Effect of saliva processing on bacterial DNA extraction

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SUMMARY

More than 700 bacterial species inhabit oral cavity of humans. Various oral diseases are related to changes in the structure of this complex community. Their pathogenesis can, thus, be better understood by study of oral microbial flora. As many bacteria are refractory to cultivation, molecular approaches based on PCR followed by downstream analysis are more suitable for community analysis than culture dependent methods. Effective DNA extraction from the sample matrix is a fundamental part of the pre-analytical phase but it can be influenced by processing of the starting material.

The aim of this study was to analyze the effects of saliva processing on DNA extraction using several non-commercial isolation procedures. Bacterial chromosomal DNA was extracted from three different sample matrices: fresh saliva, diluted saliva and pelleted saliva using four different extraction methods: phenol chloroform protocol, benzyl-chloride protocol, extraction with Chelex-100 and extraction with Triton X. Extraction from different saliva samples and the use of different extraction methods significantly affected the effectiveness of DNA extraction.

The most suitable material for bacterial DNA extraction for molecular analysis is a fresh saliva sample. The most effective methods for isolating salivary DNA are the benzyl-chloride protocol and Chelex-100 extraction. Our results have implications for studies concentrating on salivary microbiome and its role in the pathogenesis of oral diseases.

KEY WORDS: Saliva, DNA extraction, Microbiomics, T-RFLP

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INTRODUCTION

Microbial flora of oral cavity consists of more than 700 bacterial species (Aas *et al.,* 2005). The composition of oral microflora is influenced by factors such as diet, host defense, antibiotic therapy and other endogenous and exogenous factors (Ruby and Barbeau, 2002). Due to a dynamic balance resulting from the interaction between microorganisms and their host the oral microflora remains relatively stable. Changes in structure or diversity of oral microflora can participate in the pathogenesis of several oral diseases or be induced by pathological conditions such as squamous carcinoma. This might be explained by changes in present adhesive molecules and bacterial tropism (Mager *et al.,* 2005). The variability of microbial flora is associated with the most prevalent oral diseases (periodontitis and dental caries), although the exact causal relationships are far from being clear (Takeshita *et al.,* 2008). In addition, epidemiological evidence shows that oral microbiota are related to systemic diseases including cardiovascular diseases or allergy (Arbes *et al.,* 2006; Mattila *et al.,* 2005; Pussinen *et al.*, 2003). Although the majority of bacteria living in the human oral cavity are commensals, some of them can act as opportunistic pathogens and cause systematic disease, e.g. bacterial endocarditis (Berbari *et al.,* 1997) or aspiration pneumonia (Paju and Scannapieco, 2007).

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Evaluation of structural and dynamic changes in oral microflora has, thus, diagnostic value and enables better understanding of the pathogenesis and a choice of treatment based on specific diagnostics and analysis of its effectiveness. Cultivation dependent and cultivation independent methods are used to study oral microbial flora. The first group has several limitations. Many microorganisms are refractory to cultivation: it is estimated that more than 98% of bacteria from the environment cannot be cultured in vitro. In the case of oral microflora about 50% of bacterial species are uncultivable according to published studies (Aas *et al.,* 2008; Wade, 2002). This leads to underestimation of diversity of bacterial community using cultivation dependent approaches (Amann *et al.,* 1995). Moreover, any change in cultivation conditions can lead to selective growth of some bacteria and can, thus, affect the final result of structure analysis. Cultivation independent techniques are more suitable for microbial community analysis. Molecular approaches are based on PCR followed by downstream analysis of various genetic markers such as the 16S rDNA (Auguet *et al.,* 2008; Terefework *et al.,* 2008). Recent advances in high-throughput sequencing have induced efforts to use this technology to analyze oral microbiome in health and disease (Dewhirst *et al.*, 2008; Nasidze *et al.,* 2009). Regardless of the type of downstream analysis, effective DNA extraction from the sample matrix is a fundamental part of the pre-analytical phase considerably influencing the outcome of studies of microbial diversity (Hartmann and Widmer, 2008).

Saliva contains DNA from microbes residing on all oral surfaces making it an optimal diagnostic material for the analysis of the microbial flora (Sakamoto *et al.,* 2003). Moreover, the collection of saliva is non-invasive, can be repeated and is well tolerated by most patients. Saliva can be also used to analyze various biochemical parameters such as steroid levels (Fassett *et al.,* 2008), oxidative stress markers (Eisenberg *et al.,* 2008), and markers of inflammation such as C-reactive proteins, C3 and C4 complement components (Aurer *et al.,* 2005). These analyses require processing of saliva samples including freezing and thawing, diluting or centrifugation, especially to decrease the viscosity and to ease handling of samples.

The aim of this study was to analyze how processing of saliva samples affects DNA extraction using various isolation procedures.

MATERIALS AND METHODS

Sample collection and processing

Fresh saliva samples were collected in sterile plastic tubes from 10 healthy volunteers (without any symptoms of oral or systemic diseases) without stimulation and immediately submitted to DNA extraction.

Diluted saliva samples were obtained from 10 stomatologic patients recruited in a private stomatologic practice. Samples were transferred into sterile plastic microtubes, diluted 5x with sterile water and kept frozen at -20°C until DNA extraction.

The pelleted samples were collected in sterile tubes from 10 healthy volunteers without stimulation. Saliva pellets obtained by centrifugation (3000 g, 10 min) and removal of supernatant were kept frozen at -20°C until DNA extraction.

DNA extraction

Four different methods were used for DNA isolation: modified phenol chloroform protocol according to Sakamoto *et al.* (Sakamoto *et al.*, 2004), modified benzyl-chloride protocol according to Zhu *et al*. (1993), DNA extraction using Chelex-100 and DNA extraction with Triton X.

All chemicals were purchased from Sigma-Aldrich and Merck, if not specified differently.

Modified phenol chloroform protocol according to Sakamoto et al. (Sakamoto *et al.,* 2004)

Aliquots (200 µl) of saliva samples were diluted in 400 µl of TE buffer (10 mM Tris/HCl, 50 mM EDTA, pH 8.0). Bacterial cell pellet obtained by centrifugation (6800 g, 5 min, 4°C) was resuspended in 200 µl of the same buffer containing lysozyme (final concentration 5 mg ml^{-1}) and samples were incubated at 37 °C for 60 min. Proteinase K and SDS (sodium dodecyl sulphate) were added to final concentrations of 2 mg ml^{-1} and 1% (v/w), respectively, and mixtures were incubated at 50°C for 2 h. Nucleic acids were released by three cycles of freezing at -20°C followed by thawing at 65°C. Then 200 µl of phenol (saturated with 10 mM Tris/HCl, pH 8.0) were added to each mix-

ture. Samples were vortexed and centrifugated (20800 g, 5 min, 4°C). The aqueous phase was recovered and then vortexed and centrifugated (20800 g, 5 min, 4°C) with equal volume of chloroform/isoamylalcohol (24:1) (20800 g, 5 min, 4°C). Nucleic acids from the aspirated supernatant were precipitated with isopropanol. The precipitate was then washed with 70 % ethanol and resuspended in 20 µl of deionized water.

Modified benzyl-chloride protocol according to Zhu et al. (Zhu *et al.,* 1993)
Aliquots (200 µl) of saliva samples were diluted 1:2 with TE buffer (10 mM Tris/HCl, 50 mM EDTA, pH 8.0). The bacterial cell pellet obtained after centrifugation (6800 g, 5 min., 4°C) was resuspended in 0.5 ml of the same buffer with 100 µl of 10% SDS and 300 µl of benzyl chloride. The mixture was vortexed and incubated 30 min at 50°C with repeated vortexing at 5 min intervals. Then 300 µl of 3 M NaOAc (pH 5.0) were added and the mixtures were kept on ice for 15 min. After centrifugation (6000 g, 15 min, 4°C) the supernatant was transferred to a new microtube. DNA was precipitated with isopropanol and washed with 70% ethanol. The precipitate was then resuspended in 20 µl of deionized water.

DNA extraction with Chelex-100
Aliquots (200 µl) of saliva samples were mixed with 300 µl of 10% Chelex-100 solution (Bio-Rad Laboratories, CA). The mixtures were vortexed, centrifugated (20800 g, 1 min, 4°C) and incubated at 95°C for 20 min. After vortexing and centrifugation the supernatant was washed with 70% ethanol and resuspended in 20 µl of deionized water.

DNA extraction with Triton X
Aliquots (200 µl) of saliva samples were diluted in the same volume of extraction buffer (10 mM Tris/HCl, 1 mM EDTA, 1% Triton X), vortexed and incubated at 100°C for 10 min. The mixtures were centrifugated (16000 g, 15 min, 4°C) and the supernatant was used for DNA preparation. Bulk nucleic acid was precipitated from solution with isopropanol followed by centrifugation (20800 g, 10 min, 4°C). The precipitate was then washed with 70 % ethanol and resuspended in 20 µl of deionized water.
All samples of isolated DNA were kept frozen at -20°C until spectrophotometric measurement and PCR amplification.

Measurement of concentration and purity of extracted DNA
Concentration and purity of extracted DNA was measured spectrophotometrically at 260 and 280 nm (NanoDrop 1000, Thermo Scientific).

PCR and agarose gel electrophoresis
The primers used for PCR amplification of the hypervariable area of genomic DNA encoding 16S rRNA were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5' GGTTACCTTGTTACGACTT-3') (Sakamoto *et al.*, 2004). Primer 27F was labeled at the 5' end with 6'-carboxyfluorescein (6-FAM). Amplification reactions were performed in a total volume of 20 µl containing 10 µl of Sigma-Aldrich REDTaq® ReadyMix™ PCR Reaction Mix (product number R2523), 1 µl of each (forward and reverse) primer and 2 µl of extracted DNA. PCR was performed on the Eppendorf Mastercycler Gradient S using the program according Sakamoto *et al.,* 2004: 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 1.5 min, with a final extension at 72°C for 10 min. The presence of specific amplicons was verified by agarose gel electrophoresis of aliquots of PCR mixtures (5 µl) in 1% agarose gel in 1x TBE buffer. DNA was visualized under short-wavelength UV light on a transluminator.

Statistical analysis
Statistical analysis of the results was performed using two-way ANOVA with isolation method and source material as analyzed factors potentially influencing DNA concentration and purity as endpoint parameters. Results are presented as mean + standard deviation.

RESULTS

DNA concentration
The highest concentrations of extracted DNA were obtained by isolation from fresh samples (Figure 1). Average concentrations of extracted DNA ranged from 111 ng μl^{-1} (Chelex-100) to 700 ng μl^{-1} (modified benzyl-chloride protocol). Although highest yield was achieved by the ben-

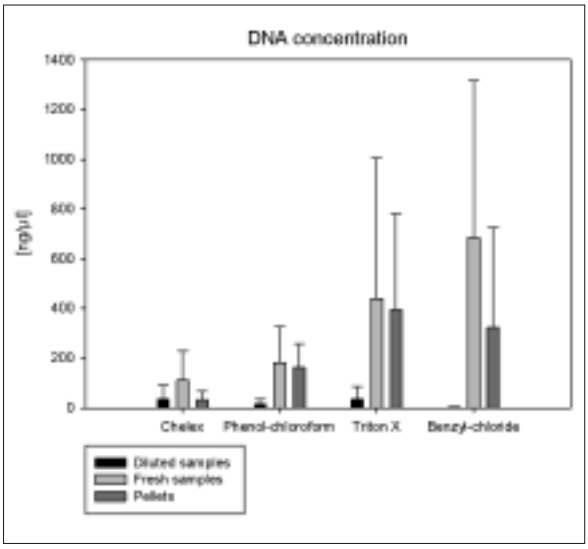

FIGURE 1 - *DNA concentration obtained from different sample matrices and using various extraction methods.*

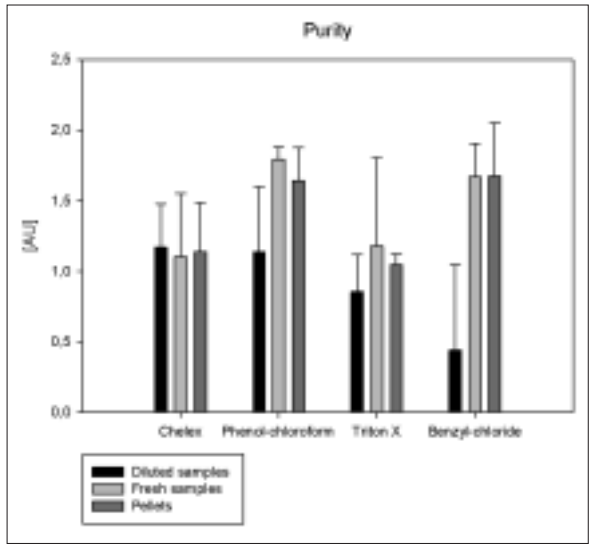

FIGURE 2 - *Purity of extracted DNA using 260/280 ratio obtained from different sample matrices and using various extraction methods.*

zyl-chloride extraction, yields from all used methods were acceptable.

According to the results the centrifugation pellet is also suitable for DNA extraction, but the achieved concentrations were lower. The highest DNA concentrations, 395±385 ng μl^{-1} (mean ± SD) were measured in samples extracted with Triton X. The concentrations of DNA isolated from diluted samples were very low. The average yields obtained by different methods range from 3±2 ng μl^{-1} using benzyl-chloride extraction to 37±55 ng μl^{-1} achieved by isolation with Chelex-100.

DNA purity

The protein contamination of dissolved DNA was assessed as the A260/A280 ratio. The highest purity was achieved by DNA isolation from the fresh samples (Figure 2). The average values obtained by different methods range from 1.1±0.4 AU (mean ± SD) with Chelex-100 isolation to 1.8±0.1 AU with phenol chloroform protocol. Similar values were obtained also using centrifugation pellets as starting material. The worst result, mean 1.1±0.2 AU, was obtained from samples isolated by Triton X extraction, the best one, 1.7±0.1 AU, was achieved by benzyl-chloride extraction.

Elimination of protein contamination of DNA extracted from diluted samples was poor. The best obtained ratio was 1.2±0.3 AU, achieved with Chelex-100 extraction. The phenol chloroform extraction eliminates on average only 3.5 % less of contamination comparing with Chelex-100 extraction. Samples isolated using Triton X protocol achieved 27% lower purity. The worst purity, 0.4±0.3 AU (62% lower than samples extracted with Chelex-100), was found in samples isolated with benzyl-chloride.

Effectiveness of DNA extraction

The effectiveness of DNA extraction was evaluated as the proportion of samples with successful PCR amplification from all samples. Samples with a specific PCR amplification product in agarose gel electrophoresis were considered positive. Failure of amplification was related primarily to the extraction method. The significant influence of sample processing was not confirmed. The most successful was the amplification with isolates from fresh saliva as template (Figure 3). All samples were positive using Chelex-100 and benzyl-chloride extraction. In case of phenol-chloroform extraction and triton X extraction the PCR effectiveness was 80% and 12.5% respectively.

The highest PCR positivity reached using DNA isolated from pellets was 90%. This effectiveness was achieved by phenol-chloroform and benzyl-chloride extractions. The worst results gave the triton X isolation, where only 10% of amplifications were successful.

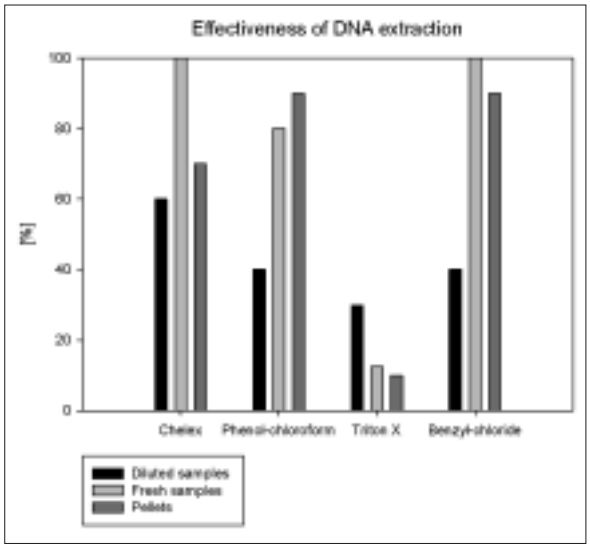

FIGURE 3 - *PCR positivity. Proportion of isolates with specific amplification product.*

The least suitable for the DNA extraction according this evaluation are diluted samples. The effectiveness ranged from 30% to 60% using different extraction procedures.
The statistical analysis demonstrated that the use of different extraction method as well as diverse processing of saliva influence the obtained DNA concentration and its purity significantly ($p<0.01$). Moreover, the effect of DNA isolation on PCR amplification was proved to be significant ($p<0.05$).

DISCUSSION

Extraction of DNA from a complex sample is an essential step inevitable for molecular analyses of microbial communities. Several studies proved that use of different procedures in this step can affect the result of analysis (Hartmann and Widmer, 2008; Li *et al.,* 2007). Therefore, we decided to evaluate the influence of different sample matrices on the effectiveness of four standard non-commercial isolation methods. To ensure accurate interpretation of obtained results DNA concentration, purity and positivity of PCR amplification were assessed.
The DNA yield should be interpreted with caution because saliva samples contain also epithelial cells and so part of the extracted DNA is of human origin (Quinque *et al.,* 2006). The interpretation of DNA concentration measured spectrophotometrically is valid only taking into account other evaluated parameters. Alternatively, human and bacterial DNA yield might be analyzed using real time PCR (Horz *et al.,* 2008).
Statistical analysis demonstrates that both processing of saliva sample and extraction method affected the DNA yield and PCR positivity significantly. The highest proportion of positive PCR runs with specific amplification product was achieved with templates isolated from fresh saliva, especially if extracted using the modified benzyl-chloride protocol. In line with this result are the findings of DNA concentration and purity. The PCR positivity with Chelex-100 extracts is 100% but the DNA yield is more than 80% lower in comparison to the benzyl-chloride protocol. Chelex-100 does not chemically isolate DNA but it exerts a reversing effect on PCR inhibitors, which might be especially important in saliva (Matto *et al.,* 1998; Ochert *et al.,* 1994). Triton X extraction gave very poor PCR results, although measured DNA concentrations were high. This might be in contrast to Chelex-100 explained by the inability of triton X to reverse the inhibiting effect of some contaminants present in saliva or by the fragmentation of nucleic acids (Aldous *et al.,* 2005).
Centrifugation pellets were surprisingly shown to be a more difficult matrix than fresh saliva samples. A higher concentration of nucleases, PCR inhibitors and the inability of used methods to inactivate them might be an explanation. The most suitable of applied extraction methods for this matrix were the modified phenol-chloroform and the modified benzyl-chloride protocols. Discrepancies between the DNA concentration and the PCR amplification in case of Chelex-100 and Triton X can be explained as noted in the case of fresh samples.
From our results we conclude that the most suitable material for bacterial DNA extraction for molecular analysis is an unprocessed saliva sample. The most effective methods for isolating salivary DNA are the benzyl-chloride protocol and Chelex-100 extraction.
The results demonstrated that diluted samples were not a suitable material for DNA extraction for molecular analyses. The highest PCR positivity achieved was only 60% reached using the

Chelex-100 extraction method. The measured concentrations did not reach even one tenth of concentrations gained by isolation from another sample types. If analysis of pelleted samples is required, taking into account the hands-on labor intensive work time the most appropriate method is the extraction using the modified benzyl-chloride protocol according to Zhu *et al.,* 1993. These results should be taken into account in studies analyzing oral microbial flora from saliva to prevent at least some laborious optimization of saliva sample processing and DNA isolation. Although clear differences were found between the extraction methods used, even the best results were less than ideal. Saliva represents a very interesting diagnostic tool, but also a complex matrix. The procedure for salivary RNA has been commercialized recently (Park *et al.,* 2006). A similar easy, cheap, effective and reproducible kit using novel approaches for microbial DNA isolation specifically from saliva is needed as oral health status is studied in association with salivary microbiome (Nadkarni *et al.,* 2009; Nasidze *et al.,* 2009).

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