3.59	2.93 ± 2.76	2.02 ± 1.52	*(Imm, 5 M), *(1 M, 5 M)
Purity	1.87 ± 0.12	1.99 ± 0.11	1.78 ± 0.16	1.91 ± 0.14	*(Imm, 1 M), *(Imm, 3 M), *(1 M, 3 M), *(1 M, 5 M), *(3 M, 5 M)

*: $p<0.05$

Imm: immediately; M: Month after collection time

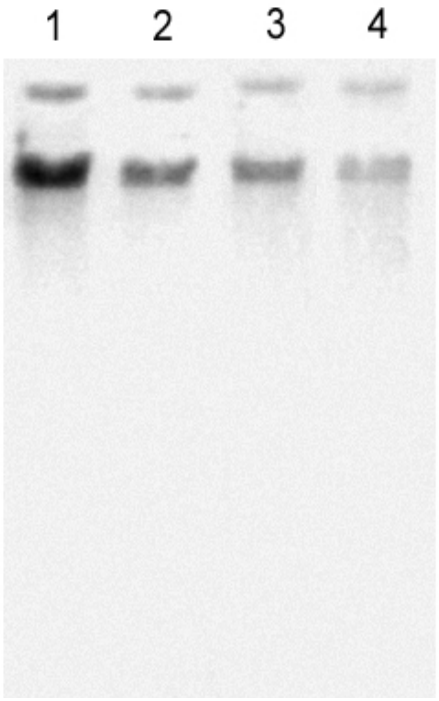

Fig. 3. Electrophoretic pattern of high molecular mass genomic DNA from stimulated whole saliva stored at 4℃. Lane 1: 0 day, lane 2: 1 month, lane 3: 3 months, lane 4: 5 months. Electrophoresis were performed on 1% agarose submarine gel at constant 100 V in TBE buffer for 1 h, with ethidium bromide.

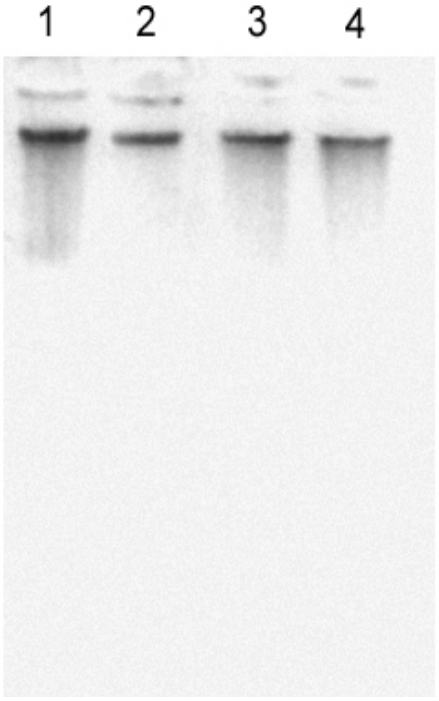

Fig. 4. Electrophoretic pattern of high molecular mass genomic DNA from stimulated whole saliva stored at -20℃. Lane 1: 0 day, lane 2: 1 month, lane 3: 3 months, lane 4: 5 months. Electrophoresis were performed on 1% agarose submarine gel at constant 100 V in TBE buffer for 1 h, with ethidium bromide.

Table 4. Differences of concentration and purity of DNA from stimulated whole saliva according to period of storage at -20℃

Sample (n = 20)	Time of DNA extraction				Significance
	Imm	1 M	3 M	5 M	
Concentration (μg/mL)	4.73 ± 4.67	5.36 ± 2.76	4.34 ± 3.10	2.68 ± 1.92	*(Imm, 5 M), *(1 M, 5 M), *(3 M, 5 M)
Purity	1.87 ± 0.12	1.91 ± 0.10	1.81 ± 0.18	1.88 ± 0.12	*(1 M, 3 M)

*: $p<0.05$
Imm: immediately; M: Month after collection time

Table 5. Differences of concentration and purity of DNA from stimulated whole saliva according to period of storage at -70℃

Sample (n = 20)	Time of DNA extraction				Significance
	Imm	1 M	3 M	5 M	
Concentration (μg/mL)	4.73 ± 4.67	5.34 ± 4.25	4.89 ± 4.32	3.17 ± 2.15	N.S.
Purity	1.87 ± 0.19	1.77 ± 0.18	1.88 ± 0.13	1.88 ± 0.13	*(Imm, 1 M), *(1 M, 3 M), *(1 M, 5 M)

*: $p<0.05$; N.S.: no significance
Imm: immediately; W: Week after collection time; M: Month after collection time

Table 6. Differences of concentration and purity of DNA from stimulated whole saliva according to period of storage at room temperature after dry out

Sample (n = 20)	Time of DNA extraction						Significance
	Imm	ID	2 W	1 M	3 M	5 M	
Concentration (μg/mL)	4.73 ± 4.67	2.86 ± 2.48	0.37 ± 0.23	0.28 ± 0.17	0.27 ± 0.24	0.27 ± 0.29	*(Imm, ID), **(Imm, 2 W), **(Imm, 1 M), **(Imm, 3 M), **(Imm, 5 M), *(IID, 2 W), *(ID, 1 M), *(ID, 3 M), *(ID, 5 M)
Purity	1.87 ± 0.19	1.92 ± 0.15	2.04 ± 0.30	1.70 ± 0.21	1.33 ± 0.21	1.70 ± 1.04	*(Imm, 3 M), *(ID, 3 M), *(2 W, 1 M), **(2 W, 3 M), *(2 W, 5 M), *(1 M, 3 M), *(3 M, 5 M)

**: $p<0.01$; *: $p<0.05$
Imm: immediately; ID :immediately after dry out
W: Week after collection time; M: Month after collection time

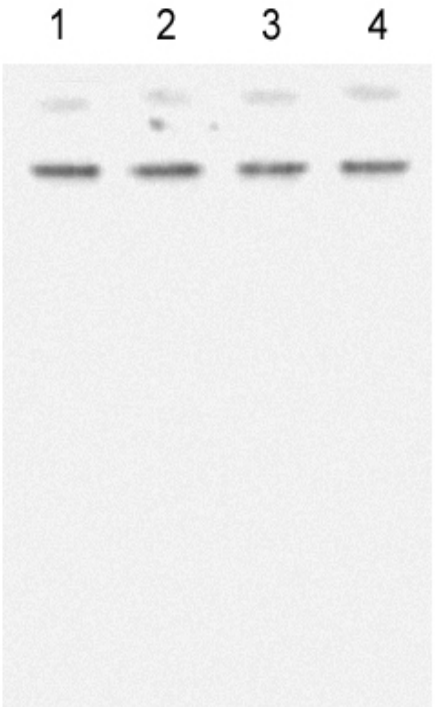

Fig. 5. Electrophoretic pattern of high molecular mass genomic DNA from stimulated whole saliva stored at -70℃. Lane 1: 0 day, lane 2: 1 month, lane 3: 3 months, lane 4: 5 months. Electrophoresis were performed on 1% agarose submarine gel at constant 100 V in TBE buffer for 1 h, with ethidium bromide.

Fig. 6. Electrophoretic pattern of high molecular mass genomic DNA from stimulated whole saliva stored at room temperature after dry out. Lane 1: immediately after collection, lane 2: 0 day after dry out, lane 3: 2 weeks, lane 4: 1 month, lane 5: 3 months, lane 6: 5 months. Electrophoresis were performed on 1% agarose submarine gel at constant 100 V in TBE buffer for 1 h, with ethidium bromide.

gradual reduction was occurred. There were marked reduction in concentration of DNA in 3, 5 months ($p<0.05$, $p<0.01$). Purities of DNA from saliva stored for 3 and 5 months showed significant differences with those of fresh saliva and stored saliva for 1 month ($p<0.05$). Eletrophoretic pattern of high molecular mass of genomic DNA showed degradation of DNA according to storage period, and especially in 3, 5 months, the characteristic band of high molecular mass genomic DNA were almost disappeared (Fig. 2).

Concentration of genomic DNA from saliva stored at 4℃ for 1 month did not differ from that of fresh saliva (Table 3). Concentration of DNA showed decreasing tendency in 3 months, but there was no significant difference either. Concentration of DNA decreased significantly ($p<0.05$) in 5 months. In electrophoretic pattern of genomic DNA, there were gradual degradation of DNA according to period of storage (Fig. 3).

In the case of saliva stored at -20℃, there were no changes of concentration of genomic DNA in 3 months. Concentration of DNA decreased significantly ($p<0.05$) (Table 4) in 5 months. In a results of electrophoresis, the band of high molecular mass genomic DNA did not differ each other except for that in 5 months which showed some degradation (Fig. 4).

Table 7. Differences of concentration and purity of DNA from stimulated whole saliva according to period of storage at room temperature after lyophilization

Sample (n = 20)	Time of DNA extraction				Significance
	Imm	1 M	3 M	5 M	
Concentration (μg/mL)	4.73 ± 4.67	4.04 ± 3.54	4.74 ± 4.70	3.29 ± 2.35	N.S.
Purity	1.87 ± 0.19	1.91 ± 0.13	1.82 ± 0.11	1.94 ± 0.22	*(3 M, 5 M)

*: $p<0.05$; N.S.: no significance
Imm: immediately; M: Month after collection time

Table 8. PCR success rates to amplify ß-globin gene fragments(989-bp) of DNA from stimulated whole saliva stored under various storage conditions

Storage conditions	PCR success rate(%)				
	Time of DNA extraction				
	ID	2 W	1 M	3 M	5 M
Room temperature	-	10	0	0	0
4℃	-	-	30	10	0
-20℃	-	-	100	60	50
-70℃	-	-	100	90	90
DO	50	0	0	0	0
Lyo	-	-	100	100	100

DO: room temperature after dry out; Lyo: room temperature after lyophilization
ID: immediately after dry out; W: Week after collection time
M: Month after collection time

Although concentration of genomic DNA from saliva stored at -70℃ for 5 months decreased as compared with that from fresh saliva, there were no differences according to storage period (Table 5). Purity of DNA from saliva stored for 1 month differed with those for other period ($p<0.05$). In electrophoretic pattern of high molecular mass of genomic DNA, there were no evidences of degradation for 5 months (Fig. 5).

To examine change of DNA after drying, 600 μL of stimulated whole saliva was inoculated into the Petri dish and dried out naturally at room temperature. Concentration of genfomic DNA immediately extracted from saliva dried on Petri dish were 60% compared with that of fresh saliva (Table 6). Concentration of DNA from dried saliva showed rapid reduction within 2 weeks ($p<0.05$), and showed no differences later than 2 weeks. In electrophoretic pattern, there was marked degradation of genomic DNA after 2 weeks (Fig. 6).

There were no significant differences of concentration of genomic DNA from lyophilized saliva according to storage period. Concentration of DNA showed decreasing tendency in 5 months (Table 7).

To evaluate integrity of DNA from saliva stored

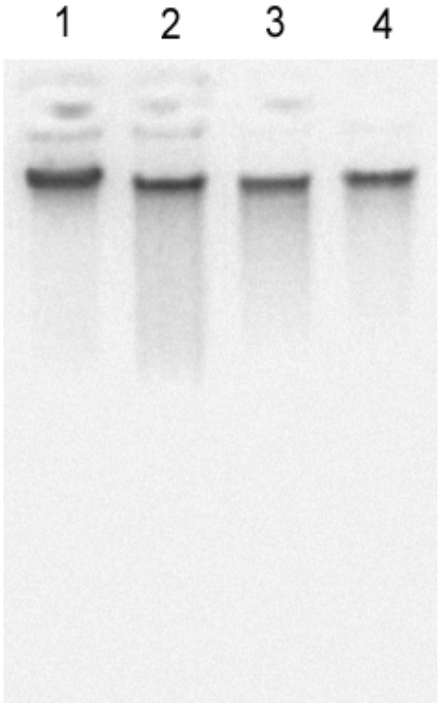

Fig. 7. Electrophoretic pattern of high molecular mass genomic DNA from stimulated whole saliva stored at room temperature after lyophilization. Lane 1: 0 day, lane 2: 1 month, lane 3: 3 months, lane 4: 5 months. Electrophoresis were performed on 1% agarose submarine gel at constant 100 V in TBE buffer for 1 h, with ethidium bromide.

under various storage conditions, amplification of ß-globin gene fragments (989-bp) by PCR was also attempted. In most saliva stored at room temperature and 4℃ and dry-out saliva, amplification of ß-globin gene by PCR was failed. Although all saliva stored at -20℃ for 1 month were able to amplify ß-globin gene fragments, success rate of amplification decreased to 60% and 50% in 3 and 5 months, respectively. In all saliva stored at -70℃ for 1 month ß-globin gene was successfully amplified, success rate of amplification decreased slightly to 90% in 3 and 5 months. Amplification of ß-globin gene using PCR was successful in all lyophilized saliva stored for 5 months.

Ⅳ. DISCUSSION

Diagnostic tests based on fluid generally use blood and urine, and less frequently the esoteric fluids such as saliva, sweat, and tears. Sweat and tears, however, are difficult to obtain in sufficient quantities for routine testing, and urine will always lack the charisma of the other fluids. Saliva, therefore becomes the most favoured alternative to blood and it is more practical in the dental field[22]. It has been paid attention to possibility of saliva as diagnostic analyte by simplicity and safety of collection that is the greatest advantage in the laboratory as well as clinic, with appearance of incurable diseases such as AIDS[19]. Most molecules present in blood or urine can also be detected in salivary secretions. Their concentrations in saliva are usually one tenth to one thousandth of those in blood. Although highly sensitive methods of detection are required, technical advances have made this feasible[22]. Recently, remarkable advance in diagnostic procedure are increased sensitivity and specificity due to the development of micrometho-dologies and increasing availability of immunological and molecular biological reagents, so amount of analyte or concentration of molecule are no longer limitation of diagnostics. Therefore, it is becoming increasingly apparent that saliva has many diagnostic uses.

The object of clinical investigation for the pathological basis of disease has been expanded to include a new analyte: nucleic acid.[23] In 1976, one of the first application of DNA hybridization technology to detect α-thalassemia in utero was reported.[24] Extraction of high molecular weight genomic DNA from human blood and saliva under various conditions and its recovery after long-term storage in an intact form are of great interest to studying genetic diseases[25].

The purpose of the present study was to compare DNA from saliva with those from blood and buccal swab, to investigate the changes of genomic DNA in saliva according to the storage temperature and period of saliva samples, and to evaluate the

integrity of the DNA from saliva stored under various storage conditions by PCR analysis. Gender and age differences were not incorporated in this study, because yield of salivary DNA was not influenced by either gender or ethnic background.[20)]

Many scientists have studied the concentration of high molecular weight DNA from blood using various methods and reported ranging from 18.7 to 40 μg/mL[26,27,28)]. In the present study, concentration of genomic DNA extracted from blood was 20.23 ± 8.68 μg/mL. Compared with data from the previous studies, concentration of genomic DNA from blood in the present study showed a similar results. Several studies have reported concentration of genomic DNA extracted from saliva using various methods. Walsh and coworkers[21)] isolated DNA from saliva using phenol/chloroform extraction, reporting recoveries of 3-14 μg/mL of saliva from an unspecified number of subjects. van Schie *et al.*[20)] also isolated DNA from whole saliva collected from 69 subjects using a commercial DNA isolation kit and reported that the mean yield of DNA recovered per mL of saliva was 19.2 ± 14.1 μg, with a minimum value of 4.3 μg and a maximum value of 71.4 μg. In this present study, the concentration of DNA from stimulated whole saliva was 6.16 ± 2.78 μg/mL, and slightly lowered than those from previous studies. Subtle difference in the concentration could be considered due to the differences in sample volume and experimental method. There was a tendency that concentration of DNA from stimulated whole saliva was higher than that from unstimulated whole saliva. It means that the amount of buccal cells shed from buccal cheek by mechanical rubbing during gum chewing overcome dilution effect by increased salivary flow rate by stimulation.

Biological molecule are known to be stable to some extent in tissues under mummified or dehydrated conditions, as exemplified by successful DNA amplification from archaeological and paleontological samples[29,30)]. Freeze drying yields dehydrated conditions, and is a convenient means of stabilizing biological materials as it eliminates the need for cryogenic storage[31)]. It is now suggested that lyophilization ("freeze-drying") is a suitable method for preserving tissue sample for DNA profiling[31,32,33)]. In this present study, the effects of lyophilization on genomic DNA were also investigated. Concentration of DNA immediately extracted from lyophilized stimulated whole saliva was 5.38 ± 3.45 μg/mL, about 87% of that from fresh saliva, but there was no significant difference.

Desquamated buccal epithelial cells in whole saliva provide an accessible source of germ-line DNA. Buccal cells can be also obtained for DNA isolation using cytobrushes or swabs. Reported DNA yields range from 2 to 111 μg of DNA for brushes or swabs[34,35,36,37)]. In this present study, total amount of DNA yields scraped 3 times by metal spatula was 0.22 ± 0.08 μg, lowered than reported yields. It is likely that sample in the present study could be diluted excessively. Actually, differences in number and extent of scraping on buccal cheek and degree of following dilution affect the concentration of DNA from buccal swab significantly.

Although isolated DNA is stable at 4℃ for at least 3 years[23)], genomic DNA in tissue is known to be unstable, because of degradative activity of DNases. Ultraviolet light, extremes in pH, and severe heat are the most problematic. Direct microbial contamination or environments that foster microbial growth, such as high humidity can damage the molecular arrangement of DNA and make it unsuitable for analysis[15)]. The effects of storage of sample on the integrity of DNA have been investigated mainly on blood[23,25,26)]. It is generally recommended that DNA samples be kept below the freezing point of water for long-term storage, because freezing serve to minimize the degradative activity of DNases.

Whole saliva is a product of the secretions of the parotid, submandibular and sublingual glands, but also contains constituents of gingival crevicular fluids, desquamated epithelial cells, blood, bacteria, viruses, fungi, and possibly food residues. Therefore, the progressive degradation of the human genomic DNA could happen. Stimulated whole saliva was used in the present additional study on the effects

of various storage conditions on genomic DNA, because stimulated whole saliva is easily gained than unstimulated whole saliva and concentration of DNA from stimulated whole saliva was higher than that of unstimulated one. In this present study, the effects of temperature and period of storage on integrity of genomic DNA showed better results as storage temperature was getting lowered. Concentrations of genomic DNA from saliva stored at room temperature and 4℃ were decreased gradually from the beginning of storage, in a greater or less degree. In the case of saliva stored at -20℃, there was no change of concentration of genomic DNA in 3 months. But in 5 months, concentration of DNA decreased significantly. Concentration of genomic DNA from saliva stored at -70℃ for 5 months did not show any significant difference compared that of fresh saliva. The effects of drying on genomic DNA were also investigated. Concentration of genomic DNA immediately extracted from saliva dried on Petri dish were 60% compared with that of fresh saliva. Concentration of DNA from dried saliva showed rapid reduction within 2 weeks. In the case of lyophilized saliva, there was no significant change of concentration of genomic DNA for 5 months. In this study, dry-out saliva samples had been stored under lights unlike others, cause of rapid reduction of concentration of DNA is probably effects of UV light and oxygen as well as drying damage.

Integrity of DNA from saliva stored under various storage conditions was also evaluated by PCR amplification of the 989-bp sized human ß-globin gene fragments. In this study, ß-globin gene fragments from saliva stored at room temperature and 4℃ and dry-out saliva were failed to amplify in many cases. Success rate of amplification of ß-globin gene fragments from saliva stored at -20℃ for 1 month was 100%, and decreased markedly after then. In all saliva stored at -70℃ for 1 month ß-globin gene was successfully amplified, success rate of amplification decreased slightly to 90% in 3 and 5 months. Amplification of ß-globin gene using PCR was successful in all lyophilized saliva stored for 5 months. The results of amplification of ß-globin gene fragments from saliva stored under various conditions were similar to changes of concentration of genomic DNA.

There has been other studies on the effects of various storage conditions on salivary DNA. Walsh *et al.*[21] studied the stability of DNA in saliva for 3 weeks, and suggested that an adequate long-term storage condition for saliva is -20℃, but that short-term storage of saliva on cotton swabs at 4 or 20℃ can yield DNA suitable for analysis. van Schie and Wilson[20] isolated DNA from two saliva sample stored for a period of almost 6 years, and demonstrated that quantities of DNA can be obtained from saliva maintained for prolonged interval at -70℃. The results of previous studies on the effects of various storage conditions on salivary DNA correspond with the our results of storage effect. In this present study, the result of long-term storage of lyophilized saliva was similar to that of frozen sample at -70℃. It is suggested that lyophilization could serve as a substitute for storage of sample in a deep freezer. Lyophilization also allows convenient and inexpensive shipment of dried samples at ambient temperature.

It is now apparent that mutations in regulatory and structural genes as well as chromosomal anomalies are associated with approximately 14 percent of the major craniofacial-oral-dental malformations, and approximately three-quarters of all congenital malformations affect the craniofacial-oral-dental and neck regions of the newborn infant[8]. Diagnostic molecular pathology will grow in importance as test scope using the tools of molecular biology continue to expand[23]. Laboratory tests based on the analysis of DNA or RNA becomes routine examination, thus availability of DNA isolated from saliva could be increased with simplicity and safety of collection that is the greatest advantage. There will be more needs of the information for a standardized approach to saliva collection, preservation, and analysis.

V. CONCLUSIONS

The present study was performed to compare DNA from saliva with those from blood and buccal swab, to evaluate diagnostic and forensic application of saliva as analyte, to investigate the effect of various storage conditions to extraction of genomic DNA of saliva, and to evaluate the integrity of the DNA from saliva stored under various storage conditions by PCR analysis.
The results were as follows:

1. Concentration of genomic DNA extracted from saliva was lower than that from blood ($p<0.05$), but there were no significant differences among various types of saliva samples. Purities of genomic DNA extracted from stimulated whole saliva and lyophilized one were significantly higher than that from blood ($p<0.05$). Purity of genomic DNA extracted from buccal swab was lower than those from various types of saliva samples ($p<0.05$).
2. Concentration of genomic DNA from saliva stored at room temperature showed gradual reduction after 1 month, and decreased significantly in 3 and 5 months ($p<0.05$, $p<0.01$, respectively). Purities of DNA from saliva stored for 3 and 5 months showed significant differences with those of fresh saliva and stored saliva for 1 month ($p<0.05$).
3. In the case of saliva stored at 4℃ and -20℃, there were no significant changes of concentration of genomic DNA in 3 months. Concentration of DNA decreased significantly in 5 months ($p<0.05$).
4. There were no significant differences of concentration of genomic DNA from saliva stored at -70℃ and from lyophilized one according to storage period. Concentration of DNA showed decreasing tendency in 5 months.
5. Concentration of genomic DNA immediately extracted from saliva dried on Petri dish were 60% compared with that of fresh saliva. Concentration of DNA from saliva stored at room temperature after dry-out showed rapid reduction within 2 weeks ($p<0.05$).
6. Amplification of ß-globin gene using PCR was successful in all lyophilized saliva stored for 5 months. At the time of 1 month, ß-globin gene was successfully amplified in all saliva samples stored at -20℃ and -70℃, and in some saliva samples stored at 4℃. ß-globin gene was failed to amplify in saliva stored at room temperature and dry-out saliva.

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국문요약

인체타액의 보관이 DNA 분리와 안정도에 미치는 영향

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최근 진단분야에 있어서의 가장 획기적인 진보로는 향상된 진단 술식의 민감도와 특이도를 들 수 있으며 이는 다양한 면역화학물질과 분자생물학적 시약의 활용도가 증가되고 이와 더불어 진단용 기구의 수준 향상으로 가능해진 미세 술식의 발달에 따른 결과이다. 이러한 기술의 발전은 임상검사용 검체 뿐만 아니라 DNA의 공급원으로서의 타액의 진단학적 가치를 고려하게 되었다.

본 연구는 인체의 타액에서 genomic DNA를 분리하고 이를 혈액 및 협점막 swab에서 분리한 genomic DNA와 비교 검토해 봄으로써 타액 검체의 진단학적 활용도를 살펴보고, 타액 검체의 다양한 보관 과정이 genomic DNA의 분리에 미치는 영향을 살펴보고자 시행되었으며, 또한 분리된 genomic DNA의 안정도를 살펴보고자 중합효소 연쇄반응 분석법을 이용하여 ß-globin 유전자의 증폭을 시행하였다.

10명의 피검자(평균 나이: 29.9 ± 9.8 세)를 대상으로 혈액, 비자극성, 자극성 전타액 및 협점막 swab을 채취한 후 이로부터 genomic DNA를 분리하였다. 여러 다양한 보관조건이 genomic DNA에 미치는 영향을 알아보기 위하여 건강한 20명의 피검자(평균 나이: 32.3 ± 6.6 세)를 대상으로 자극성 전타액을 채취하여 실온, 4℃, -20℃, -70℃, 자연 건조 및 동결 건조 상태에서 1, 3, 5 개월 동안 보관한 후 genomic DNA를 분리, 조사하였으며, 분리된 genomic DNA의 안정도를 살펴보고자 중합효소 연쇄반응 분석법을 이용하여 989-bp의 ß-globin 유전자를 증폭한 후 전기영동 검사를 시행하여 다음과 같은 결론을 얻었다.

1. 타액으로부터 분리한 genomic DNA의 농도는 혈액의 경우에 비하여 유의하게 낮았으며 ($p<0.05$), 타액군 간에는 유의한 차이가 없었다. 자극성 전타액과 이를 동결 건조한 검체에서 분리한 genomic DNA의 순도는 혈액의 경우에 비하여 유의하게 높았으며 ($p<0.05$), 협점막 swab으로부터 분리한 genomic DNA 의 순도는 타액의 경우에 비하여 유의하게 낮게 나타났다 ($p<0.05$).
2. 실온에서 보관한 타액 검체로부터 분리한 genomic DNA의 농도는 1 개월 후부터 점차적으로 감소되었으며, 3 개월과 5 개월 동안 보관한 타액 검체에서는 유의하게 감소되었다 (각각 $p<0.05$, $p<0.01$). DNA의 순도 또한 점차적으로 감소되어 3 개월과 5 개월 동안 보관한 타액 DNA의 순도는 신선한 타액과 1 개월 동안 보관된 타액 검체의 순도보다 낮게 나타났다 ($p<0.05$).
3. 타액 검체를 4℃와 -20℃에서 보관한 후 분리한 genomic DNA의 농도는 3 개월의 보관 기간 동안 유의한 변화가 없었으나, 보관 기간 5 개월 후의 검체에서는 유의하게 감소되었다 ($p<0.05$).
4. 타액을 -70℃에서 보관한 검체와 동결 건조한 후 보관한 검체로부터 분리한 genomic DNA의 농도는 보관 기간에 따른 유의한 차이를 보이지 않았으나, 보관 후 5 개월 후의 검체에서는 DNA의 농도가 감소되는 경향을 보였다.
5. 타액을 자연 건조한 후 즉시 genomic DNA를 분리한 결과, 신선한 타액에 비하여 약 60%의 DNA를 얻을 수 있었다. 자연 건조한 후에 실온에서 보관한 타액 검체로부터 분리한 genomic DNA 농도는 보관 2 주 만에 급격하게 감소되었다 ($p<0.05$).
6. 중합효소 연쇄반응 방법을 이용한 ß-globin 유전자의 증폭은 동결 건조한 후 보관한 타액의 경우 보관 기간 5 개월까지의 모든 검체에서 가능하였으며, 보관 기간 1 개월을 기준으로 보았을 때 -20℃ 와 -70℃에서 보관한 타액의 경우 모든 검체에서, 4℃에서 보관한 타액의 경우 일부분의 검체에서만 증폭이 가능하였고, 실온에서 보관한 타액과 자연 건조 후 실온에서 보관한 타액의 경우는 증폭이 이루어지지 않았다.

주제어 : 타액, DNA, 보관, 중합효소 연쇄반응 분석법, 안정도