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Notes & Tips

DNA from oral bacteria by sodium hydroxide–paper method suitable for polymerase chain reaction

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ABSTRACT

In the oral cavity, we can find a complex mixture of microorganisms, commensals, and pathogens. The studies of normal oral microbiota, as well as the studies of much oral pathology (e.g., caries, periodontitis), involve the isolation and cultivation of these microorganisms and their molecular analysis. The aim of this study was to validate a quick, easy, efficient, and inexpensive DNA extraction method for the recovery of genomic DNA from gram-positive and gram-negative oral bacteria to be used in polymerase chain reaction amplification. This method worked great with all samples analyzed, providing an approach to extract DNA for different microorganisms.

The oral cavity is the most complex and accessible microbial ecosystem of the human body. The microorganisms present in the oral cavity are a complex mixture of commensals and pathogens [1]. Many of them are difficult to cultivate in the laboratory, and therefore molecular studies of them are complicated to do.

Many different protocols to obtain genomic DNA of bacteria have been developed recently, but all of them involve cell lysis and the subsequent DNA recovery [2]. Many factors play a role in the efficiency of bacterial cell lysis, including the constitution of the cell wall, the physiological state of the cell, and cell concentration [2]. As a consequence, most DNA extraction methods are optimal for just one or a group of bacterial species.

The aim of this study was to validate a suitable DNA extraction method for the recovery of genomic DNA from oral bacteria to be used in polymerase chain reaction (PCR)[1] amplification. We used as model organisms the gram-negative bacteria *Porphyromonas gingivalis* W83 (ATCC BAA-308) and *Aggregatibacter actinomycetemcomitans* ATCC 29522 and the gram-positive bacteria *Streptococcus mutans* ATCC 21575 and *Lactobacillus casei* ATCC 334. We used a simple but efficient procedure for extracting genomic DNA from these bacterial samples based on a method originally described by Zhou et al. to obtain genomic DNA from dried blood samples [3].

S. mutans ATCC 21575, *P. gingivalis* W83 (ATCC BAA-308), and *A. actinomycetemcomitans* ATCC 29522 cells were each inoculated into brain heart infusion (BHI) broth (Media Products, Groningen, The Netherlands) and grown at 37 °C in a candle jar as described previously [4], and *L. casei* ATCC 334 was grown in a candle jar into MRS broth (Oxoid, Basingstoke, UK) at 37 °C [5]. All liquid cultures were grown to reach an optical density near 1.0 at 600 nm. Liquid culture (100 μl) was deposited in the center of a 4-cm-diameter circle of 3MM filter paper (Whatman). All filters were dried at room temperature (~25 °C) for 1 h. Duplicate 1.2-mm diameter disks were punched from each bacteria-containing filter paper, and each disk was transferred to a 0.2-ml PCR tube. To extract DNA, 20 mM sodium hydroxide (NaOH, 200 μl) was added to each tube and incubated for 30 min (37 °C). The tube was inverted occasionally during incubation. The solution was then discarded, and the disk was washed in 200 μl of TE buffer (10 mM Tris–HCl and 0.1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0) for 2 min. After the removal of the TE buffer, the disk was air-dried at 37 °C. The presence of genomic DNA in filter papers was determined by PCR amplification of a DNA fragment specific to each microorganism. The oligonucleotide primers used are listed in Table 1. Each PCR was performed in a 20-μl final volume containing genomic DNA on one 1.2-mm filter disk, 1 μM required primers, 0.2 mM of each dNTP (Fermentas), 0.05 U/μl *Taq* DNA polymerase (Fermentas), and 1× PCR buffer containing 1.5 mM $MgCl_2$. Amplification was carried out in a GeneAmp PCR System 2400 (PerkinElmer) and the thermal profile consisted of initial denaturation at 94 °C for 2 min,

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[1] *Abbreviations used:* PCR, polymerase chain reaction; BHI, brain heart infusion; NaOH, sodium hydroxide; EDTA, ethylenediaminetetraacetic acid; rRNA, ribosomal RNA; CFU, colony-forming unit.

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Table 1
Primers used in this study.

Microorganism and target gene	Primer name and sequence (5′–3′)	Source or reference
Aggregatibacter actinomycetemcomitans ATCC 29522/16S RNA	Fw: ATTGGGGTTTAGCCCTGGTG Rev[a]: ACGTCATCCCCACCTTCCTC	Tran and Rudney (1999) [4]
Porphyromonas gingivalis W83 (ATCC BAA-308)/16S RNA	Fw: TGTAGATGACTGATGGTGAAAACC Rev[a]: ACGTCATCCCCACCTTCCTC	Tran and Rudney (1999) [4]
Streptococcus mutans ATCC 25175/*htrA*	Fw: TCGCGAAAAAGATAAACAAACA Rev: GCCCCTTCACAGTTGGTTAG	Chen et al. (2007) [7]
Lactobacillus sp./16S RNA	Fw: TGGAAACAGATGCTAATACCG Rev: GTCCATTGTGGAAGATTCCC	Caufield et al. (2007) [9]
Lactobacillus casei ATCC 334/16S RNA	Fw: GAACCGCATGGTTCTTGGCTGAAA Rev: ATACCGTCACGCCGACAACAGTTA	This study[b]
Lactobacillus casei ATCC 334/LSEI_0030	Fw: GATTCTCGCGCAACCAAATGTCC Rev: GAAAGCCATGGTCAGTTGCAGGT	This study[b]
Lactobacillus casei ATCC 334/LSEI_0869	Fw: AGGCTGGCTATGAATCAGG Rev: TGGACTCAATTACTTCATACC	This study[b]
Lactobacillus casei ATCC 334/LSEI_1990	Fw: GATCCATCAATTGGCGGGATTGC Rev: AGCGGCAACTAGATGCACATTGG	This study[b]
Lactobacillus casei ATCC 334/LSEI_2025	Fw: ATGGATAACACAACGATTGC Rev: CATCAACATGTACATGCTCG	This study[b]
Lactobacillus casei ATCC 334/LSEI_2413	Fw: TGGATCCTGACTGAGTTGGCCAT Rev: TCGGATCGGACAAAGCAACCTGA	This study[b]
Lactobacillus casei ATCC 334/LSEI_0391-0393	Fw: GGTGTATGCAGCTTTGGCG Rev: ACACTCAAAATCGTCTTGCC	This study[b]
Lactobacillus casei ATCC 334/LSEI_1820-1822	Fw: ACGGATAGCTGCGGAATGG Rev: ACTGGCATGCTACCGTGG	This study[b]

Note: Fw, forward primer; Rev, reverse primer.
[a] Reverse primer is conserved to amplify 16S RNA gene from *A. actinomycetemcomitans* and *P. gingivalis*.
[b] Primers were designed by PrimerQuest program, available on IDT website (Integrated DNA Technologies, http://www.idtdna.com/site).

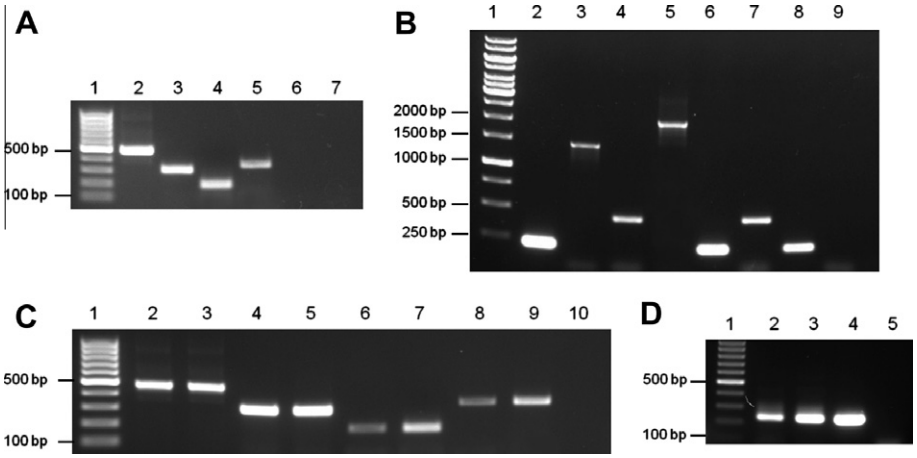

Fig.1. PCR products obtained using genomic DNA extracted with the NaOH method. Different amplicons were subjected to 1% agarose gel electrophoresis. (A) PCR products obtained from genomic DNA of different bacteria. Lane 1: molecular size marker, 100-bp DNA ladder (Fermentas); lane 2: 479 bp PCR product of *htrA* gene of *S. mutans* filter papers; lane 3: 309 bp of 16S RNA gene of *L. casei*; lane 4: 197 bp of 16S RNA gene of *P. gingivalis*; lane 5: 360 bp of 16S RNA gene of *A. actinomycetemcomitans*; lanes 6 and 7: negative control, filter papers with MRS and BHI broth, respectively. (B) Different PCR products obtained from *L. casei* genomic DNA. Lane 1: molecular size marker, 1-kb DNA ladder (Fermentas); lane 2: 248 bp PCR product of *LSEI0030* gene; lane 3: 1437 bp PCR product of a fragment between *LSEI0391–LSEI0393* genes; lane 4: 460 bp PCR product of *LSEI0869* gene; lane 5: 2118 bp PCR product of a fragment between *LSEI1820–1822* genes; lane 6: 235 bp PCR product of *LSEI1990* gene; lane 7: 475 bp PCR product of *LSEI2025* gene; lane 8: 241 bp PCR product of *LSEI2413* gene; lane 9: amplification negative control. (C) PCR products obtained from genomic DNA samples extracted from different bacterial colonies. Lane 1: molecular size marker, 100-bp DNA ladder (Fermentas); lanes 2, 4, 6, and 8: PCRs performed using as DNA template one disk of 1.2-mm of *S. mutans*, *L. casei*, *P. gingivalis*, and *A. actinomycetemcomitans*, respectively; lanes 3, 5, 7, and 9: PCRs using two disks of *S. mutans*, *L. casei*, *P. gingivalis*, and *A. actinomycetemcomitans*, respectively; lane 10: amplification negative control. (D) A 232 bp fragment of 16S RNA gene of *Lactobacillus* sp. amplified by PCR with genomic DNA extracted from saliva samples. Lane 1: molecular size marker, 100-bp DNA ladder (Fermentas); lane 2: PCRs using one disk of saliva sample; lane 3: PCRs using two disks of saliva sample; lane 4: positive control, PCR with one disk of *L. casei* culture; lane 5: negative amplification control.

followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 5 min. Aliquots of PCR products were run in 1% agarose gels using 1× TAE buffer (40 mM Tris–acetate and 1 mM Na_2EDTA) containing 500 ng/ml ethidium bromide and visualized by ultraviolet transillumination.

First, we analyzed the presence of genomic DNA in the filter papers. The results of PCR amplification are shown in Fig. 1A. A fragment of the 16S ribosomal RNA (rRNA) gene was obtained by amplifying in *A. actinomycetemcomitans* and *P. gingivalis* genomic DNA samples [4] and in *L. casei* genomic DNA sample (primers designed in this study based on the sequence published by Makarova et al. [6]). In *S. mutans*, we used species-specific PCR primers to amplify the *htrA* gene as reported previously [7].

The expected product lengths were 479 bp for *S. mutans*, 309 bp for *L. casei*, 197 bp for *P. gingivalis*, and 360 bp for *A. actinomycetemcomitans*. All of the amplifications were highly specific, exhibiting just a band of the expected size in each case (Fig. 1A). Filter papers with either MRS or BHI broth were used as negative controls, which did not generate amplicons, showing that the filter method is clean (Fig 1A, lanes 6 and 7).

To confirm the quality and integrity of the genomic DNA extracted, PCR amplification of several genome regions was performed with *L. casei* filter papers. Seven regions of various sizes and located in different sites of the *L. casei* chromosome were selected: *LSEI0030* (248 bp), *LSEI0391–LSEI0393* (1437 bp), *LSEI0869* (460 bp), *LSEI1820–1822* (2118 bp), *LSEI1990* (235 bp), *LSEI2025* (475 bp), and *LSEI2413* (241 bp). The PCR fragments obtained were analyzed by agarose gel electrophoresis, and the results showed highly specific amplifications, exhibiting a band of the expected size in each case (Fig. 1B). These results suggest that the genomic DNA extracted by this method was of high integrity.

Many studies of presence, prevalence, and/or genotyping of bacteria in the oral cavity involve the isolation of these microorganisms from saliva samples on a specific solid medium [8]. The colony-forming units (CFUs) grown are counted and collected from the culture plates for genotypic analysis but must be previously inoculated and grown in liquid medium because most genomic DNA extraction kits need a large number of cells to obtain genomic DNA [5]. In this context, we performed another approach to determine whether the method of NaOH is directly applicable to bacterial colonies. Plate cultures of *A. actinomycetemcomitans*, *P. gingivalis*, *L. casei*, and *S. mutans* were grown independently for 48 h at 37 °C, and one colony (CFU) of each was isolated, independently resuspended in 50 µl of distilled water, and deposited in the center of a 4-cm-diameter circle of 3MM filter paper (Whatman). The DNA extraction and PCR were performed as described before.

To measure whether the bacterial DNA present in the filter papers was sufficient to be used in PCR amplification, we used the previously described primers in PCR amplifications. The results are shown in Fig. 1C. Lanes 2, 4, 6, and 8 show amplification obtained when the PCRs were performedusing as DNA template one disk of 1.2-mm of *S. mutans*, *L. casei*, *P. gingivalis*, and *A. actinomycetemcomitans*, respectively. The results shown in lanes 3, 5, 7, and 9 were obtained using (in each PCR) two disks of *S. mutans*, *L. casei*, *P. gingivalis*, and *A. actinomycetemcomitans*, respectively. All lanes show the expected size amplicons, and qualitative differences do not exist when using one versus two disks; however, particularly for the *P. gingivalis* and *A. actinomycetemcomitans* samples, we observed more amplification when using two disks. This indicates that a single disk from those species had insufficient DNA to saturate the reaction; this result might be related to the smaller size of these colonies.

Finally, we made a preliminary approach to determine whether the NaOH method could be used for DNA extraction from dried saliva samples. For this purpose, we used a saliva sample whose bacteria content was previously determined using conventional microbiological methods of isolation and quantification [7–9]. This saliva sample possessed 1.75×10^5 ($\pm 3.1 \times 10^4$) UFC of *Lactobacillus* sp. and 1×10^5 ($\pm 2 \times 10^4$) UFC of *S. mutans*. An aliquot of 50 µl of saliva sample was deposited in the center of a 4-cm-diameter circle of 3MM filter paper (Whatman) and dried at room temperature (~25 °C) for 1 h. The DNA extraction and PCR were performed as described before. To determine the presence of the genomic DNA of *Lactobacillus* sp., we used a pair primers described to amplify a 232 bp fragment of the 16S rRNA gene in *Lactobacillus* sp. strains [9]. The result is shown in Fig. 1D. Lanes 2 and 3 show amplification obtained when the PCRs were performed using as DNA template one or two disks of 1.2-mm of saliva sample in each PCR, respectively. Lane 4 shows amplification obtained with one disk of 1.2-mm of *L. casei* as a positive control. The same approach was performed to amplify the *htrA* gene of *S. mutans*, as mentioned previously, from these filter papers with saliva sample obtaining a positive result but using two disks of 1.2-mm (data not shown). These outcomes allow one to indicate that it could be possible to obtain genomic DNA suitable for PCR, directly from saliva samples, for the NaOH method.

In this article, we have described a simple but efficient method (called the NaOH method for short [3]) for extracting genomic DNA from bacterial samples. Using the NaOH method on 3MM filter papers, amplicons of the expected size were obtained for all of the genomic samples analyzed, and other amplicons were not seen, indicating that the method is clean.

We used the gram-negative bacteria *P. gingivalis* W83 (ATCC BAA-308) and *A. actinomycetemcomitans* ATCC 29522 and the gram-positive bacteria *S. mutans* ATCC 21575 and *L. casei* ATCC 334 as model microorganisms present in the oral cavity. These bacteria have different cell wall constitutions (gram-positive vs. gram-negative), different cellular shapes (cocci vs. rods), different GC content in their genomes (36.82% *S. mutans* ATCC 21575, 46.57% *L. casei* ATCC 334, 48.28% *P. gingivalis* W83, and 44.67% *A. actinomycetemcomitans* ATCC 29522) and different physiological characteristics (e.g., *S. mutans* and *L. casei* are acidophilic and acidogenic microorganisms), covering a range of different factors that can play a role in the efficiency of DNA purification by traditional methods. The method worked great with all samples analyzed—bacteria grown in liquid medium, bacteria taken from solid surfaces, and even saliva samples with mixtures of different microorganisms. In addition to performing the experiments described here, we used the same approach with *Escherichia coli* K-12 cultures (gram-negative bacteria, GC content 50.78%), *Acidithiobacillus ferrooxidans* ATCC 23270 cultures (gram-negative, GC content 58.77%), and *Candida albicans* colonies (yeast, GC content 33.51%) and obtained satisfactory results (data not shown), suggesting that similar outcomes could be obtained with other microorganisms.

The sample processing proposed here offers DNA free of amplification inhibitors at a substantial savings in time, effort, and cost compared with traditional DNA extraction methods. Furthermore, the DNA is useful for PCR amplifications, generating specific products that also can be used for molecular biology experiments such as cloning and sequencing.

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