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ORIGINAL ARTICLE

The impact of different DNA extraction methods on the analysis of microbial diversity of oral saliva from healthy youths by polymerase chain reaction-denaturing gradient gel electrophoresis

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Abstract *Background/purpose:* Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), as a conventional molecular technique, was utilized to analyze the diversity of oral microbiota. However, studies found that the results of PCR-DGGE were affected by the DNA isolation method. This study compared QIAamp DNA Micro Kit extraction method with the phenol and chloroform extraction method for DNA isolation of saliva of healthy youths and analyzed PCR-DGGE fingerprints.
Materials and methods: In the first stage, samples were divided into two after collection from eight health youths. Two methods were used to isolate the DNA for PCR-DGGE analysis. In the second stage, another 16 samples were collected from 14 youths. The better method, QIAamp DNA Micro Kit, was used to isolate the DNA for PCR-DGGE analysis. The cluster analysis was performed with unweighted pair-group method with arithmetic means.
Results: The results in the first stage showed that the QIAamp DNA Micro Kit extraction method was more suitable for DNA extraction of saliva than the phenol-chloroform extraction method. In the second stage, the bands were changed into numbers "0", "1", and "2" to analyze the similarity of samples according to the bands' lightness. The similarity indices of different periods from the same individual showed that the microbiological composition was very similar (>0.95), while those from different individuals varied greatly (<0.90).

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Conclusion: PCR-DGGE was more accurate in assessing oral microbial diversity by QIAamp DNA Micro Kit. Different individuals had large differences in oral microbial diversity but also had some common microbial dominant communities.

Introduction

Oral microbiota, which consist of >700 bacteria, are among the most complex microbial communities in humans. Oral microbial imbalance can influence oral health and even general health. Generally, microbial research depends on cultivation, which has many distinct limitations. Staley and Konopka[1] reported that the cultivable number of microorganisms was only <1%, suggesting that studies based on culture are unfit for determining oral microbial diversity and genes. Cultivation seems to be a time-consuming method to utilize in microbial research, and it will gradually incur high financial costs when applied to a large number of biochemical identifications. Molecular techniques have frequently emerged in the field of microbial research in recent decades. polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE), as a technique based on electrophoretic analysis of PCR products, is one of the more common research methods.

Some studies[2–4] have found that different DNA extraction methods affected PCR-DGGE fingerprint results. Nevertheless, these reports did not draw attention from researchers, and many articles[5,6] on PCR-DGGE analysis have lacked explanations for why the authors chose this method to extract DNA from samples. Therefore, the optimization method of DNA isolation for PCR-DGGE has not had unified standardization. There is potential bias in the results of PCR-DGGE analysis. The optimal method for DNA extraction should be considered before analyzing the microbiota in a new environment by PCR-DGGE.

In this study, we compared two different methods for DNA extraction and analyzed their impacts on PCR-DGGE results. According to the results, we provided a basis for understanding the characteristics of oral microbiota, and presented a rationale for DNA extraction in oral microbial research. In addition, saliva samples in different individuals and from different periods in the same individuals from healthy youths were analyzed with PCR-DGGE. Our results provided information for the application of PCR-DGGE in the oral microbial diversity of healthy youths in southern China.

Materials and methods

Participants, sample collection, and experimental design

The students involved in this study provided verbal informed consent and the protocol was approved by the ethical committee of the Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, China. The research was conducted in full accordance with the World Medical Association Declaration of Helsinki.

First stage

Eight healthy youths were randomly selected from the College of Stomatology, Southern Medical University. All eight of the students whom we selected met the following inclusion criteria: (1) age between 21—25 years; (2) no systemic diseases, female participants not pregnant or taking oral contraceptives; (3) no antibiotics taken in previous 3 months; and (4) no smoking.

Oral examinations were conducted strictly according to the inclusion criteria and the participants were required to gargle with water 30 minutes before sample collection. Unstimulated whole saliva samples, which were all secreted naturally from the mouth, were obtained. Every single individual expectorated 2 mL of saliva and all of the samples were divided into two (1 mL each). Sixteen samples in total were obtained and allocated into two groups (Groups A and B) equally. The samples in Group A, which were submitted to the phenol and chloroform extraction method, were labeled A1 to A8, while those in Group B, which used QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) extraction method, were labeled from B1 to B8.

For double blinding and the convenience of further experiments, the sequences of the 16 labeled samples in Group A and Group B were rearranged and relabeled as numbers 1—16 by another graduate student who did not participate in this study. Numbers 1—16 corresponded to A5, A7, B4, B5, B2, B7, B1, A3, B8, A4, A2, B3, B6, A6, A1, and A8.

Second stage

Fourteen healthy youths were randomly selected from College of Stomatology, Southern Medical University. All of the students met the inclusion criteria described in the first stage.

All of the participants expectorated 2 mL of saliva and these 14 samples in total were allocated into Group A (labeled A1—A14). Then, two participants were randomly selected to provide 2 mL saliva samples again after 3 months and these two samples were allocated as Group B (these 2 randomly selected students were participant numbers 2 and 3, which corresponded to samples A2 and A3, so these two samples collected at a different time points in Group B were labeled B2 and B3). All samples in the second stage were submitted to the QIAamp DNA Micro Kit extraction method.

For double blinding and the convenience of further experiments, the sequences of 16 labeled samples in Groups A and B were rearranged and relabeled as numbers 1—16 by another graduate student who did not participate in this study. Numbers 1—16 corresponded to A7, B3, A3, A2, A4, B2, A1, A5, A10, A13, A14, A9, A6, A8, A11, and A12.

Total genomic DNA extraction

Modified phenol-chloroform extraction method

This protocol for DNA extraction was modified to fit the optimal conditions for saliva samples according to the protocol of Zoetenal et al.[7] Saliva samples were thawed at room temperature.

QIAamp DNA Micro Kit extraction method

The QIAamp DNA Micro Kit (Qiagen) was used following the manufacturer's recommendations. Samples were thawed at room temperature before pretreatment conducted. Then, DNA was extracted from the saliva samples according to the procedure provided in the QIAamp DNA Micro Handbook (Qiagen).

PCR-DGGE assay

PCR was performed using the ABI 9700 GeneAmp PCR System (PE Applied Biosystems, Foster, CA, USA). A set of universal bacterial primer of 16s rDNA was used for amplification according to Lazarevic et al.[8] The primer 784DEG (5′-GGMTTAGATACCC) and 880RDEG (5′-CRTACTHCHCAGGYG) sequences, flanking the V5 region to match most sequences, produced 740 and 745 hits, respectively.

DGGE was performed with the Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The DGGE operational procedure was performed following the manufacturer's recommendations with minor modifications. Stock solution was prepared for an acrylamide:bis-acrylamide ratio of 37.5:1.

The similarities of the PCR-DGGE DNA profiles were analyzed using Phyltools Analysis Software (Joseph Felsenstein, University of Washington, version 1.32). Dendrograms were constructed by the unweighted pair-group method with arithmetic mean and unweighted pair-group method with arithmetic cluster analysis of the microorganism community structure was performed with a multivariate statistical package.

Statistical analyses

Statistical analyses were performed with SPSS software, version 16.0 (SAS Institute, Cary, NC, USA). The paired *t* test was used to assess the results and the differences were considered significant at $P < 0.05$.

Results

Impact of two different DNA extraction methods on PCR-DGGE

The 16s rDNA was well-amplified with the universal primers pairs (784DEG/880RDEG) in this study. In the first stage, the numbers of detectable bands of 16 different samples, which were labeled numbers 1—16, were 4, 9, 10, 10, 10, 14, 10, 8, 10, 3, 7, 10, 10, 8, 7, and 7, respectively (Figure 1). The mean number of bands in Group A (phenol-chloroform extraction method) was 6.63 ± 2.07, while that in Group B (QIAamp DNA Micro Kit extraction method) was 10.50 ± 1.41. The mean species richness of the DNA samples of microbiota in Group B was greater than that in Group A. The difference was statistically significant ($P = 0.001$).

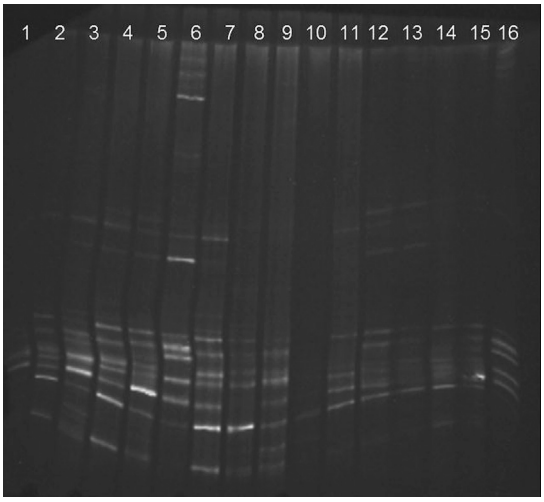

Figure 1 Denaturing gradient gel electrophoresis of 16S rDNA gene amplification products from samples from the first stage.

Analysis of microbial diversity of oral saliva from healthy youths by PCR-DGGE

In the second stage, the PCR products of DNA samples were well-amplified with a single band using agarose gel

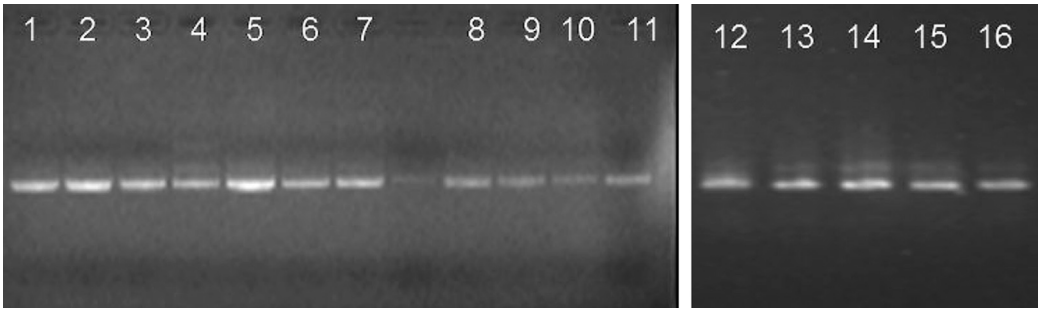

Figure 2 Polymerase chain rteaction replication of oral microbiome 16S rDNA from samples from the second stage.

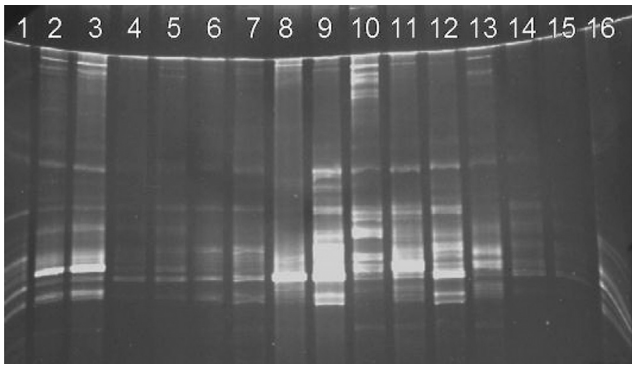

Figure 3 Denaturing gradient gel electrophoresis of 16S rDNA gene amplification products from samples from the second stage.

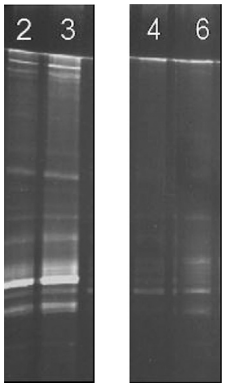

Figure 4 Denaturing gradient gel electrophoresis of 16S rDNA gene amplification products from the different periods of the second stage.

electrophoresis (Figure 2). The numbers of detectable bands of the sixteen different samples displayed on the DGGE fingerprint were 13, 18, 19, 9, 10, 10, 12, 13, 17, 21, 18, 16, 15, 10, 7, and 13 (Figure 3). For the same individual in different periods, lane numbers 2 and 3 from the same individual displayed 18 and 19 detectable bands, respectively, while numbers 4 and 6 from the same individual displayed 9 and 10 bands on the DGGE fingerprint (Figure 4), respectively.

The similarity indices of different individuals were all <0.90, while the similarity indices at different time points from the same individual were all >0.95 (Figure 5).

Discussion

The composition and variation of oral microbial communities have been studied by PCR-DGGR for many years and a large number of studies have been published. Despite total DNA extraction having been performed in these studies, the reason for the choice of these extraction methods of the standards have often been missing.

Our results indicated that the DNA extraction method impacted the DGGE analysis of the oral microbial community, expressed as the detectable number and the comparative brightness of bands on DGGE gels. Kennedy et al[9] and Wagner Mackenzie et al[10] demonstrated similar results in their studies of microbiota in other microenvironments. Considering these previous studies and our results, potential bias exists in PCR-DGGE analysis due to the total DNA extraction. In our study, the agent and programs utilized in the PCR system with the two different extraction methods were identical, except for the DNA template (extracted from 2 different DNA extraction methods). To avoid any interference, the order of the samples was also randomly shuffled before the PCR program started by a graduate student who did not otherwise participate in this study. Therefore, the reason for the different results in PCR-DGGE analysis might have been the DNA templates. The cause could be expressed in two aspects. Firstly, the abundance of the microbiota in the initial samples might have changed after DNA extraction.[11] This change might be relevant to the efficiency of the lysis buffer and the

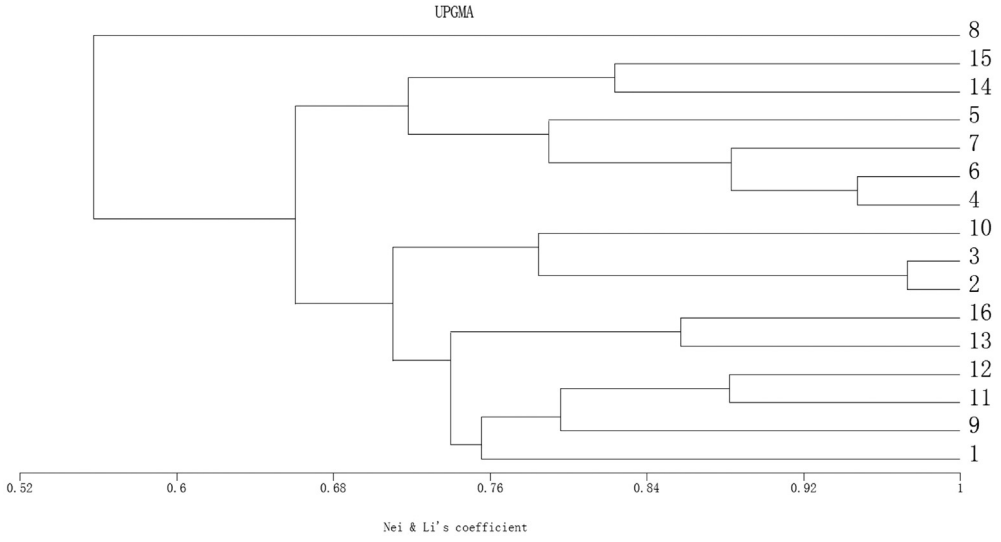

Figure 5 Unweighted pair group method with arithmetic mean cluster analysis of the microorganism community structure from samples from the second stage. UPMGA = Unweighted pair group method with arithmetic mean cluster analysis.

sensitivity of various microbiota to the lysis buffer from different extraction methods. This finding, which was consistent with the results of Gelsomino et al,[12] indicated that less abundant bacteria species are much less impacted by different extraction methods. Secondly, the PCR inhibitor concentration of DNA samples might be impacted by different extraction methods.[13] The samples collected often had various PCR inhibitors, as organisms do. Thus, the different PCR inhibitor concentrations of these samples could cause interference with PCR amplification.[14,15]

For the second stage of the study, the diversity of oral microbiota was very similar between samples from the same individuals of different periods. The oral microbiota from different individuals were unique. Nonetheless, common dominant microbiota also existed among different individuals. These findings were consistent with the results of Rasiah et al.[16] However, our results were different from those of McBain et al.[17] Their study indicated that the dominant microbiota varied between individuals. Analysis of this inconformity in our study was based on the nonculture method, while that by McBain et al[17] was based on cultivation.

In conclusion, DNA extraction methods impacted the analysis of PCR-DGGE for the study of oral microbial diversity. Our study indicated that DNA extraction methods should be considered and chosen according to different microbiota before PCR-DGGE analysis. Therefore, the detectable mass abundant bands could be obtained on DGGE gels. In our study, the QIAamp DNA Micro Kit extraction method was appropriate for oral saliva sample extraction. In addition, we will conduct further studies of the dominant microbiota in oral saliva to provide evidence for the prevention and treatment of oral diseases.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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