Oral Microbiology Immunology 2005: 20: 128–130
Printed in Singapore. All rights reserved

Oral Microbiology and Immunology

Short communication

Impact of sample storage on detection of periodontal bacteria

J. Katsoulis[1,2], L. J. R. Heitz-Mayfield[1], M. Weibel[1], R. Hirschi[1], N. P. Lang[1], G. R. Persson[1,3,4]

[1]Department of Periodontology and Fixed Prosthodontics, School of Dental Medicine, University of Bern, Switzerland, [2]Department of Removable Prosthodontics, School of Dental Medicine, University of Bern, Switzerland, [3]Department of Periodontics, University of Washington, Seattle, WA, USA, [4]Department of Oral Medicine, University of Washington, Seattle, WA, USA

Katsoulis J, Heitz-Mayfield LJR, Weibel M, Hirschi R, Lang NP, Persson GR. Impact of sample storage on detection of periodontal bacteria.
Oral Microbiol Immunol 2005: 20: 128–130. © Blackwell Munksgaard, 2005.

Background/aims: Information on the impact of sample storage prior to analysis by DNA methods is limited. The aim of this study was to investigate the effect of subgingival sample storage on bacterial detection and enumeration.
Material and methods: Subgingival plaque samples were studied by a) checkerboard DNA–DNA hybridization by immediate processing, b) storage at +4°C for 6 weeks, c) storage at −20°C for 6 months or d) storage at −20°C for 12 months.
Results: No differences in total DNA were found between protocol 1 and 2, or between protocol 3 and 4. Protocol 1 yielded 2.4 times more total bacterial DNA than did protocol 3 ($P < 0.001$). *Actinobacillus actinomycetemcomitans* and *Campylobacter gracilis* were detected in 21.1% of the immediately processed samples but only in 6.6% of the samples after 12 months of storage. Similar changes were noticed for *Treponema denticola,* which was detected in 22.3% and 9.2%, respectively. Streptococci spp., *Fusobacterium nucleatum* and *Tannerella forsythia* did not seem to be affected by storage. In contrast, the level of *Campylobacter rectus* detection frequency changed from 2.6% if processed immediately to 15.8% if samples were stored for 12 months.
Conclusions: In longitudinal clinical studies including microbiological samples and processed with DNA–DNA hybridization methods, samples should be stored for the same period of time before processing to avoid loss of microbiological information.

Key words: checkerboard DNA–DNA hybridization; microbiology; periodontitis; storage; subgingival plaque

G. Rutger Persson, DDS, PhD, Department of Periodontology and Fixed Prosthodontics, School of Dental Medicine, University of Bern, Freiburgstrasse 7, CH-3010 Bern, Switzerland
Tel.: +41 31 632 2542;
fax: +41 31 632 4915;
e-mail: rutger.persson@zmk.unibe.ch
Accepted for publication November 9, 2004

Periodontitis is an infection caused by a mixed flora of pathogens described as a biofilm (8). The high concentration of potent enzymes in a subgingival biofilm is of concern. Degradation of DNA may occur, which may interfere with the detection of bacteria (5–7, 9). The preservation of the bacterial genome is most important in assays that utilize DNA assays for information (10). It is known that samples can be stored for a long time if they are free from bacteria and are stored by noncryogenic preservation. Such samples can be analyzed with DNA detection methods at a later time (3). It is also known that storage time and temperature affects the yield of DNA from human blood samples (2).

Many bacteria can cause degradation of exogenous DNA (4, 9). It is, however, generally believed that samples in TE buffer with NaOH and properly stored will not undergo major changes. Storage of bacterial samples in transport media (i.e. 10 mM Tris-HCl, 1 mM EDTA, pH 7.6 and NaOH) may, however, result in fragmentation of bacterial DNA (1). Long-term storage of mixed bacterial samples may therefore result in false test results. After storage of samples for 2 or more years at −70°C, Moncla et al. (5) reported a loss of nucleic acid of up to 15%. Studies have also shown that if stored in weak ionic solutions, the stability of *Treponema pallidum* mitochondrial DNA fragments is affected (1). The complex mixture of bacteria in subgingival plaque samples provides a wide variety of DNA degrading products that might have an impact on the detection of bacteria by DNA processes.

Logistics issues may prevent immediate processing of microbial samples from longitudinal dental studies. Samples may therefore be in storage for different lengths of time before they can be analyzed. The aim of this pilot study was to investigate how storage of subgingival plaque samples over time affects species identification and semiquantification when analyzed by the checkerboard DNA–DNA hybridization technique.

In this pilot study, bacterial plaque samples were collected from seven subjects diagnosed with chronic periodontitis. They were scheduled to receive periodontal therapy at the Department of Periodontology and Fixed Prosthodontics, University of Bern, Switzerland. No subject-identifiable information was gathered for the purpose of study. All subjects had

at least five sites with a probing depth of 6 mm. No periodontal therapy was performed before sampling. After removal of supragingival plaque, the subgingival plaque samples were collected by sterile curettes from five different sites with a probing depth of 6 mm. Samples from each subject were pooled into an Eppendorf tube containing 0.75 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). Within 30 min after sampling, 0.5 ml 5 M NaOH was added to each tube ($n = 19$). The contents of these Eppendorf tubes were then equally allocated to four Eppendorf tubes (19×4) with 0.25 ml in each. Samples were then processed according to one of the following protocols:

1 the same day as sampled;
2 after 6 weeks of storage at +4°C;
3 after 6 months of storage at −20°C;
4 after 12 months of storage at −20°C.

A total of 40 bacterial strains in 76 samples per storage protocol were included in the analysis. Samples were processed as described elsewhere (8, 9). Briefly, bacterial DNA was extracted, concentrated on nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany) and fixed by cross-linking using ultraviolet light (Stratalinker 1800, Stratagene, La Jolla, CA). The membranes with fixed DNA were placed in a Miniblotter 45 (Immunetics, Cambridge, MA). A 30×45 'checkerboard' pattern was produced, as previously described (8, 9). Chemiluminescent signals were detected using the Storm Fluor-Imager (Storm 840, Amersham Biosciences, Piscataway, NJ). The digitized information was analyzed by the IMAGEQUANT software program (version 2.00, Amerham Pharmacia Biotech AB, Stockholm, Sweden). Signals were converted to absolute counts by comparisons with known standards.

The Kruskal–Wallis ANOVA AND the nonparametric Mann–Whitney *U*-test were used to delineate group differences. Linear regression analysis was performed to detect interdependence of variables. *P*-values = 0.05 were considered to suggest statistically significant differences. The SPSS 11.5.1 statistical PC software program was used for data analysis (SPSS, Chicago, IL).

Table 1 presents the variations of retrieved total microbial DNA by storage protocol. One-way ANOVA demonstrated differences between protocols for total DNA retrieved ($F = 12.8$, $P < 0.001$, Bonferroni *post hoc* test). Protocol 1 (mean value 185.2, SD 94.9, range 11.6–361.6) and protocol 2 showed no difference in total DNA. Similarly, no differences in total DNA retrieved were found between 6 (mean value 76.8, SD 39.9, range 11.2–142.6) and 12 months' storage. Thus 2.4 times less total bacterial DNA could be identified in the samples stored for 6 and 12 months compared with the immediately processed samples ($P < 0.001$).

Table 1. Total microbial DNA (counts $\times 10^5$) in each of the samples ($n = 19$), mean values and standard deviation (SD) for the four different storage protocols (1: Processed the same day. 2: Stored for 6 weeks at +4°C. 3: Stored at −20°C for 6 months. 4: Stored at −20°C for 12 months) and difference for total DNA between protocols 1 and 3 (F = Factor of decrease)

	Protocol				
Sample	1	2	3	4	$F_{(1 \text{ vs. } 3)}$
Total	3519.3	3491.2	1458.5	1225.7	2.5
Mean	185.2	183.7	76.8	64.5	2.4
SD	94.9	118.2	39.9	34.8	

Table 2 shows the frequencies of positive samples for targeted microbial species in protocols 1 and 4. The threshold detection level was defined at the 1×10^5 DNA level. *Actinobacillus actinomycetemcomitans* and *Campylobacter gracilis* were identified in 21.1% of the samples by protocol 1, but in only 6.6% by protocol 4. *Treponema denticola* was detected in 22.3% if processed immediately but in only 9.2% of the samples stored for 12 months. Various *Streptococci* spp., *Fusobacterium nucleatum* and *Tannerella forsythia* were virtually unaffected by storage. The decrease in the detection frequency for *Porphyromonas gingivalis* was 4% after 6 or 12 months' storage. In contrast, the detection frequency for *Campylobacter rectus* increased from 2.6% following protocol 1 to 15.8% following protocol 4.

The following bacteria either were found at the same detection frequency or differed only by a few observations: *Actinomyces israelii, Actinomyces naeslundii, Actinomyces viscosus, Actinomyces gerencseriae, Campylobacter gracilis, Campylobacter showae, Eikenella corrodens, Eubacterium nodatum, Eubacterium saburreum, Gemella morbillorum, Leptotrichia buccalis, Fusobacterium nucleatum sp. nucleatum, Fusobacterium nucleatum sp. polymorphum, Fusobacterium nucleatum sp. vincentii, Fusobacterium periodonticum, Micromonas micros, Prevotella intermedia, Prevotella nigrescens, P. gingivalis, Propionybacterium acnes* type I and II, *Streptococcus anginosus, Streptococcus constellatus, Streptococcus gordinii, Streptococcus intermedius, Streptococcus mitis, Streptococcus sanguis, T. forsythia,* and *Treponema socranskii.*

Figure 1 demonstrates the relationship between the total DNA in the sample and the amount of *P. gingivalis*. The R^2 values varied between 0.60 for the immediately processed sample to 0.72 for the sample stored for 6 months'. The R^2 value for the relationship for *P. gingivalis* in relation to total DNA was 0.69.

Dilution errors during the preparation of the aliquots could potentially have had an impact on the results. Linear regression analyses demonstrated that storage alone and not the bacterial content variation by vial ($n = 19$) explained the differences in DNA ($P < 0.001$). Analysis of the membranes and reanalysis of scanned reference

Table 2. Number (max. 19) of positive samples (i.e. counts > 1.0×10^5) for the four different storage protocols (1: Processed the same day. 4: stored at −20°C for 12 months) arranged in complexes of pathogens

		Protocol	
ATCC	Type strain	1	4
43718 +29523	*Actinobacillus actinomycetemcomitans b + a*	16	5
17929	*Actinomyces odontolyticum I*	14	6
33624(27)	*Capnocytophaga gingivalis*	16	5
33596(25)	*Capnocytophaga ochracea*	14	7
33238(371)	*Campylobacter rectus*	2	10
33612 (4)	*Capnocytphaga sputigena*	17	7
19696	*Neisseria mucosa*	17	10
25845	*Prevotella melaninogenica*	14	6
43541	*Selenomonas noxia*	16	6
B1	*Treponema denticola*	17	7
10790	*Veillonella parvula*	15	7

ATCC, American Type Culture Collection.

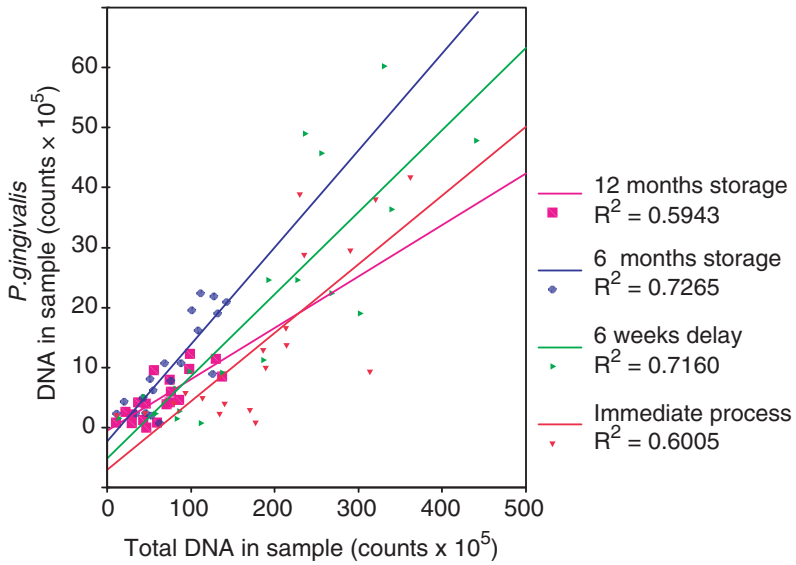

Fig. 1. Scatterplot diagram demonstrating different patterns of relationships between total DNA in samples, by the DNA–DNA checkerboard accounting for the amounts of *P. gingivalis* in samples and the effect of the four different storage models. R^2 values are provided.

standards clearly demonstrated that the laboratory processing of the reference standards showed high reproducibility, with less than 1% deviation for any of the pathogens included in the assay.

Most longitudinal clinical studies do not provide information on the length of storage before processing with checkerboard DNA–DNA hybridization methods. The present study demonstrated a significant impact on long-term storage of some subgingival bacterial samples processed with the checkerboard DNA–DNA hybridization method. The negative impact of storage differed by bacterial species. A standardized storage protocol should therefore be used to assure comparable qualitative and quantitative results by DNA–DNA hybridization with genomic probes.

Acknowledgments

The present study was supported by the Clinical Research Foundation (CRF), University of Bern, Switzerland.

References

1. Abe T, Takano H, Sasaki N, Mori K, Kawano S. *In vitro* DNA fragmentation of mitochondrial DNA caused by single-stranded breakage related to macroplasmodial senescence of the true slime mold, *Physarum polycephalum*. Curr Genet 2000: **37**: 125–135.
2. Cushwa WT, Medrano JF. Effect of blood storage time and temperature on DNA yield and quality. Biotechniques 1993: **14**: 204–207.
3. Kilpatrick CW. Noncryogenic preservation of mammalian tissues for DNA extraction: an assessment of storage methods. Biochem Genet 2002: **40**: 53–62.
4. Kruszewska H, Misicka A, Chmielowiec U. Biodegradation of DNA and nucleotides to nucleosides and free bases. Il Farmaco 2004: **59**: 13–20.
5. Moncla BJ, Braham P, Dix K, Watanabe S, Schwartz D. Use of synthetic oligonucleotide DNA probes for the identification of *Bacteroides gingivalis*. J Clin Microbiol 1990: **28**: 324–327.
6. Mousques T, Listgarten MA, Stoller NH. Effect of sampling on the composition of the human subgingival microbial flora. J Periodontal Res 1980: **15**: 137–143.
7. Sbordone L, Ramaglia L, Gulletta E, Iacono V. Recolonization of the subgingival microflora after scaling and root planing in human periodontitis. J Periodontol 1990: **61**: 579–584.
8. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. Periodontol 2000 2002: **28**: 12–55.
9. Socransky SS, Haffajee AD, Smith C, Martin L, Haffajee JA, Uzel NG, et al. Use of checkerboard DNA-DNA hybridization to study complex microbial ecosystems. Oral Microbiol Immunol 2004: **19**: 352–362.
10. Tuttle RM, Waselenko JK, Yosseffi P, Weigand N, Martin RK. Preservation of nucleic acids for polymerase chain reaction after prolonged storage at room temperature. Diagn Mol Pathol 1998: **7**: 302–309.
11. Villanueva AV, Podzorski RP, Reyes MP. Effects of various handling and storage conditions on the stability of *Treponema pallidum* DNA in cerebrospinal fluid. J Clin Microbiol 1998: **36**: 2117–2119.
12. Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. J Clin Periodontol 2000: **27**: 648–657.