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Proportional distribution of the red complex and its individual pathogens after sample storage using the checkerboard DNA–DNA hybridization technique

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Abstract

Background: Information on the impact of sample storage prior to analysis by DNA methods is limited.

Aims: To investigate the effect of microbial sample storage on bacterial detection and proportional distribution of the red complex and its individual pathogens.

Material and Methods: Subgingival plaque samples were analysed by (1) immediate processing, (2) after storage at +4°C for 6 weeks, (3) after storage at −20°C for 6 months or (4) after storage at −20°C for 12 months using the checkerboard DNA–DNA hybridization.

Results: Proportional distribution of the red complex did not differ between the first three protocols. However, the total bacterial DNA for pathogens studied decreased significantly in protocols 3 and 4. Relative amounts of *Tannerella forsythensis*, *Porphyromonas gingivalis* and *Treponema denticola* remained stable in the second protocols and changed in an unpredictable way if stored for 6 or 12 months.

Conclusions: Results from samples stored for maximum 6 months at −20°C with high proportional amounts of the red complex and *T. denticola* may be used as an indicator of persistence. All bacterial samples for DNA extraction should be processed following a standardized storage protocol (i.e. samples stored at +4°C for maximum 6 weeks) in order to get comparable qualitative and quantitative results for total DNA, bacterial complexes and individual pathogens. Most representative results are yielded if processing and hybridization could be performed immediately after sampling.

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

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The aetiology of periodontal disease has been investigated using different methods for the detection and enumeration of microbial pathogens (i.e. Listgarten 1992, van Steenbergen et al. 1993, Chen & Slots 1999). The patterns of bacterial composition in the oral cavity and in the dental plaque have a major effect on the tissues surrounding the teeth. Bacteria involved in the aetiology of gingivitis include, predominantly, species of *Streptococcus*, *Fusobacterium*, *Actinomyces*, *Veillonella*, and *Treponema* and possibly *Bacteroides*, *Capnocytophaga*, and *Eikenella*. Microbial colonization is sequential, with the complexity of the associated flora increasing with time (Page 1986). Altered environmental conditions in progressive periodontitis can be selective for individual pathogens (Slots & Ting 1999). In deep pockets with anaerobic conditions bacterial composition change towards pathogens with potency to periodontal destruction, such as *Tannerella forsythensis*, *Porphyromonas gingivalis*, *Treponema denticola*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* (Haffajee & Socransky 1986, Socransky et al. 1998). Some of the pathogens are able to invade in the surrounding tissues and are thus difficult to eradicate (Sbordone et al. 1990).

In studies evaluating different treatment modalities based on bacterial composition at infected sites, a large volume of samples from different sites have to be collected for analysis. The checkerboard DNA–DNA hybridization method was developed to allow analysis of large numbers of clinical samples. However, depending on the distance between the clinic and the microbiology laboratory and its capacity, samples may have to spend different lengths of time on the way to, or in the laboratory and be stored at the laboratory before processing. In a clinical environment it is almost impossible to process samples immediately after collection or possible to store samples in freezers with temperatures below -18°C. The checkerboard DNA–DNA hybridization technology allows for a rapid and simultaneous processing of samples (Socransky et al. 1994). The method does not require bacterial viability, which facilitates transportation of samples. There is limited to no information on how storage conditions may influence the interpretation of DNA analysis of clinical plaque samples.

Thus the aim of this study was to investigate the effect of subgingival plaque samples storage over different lengths of time and under different conditions on the proportional distribution of bacterial complexes with focus on pathogens of the red complex (Socransky & Haffajee 2002) and individual pathogens using the checkerboard DNA–DNA hybridization technique.

Material and Methods

Subjects

Periodontal examination of seven subjects was performed in the Department of Periodontology and Fixed Prosthodontics at the University of Bern to identify clinical sites to be sampled. They were recruited among the patients referred for dental therapy to the Department. Because the aim of the study was strictly confined to a comparison between samples for assessing the composition of the subgingival microbiota no particular inclusion criteria, other than diagnosis of chronic periodontitis with at least five sites with probing depth (PD) $\geqslant$6 mm and willingness to participate were applied. The examination included a full-mouth PD assessment. No periodontal therapy was performed prior to sampling, which occurred at a time point later than the initial examination.

Microbiological assessment

Sample collection and transport/ storage media

After removal of supragingival plaque, subgingival plaque samples from five different sites with PD $\geqslant$6 mm were collected by performing one gentle stroke in the pocket using a sterile curette for each site. Samples were pooled by placing them into an Eppendorf tube containing 0.75 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). All samples (19×5 sites) were collected the same day by the same clinician. Within 30 min. after sampling 0.5 ml 5 M NaOH was added to each tube ($N = 19$). The contents of the Eppendorf tubes were then equally aloquated into four Eppendorf tubes with 0.25 ml in each. The samples in these tubes (19×4) allowed for the four different methods of processing.

Protocol and storage conditions

The following four protocols were used by the checkerboard DNA–DNA hybridization method (Fig. 1):

(1) Immediate processing of the sample on the day of sampling.
(2) The sample was stored at $+4^{\circ}$C for 6 weeks before processing.
(3) The sample was stored at -20°C for 6 months before processing.
(4) The sample was stored at -20°C for 12 months before processing.

DNA probes

A total of 40 bacterial strains were included in the analysis (Socransky et al. 1994). From these bacteria the DNA was extracted and used as probes. The bacteria were grown at 35°C under anaerobic conditions (85% N_2, 5% CO_2, 10% H_2) for 3–7 days and on Trypticase soy agar supplemented with 3% defibrinated sheep blood. The bacteria were then placed in 1000 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6) and washed twice by centrifugation in TE buffer at 1027 *g* for 10 min. The DNA precipitation was obtained as described elsewhere (Socransky et al. 1994). Whole genomic DNA probes were prepared from each of the 40 test strains by labelling 1 µg DNA with Digoxigenin using a random primer technique.

DNA–DNA hybridization

The samples were analysed for their content of 40 subgingival species consistent with a publication by Socransky & Haffajee (2002). Briefly, bacterial DNA was extracted, placed in the channels of a Minislot device (Immunetics, Cambridge, MA, USA) and concentrated on nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany) by vacuum aspiration. The bacterial DNA was fixed by cross-linking using ultraviolet light (Stratalinker 1800, Stratagene, La Jolla, CA, USA). The membranes with fixed DNA were placed in a Miniblotter 45 (Immunetics). A 30×45 ''checkerboard'' pattern was produced.

Detection and enumeration

Signals were detected by chemiluminescence. Following the final laboratory preparations the signals were detected using the Storm Fluor-Imager (Storm 840, Amersham Biosciences, Piscataway, NJ, USA) and setup at 200 μm and 600 V. This technique allowed the determination of the intensity of the fluorescence.

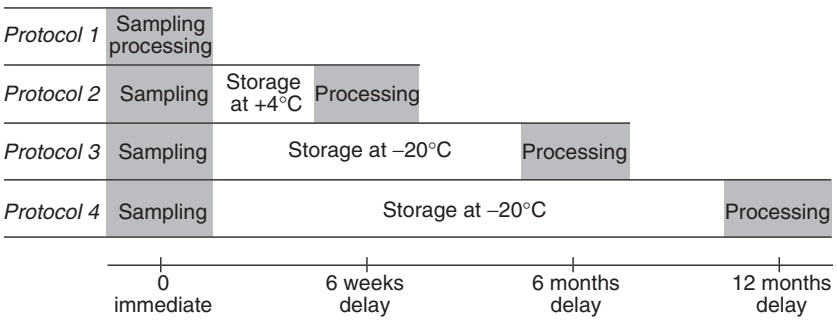

Fig. 1. Time schedule for the four different protocols and storage conditions of the samples. Each protocol used 19 samples for the checkerboard DNA–DNA hybridization method.

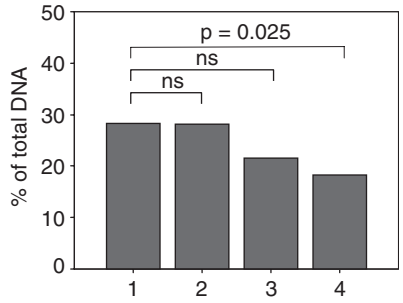

Fig. 2. Bar chart describing proportional distribution of the red complex and statistical significance by mean proportional DNA (Mann–Whitney *U*-test) for the four different storage protocols (1, immediate process; 2, 6 weeks delay +4°C; 3, 6 months delay −20°C; 4, 12 months delay −20°C; NS, no significant difference).

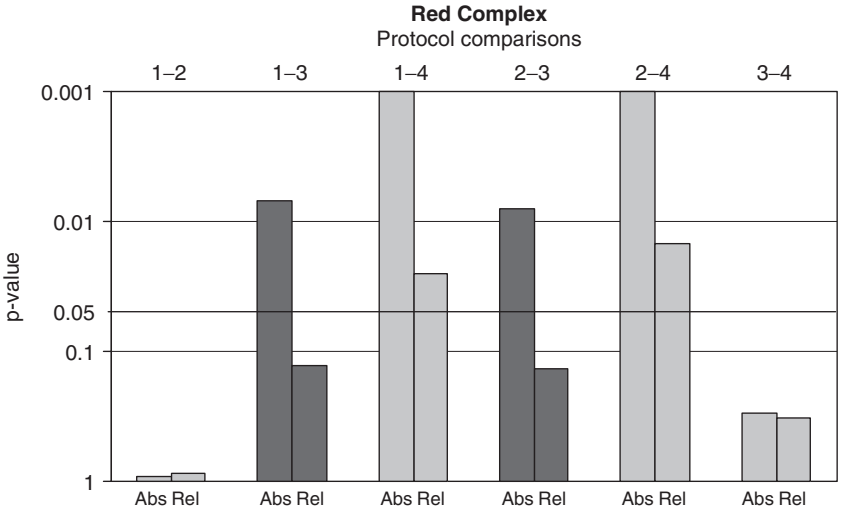

Fig. 3. Protocol comparisons showing *p*-values of the statistical analysis (non-parametric Mann–Whitney *U*-test) using absolute DNA counts (abs) and relative amounts (rel) for the red complex (1, immediate process; 2, 6 weeks delay +4°C; 3, 6 months delay −20°C; 4, 12 months delay −20°C).

After scanning the membranes, pictures of the membranes were printed for documentation purposes. In order to receive a fully detailed account and composition of the identified bacteria, the digitized information was analysed by a software program (ImageQuant, Amersham Pharmacia, Piscataway, NJ, USA). The program allowed comparison of the density 19 sample lanes for each of the 40 probes studied against the two standard lanes (10^5 or 10^6 cells). Signals were converted to absolute counts by comparisons with these standards. Quantitative results were obtained as absolute counts. The sum of DNA accounted from each individual pathogenic strain studied was defined as total DNA.

Reference standards

Channel 29 and 30 of the Minislot device were used for the two reference standards containing 1 ng (10^5 cells) and 10 ng (10^6 cells) of each of the 40 probes. Because of laboratory conditions it was necessary to process new standards for the checkerboard DNA–DNA hybridization assays for the 6 and 12 months samples. Therefore the previous and the new standards were tested against each other in replicated assays. For each of the 40 pathogens studied the signal strengths were compared and found to agree to 100% for total DNA amounts and within 99.8% to 100% for the 40 individual pathogens.

Statistical methods

The Kruskal–Wallis ANOVA and non-parametric Mann–Whitney *U*-test were used to test for group differences. Linear regression analysis was performed to detect interdependence of variables. Statistical significance was declared if *p*-values were $\leqslant 0.05$. The SPSS 11.5.1 statistical PC software program was used for data analysis (SPSS 11.5.1, SPSS, Chicago, IL, USA).

Results

Red complex

Analysis of the data based on six complexes of pathogens (Socransky & Haffajee 2002) and a seventh group (grey complex) combined for all other pathogens among the 40 strains studied demonstrated different effects on storage model used. A statistically significant difference (non-parametric Mann–Whitney *U*-test) in the proportions of the red complex was found between the first (baseline) and fourt (12 months delay at −20°C) protocol (Fig. 2). Although the mean total DNA between protocols 1 and 3 or protocols 2 and 3 decreased ($p < 0.001$), the mean proportional distribution of the red complex (i.e. *T. forsythensis*, *P. gingivalis* and *T. denticola*) did not differ significantly between the first three protocols (protocol 1: mean value 28.3 %, SD 14.2, range 1.9–54.7; protocol 2: mean value 28.1%, SD 12.9, range 2.0–46.8; protocol 3: mean value 21.5%, SD 10.1, range 1.2–36.7). However analysis of the absolute counts of the red complex showed other results for the protocol comparisons (Fig. 3).

Although total DNA between protocols 1 and 2 did not differ, hierarchical cluster analysis illustrated different patterns of interrelationship between the complexes of pathogens. Likewise although total DNA amounts were not statistically significant between protocols 3 and 4, cluster analysis suggested that different patterns between complexes of pathogens emerged (Fig. 4).

T. forsythensis, P. gingivalis, T. denticola

Linear regression analysis demonstrated that storage alone was the explanatory variable to the differences in mean total DNA for *T. forsythensis* ($p < 0.001$), *P. gingivalis* ($p < 0.01$) and *T. denticola* ($p < 0.001$). Variation in total DNA for the individual pathogens within each of the 19 sets of the four protocols remained similar. The different patterns in the absolute amounts and relative proportions of bacteria identified by the DNA–DNA checkerboard hybridization method are illustrated (Fig. 5). Different patterns of statistical significance (non-parametric Mann–Whitney *U*-test) were found between the four storage protocols for the total amounts and the proportions of the individual pathogens. Differences emerged between protocol comparisons using the total amount and those using the proportional distribution (Fig. 6). Thus, comparing protocol 1 with protocol 2 no differences in the mean total DNA (absolute counts) could be identified

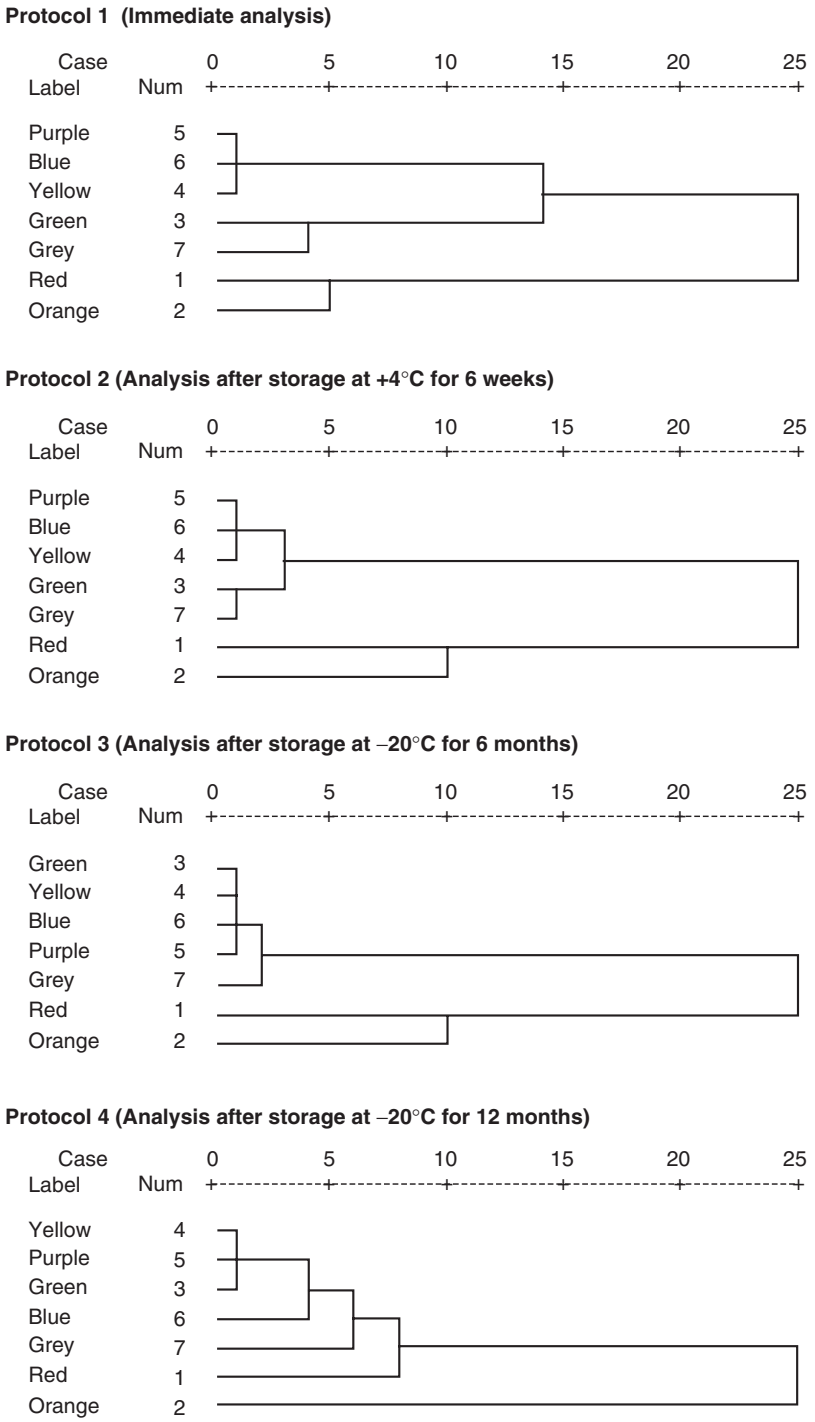

Fig. 4. Dendrograms illustrating the average linkage between groups of complexes (hierarchical cluster analysis, rescaled distance cluster combine) for the four different storage protocols.

for *T. forsythensis* ($p = 0.908$), *P. gingivalis* ($p = 0.525$) and *T. denticola* ($p = 0.201$). Likewise relative amounts of the first and the second protocol were stable (*T. forsythensis*: $p = 0.644$; *P. gingivalis*: $p = 0.172$; *T. denticola*: $p = 0.181$). Similar patterns were found for the bacteria in the other complexes (Table 1). Proportional distribution for the third protocol compared with baseline was different for *P. gingivalis* ($p = 0.006$) although total counts did not change ($p = 0.665$). In contrast, differences comparing protocol 1 (baseline) with protocol 4 were significant for the absolute counts ($p = 0.034$) but not for the relative proportion ($p = 0.583$) of *P. gingivalis*. Absolute and relative amounts of *T. denticola* were stable comparing protocol 1 with protocol 3, but both different for the comparison of protocol 1 *versus* 4 ($p = 0.001$ and 0.027). For *T. forsythensis* in all comparisons (absolutes and relatives) of protocol 1 *versus* 3, and 1 *versus* 4, respectively, significant differences emerged ($p < 0.002$).

Discussion

In the absence of available data on the effect of different bacterial storage methods and under the perception that bacterial DNA sequences in plaque samples are stable attention to details on storage length may have been ignored. Information from clinical periodontal microbiological studies on storage protocol is often not presented. Thus, samples may have been stored frozen at different temperatures varying from room temperature, $+4^{\circ}C$ to $-80^{\circ}C$ which may have an impact on the results and their interpretations (i.e. van Steenbergen et al. 1993, Piccolomini et al. 1998). In most clinics it would be very difficult to store samples at anything colder than $-20^{\circ}C$. The transport of samples also provides challenges in that dry ice and express mail services is often the only method available to deliver clinical bacterial samples to the laboratory. Such a transport procedure may in itself cause variations of storage temperature, which may have an impact on bacterial DNA stability. The present study has demonstrated that there is bacterial DNA stability in samples if samples can be transported to the laboratory including being processed within a time period of up to 6 weeks and stored at temperature $+4^{\circ}C$ in proper media and aimed for the checkerboard DNA–DNA hybridization method. It should be noted that the total DNA (sum of DNA from each pathogen studied) only reflects the DNA from pathogens selected for analysis. Thus the absolute complete amount of DNA in samples remains by this method unknown.

Assuming that baseline and study endpoint samples are processed at the same time in order to process samples under standardized conditions the baseline data will falsely demonstrate less total bacterial and individual bacterial DNA and a different pattern of bacterial distribution than the endpoint samples. The endpoint sample having lost no bacterial DNA and with a different pattern

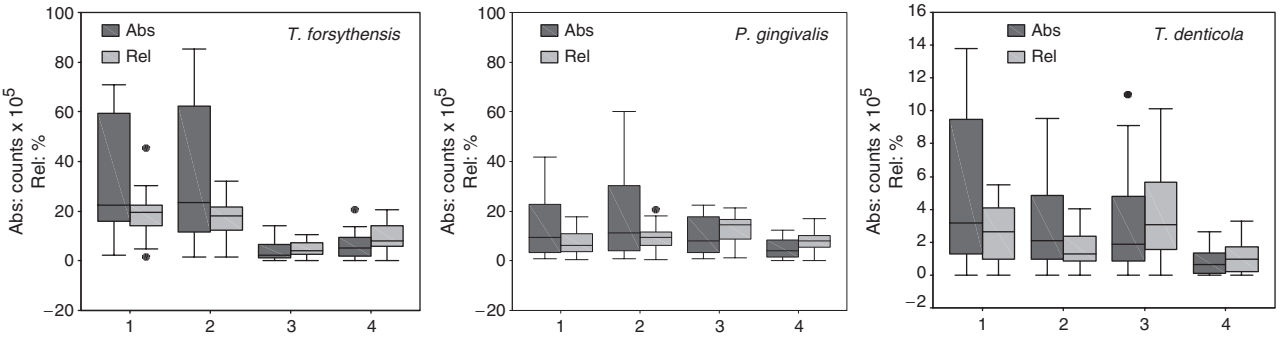

Fig. 5. Boxplot diagrams presenting total absolute DNA counts (abs) and relative amounts for *Tannerella forsythensis*, *Porphyromonas gingivalis* and *Treponema denticola* representing the red complex for the four different storage protocols (• , outlier; 1, immediate process; 2, 6 weeks delay +4°C; 3, 6 months delay −20°C; 4, 12 months delay −20°C).

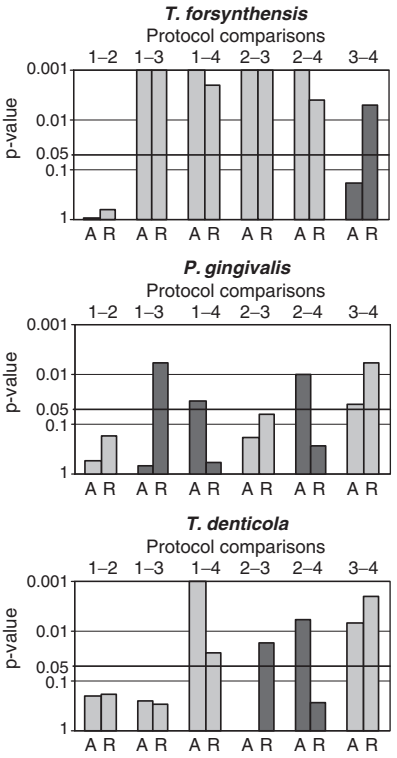

Fig. 6. Protocol comparisons showing *p*-values of the statistical analysis (non-parametric Mann–Whitney *U*-test) using absolute DNA counts (A) and relative amounts (R) for the individual pathogens (1, immediate process; 2, 6 weeks delay +4°C; 3, 6 months delay −20°C; 4, 12 months delay −20°C).

Table 1. Protocol comparisons with statistical differences by mean total DNA (one-way ANOVA, post hoc Bonferroni) and by mean absolute counts (abs) or proportional amounts (rel) of complexes and selected individual pathogens (non-parametric Mann–Whitney *U*-test) between the four different storage protocols

		1 *versus* 2		1 *versus* 3		1 *versus* 4		2 *versus* 3		2 *versus* 4		3 *versus* 4	
		+/−	*p*	+/−	*p*	+/−	*p*	+/−	*p*	+/−	*p*	+/−	*p*
Total DNA	100%		1.000	−	*0.001*	−	*0.000*	−	*0.001*	−	*0.000*		1.000
Red	Abs		0.908	−	**0.007**	−	**0.000**	−	**0.008**	−	**0.001**		0.297
	Rel		0.883		0.130	−	**0.025**		0.138	−	**0.015**		0.325
Orange	Abs		0.133	+	**0.028**	−	**0.008**	−	**0.001**	−	**0.000**		0.613
	Rel	+	**0.000**	+	**0.045**	+	**0.005**	−	**0.005**	−	**0.020**		0.503
Green	Abs	−	**0.002**	−	**0.000**	−	**0.000**	−	**0.001**	−	**0.000**		0.208
	Rel	−	**0.002**	−	**0.000**	−	**0.000**		0.285		0.123		0.330
Yellow	Abs		0.536		0.273	−	**0.019**		0.757		0.061		0.121
	Rel		0.998	+	*0.013*	+	*0.022*	+	*0.007*	+	*0.012*		0.330
Purple	Abs		0.665		0.544	−	*0.018*		0.795		0.138	−	*0.032*
	Rel		0.624	+	**0.002**	+	**0.040**	+	**0.012**		0.075		0.325
Blue	Abs		0.258		0.189		0.297	+	**0.029**		0.091		0.916
	Rel		0.271	+	*0.000*	+	*0.000*	+	*0.000*	+	*0.000*		0.685
Grey	Abs	−	*0.002*	−	*0.000*	−	*0.000*		0.533		0.313		0.653
	Rel	−	**0.007**		0.518		0.290	+	**0.014**	+	**0.001**	+	**0.032**
TF	Abs		0.908	−	**0.000**	−	**0.000**	−	**0.000**	−	**0.000**		0.191
	Rel		0.644	−	*0.000*	−	*0.002*	−	*0.000*	−	*0.004*	+	*0.005*
PG	Abs		0.525		0.665	−	*0.034*		0.191	−	*0.010*	−	*0.040*
	Rel		0.172	+	**0.006**		0.583		0.065		0.271	−	**0.006**
TD	Abs		0.201		0.258	−	**0.001**		1.000	−	**0.006**	−	**0.007**
	Rel		0.181		0.297	−	*0.027*	+	*0.017*		0.271	−	*0.002*
PI	Abs		0.246	−	*0.032*		0.065	−	*0.003*	−	*0.006*		0.751
	Rel		0.085		0.773		0.385	−	**0.050**		0.284		0.284
AA	Abs		0.298	−	**0.000**	−	**0.000**	−	**0.004**	−	**0.000**	−	**0.007**
	Rel		0.245		0.143	−	**0.000**		0.663	−	**0.001**	−	**0.001**

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; TF, *Tannerella forsythensis*; PG, *Porphyromonas gingivalis*; TD, *Treponema denticola*; PI, *Prevotella intermedia*; AA, *Actinobacillus actinomycetemcomitans*.

of bacterial distribution together with the baseline data falsely suggest demonstrate that a specific antibacterial clinical procedure might be ineffective whereas in reality it was effective. The present data suggested that, at least, a standardized storage protocol should be used for longitudinal studies of microbiological samples aimed for the checkerboard DNA–DNA hybridization method.

Another major problem demonstrated in the present study was the fact that there appears to be different storage effect impact on different types of bacteria. This may lead to misinterpretation of study results based on proportional distributions of bacteria involving microbial samples consisting of bacterial samples containing large volumes of mixed bacteria. However, the proportional distribution within the red complex and its individual pathogens (*T. forsythensis*, *P. gingivalis* and *T. denticola*) remains stable as demonstrated between baseline and 6 weeks samples. This would be of importance to clinicians in private practice because they are depending on quick analysis for the therapy to be continued as soon as possible. However, comparing baseline

with 6 or 12 months data for pathogens in the red complex would yield different results. Thus, *T. denticola* would yield similar results and remain stable but the proportional distribution of *P. gingivalis* would increase and the proportion of *T. forsythensis* would decrease in relation to total DNA in sample. This would have significant consequences for longitudinal studies focusing on the distribution of these three pathogens.

Bacteria seem to influence each other during storage. Studies have shown that several bacteria possess the capacities to degrade exogenous DNA to nucleic bases or their derivates (Ahmad et al. 1995, Kruszewska et al. 2004). Such degradation may initiate quickly or may proceed over some time. Subgingival plaque samples constitute one of the largest varieties of pathogens known. It is quite possible that among these pathogens there may be those that have the capacity to alter the DNA to such an extent that the checkerboard DNA–DNA hybridization methods fails to recognize target pathogens once the DNA is degraded.

Research in medical genetics may frequently involve freezing of large numbers of blood samples for subsequent DNA isolation and analysis. Storage time and temperature affects the yield of DNA from human blood apparently consistent with the present findings regarding loss of microbial DNA (Cushwa & Medrano 1993). There are no studies that have explored what causes such changes. The design of the present study did not allow analysis to determine whether changes in DNA accounted for was the result of storage time only or a combination freezing condition and storage. Further studies are necessary to determine storage conditions that would allow long-term preservation of bacterial DNA and without distortion of individual bacterial strain DNA. In our laboratory, however, purified bacterial strains can be stored frozen for 2 or more years without or with minimal loss of bacterial DNA. The changes in bacterial DNA reported here is most likely a result of unknown proteolytic activities that degrade bacterial DNA. This degradation would then differ from sample to sample dependent on the composite degradation activities as a result of bacteria present in the sample. Hence, it might not be possible to control for such degradation of bacterial DNA.

The impact of inadvertently collected blood together with the bacterial sample is not clear. One might speculate that blood could provide selective growth conditions for specific pathogens. The presence of blood in sample may have an impact on the absolute and relative amounts of some pathogens.

It can be concluded that the proportional distributions for the red complex and its individual pathogens (*T. forsythensis*, *P. gingivalis* and *T. denticola*) remains constant comparing the immediately processed samples within 6 weeks of storage but will differ if stored for 6 or 12 months. All bacterial samples for DNA extraction should be processed following a standardized storage protocol (i.e. samples stored at +4°C for maximum 6 weeks) in order to get comparable qualitative and quantitative results for total DNA, bacterial complexes and individual pathogens.

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