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Rapid Detection of Pathogens Associated with Dental Caries and Periodontitis by PCR Using a Modified DNA Extraction Method

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Abstract

DNA extraction is a prerequisite for the identification of pathogens in clinical samples. Commercial DNA extraction kits generally involve time-consuming and laborious multi-step procedures.

In the present study, our modified DNA isolation method for saliva samples allows for the quick detection of pathogens associated with dental caries or periodontitis by PCR within 1 h. To release DNA from the bacteria, 1 min of boiling was adequate, and the resulting isolated DNA can be used many times and is suitable for long term storage of at least 13 months at 4℃, and even longer at -20℃.

In conclusion, our modified DNA extraction method is simple, rapid, and cost-effective, and suitable for preparing DNA from clinical samples for PCR for the rapid detection of oral pathogens from saliva.

Key words : DNA extraction, Oral pathogens, Saliva, Polymerase chain reaction(PCR)

Ⅰ. Introduction

Dental caries is one of the most common diseases in humans, and results from demineralization of tooth enamel by acids produced by oral bacteria, with *Streptococcus mutans* implicated as the main causative organism. The detection of dental caries-related pathogens (*Streptococcus mutans* and *Streptococcus sobrinus*) and periodontal disease-related pathogens (*Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella intermedia, Tannerella forsythia*, and *Aggregatibacter actinomycetemcomitans*) is particularly important in children since they may be at risk of developing dental caries and periodontitis[1,2]. They are more easily curable when diagnosed at the early stage. A number of studies have suggested that a polymerase chain reaction (PCR) assay can detect low numbers of bacteria[3-7]. However, it is difficult to find the existence of the bacteria in saliva or in plaque without culturing them and it takes over 48 hr and commercial DNA extraction kits that are used to prepare genomic DNA for PCR cost between 100,000 won and 300,000 won and take about 30 min to extract DNA from an organism. They are not convenient when there are a lot of samples and when they are not available but a researcher/doctor needs to detect a certain bacteria and diagnose a disease for a short period of time since it takes time to order. Several DNA extraction methods without using commercial kits have been developed for PCR[8-10]. They have been suggested as simple and rapid methods for the identification of an organism, yet, they still contain several steps and use toxic reagents such as phenol and chloroform[11]. Phenol is a hazardous organic compound that causes chemical burns and chloroform is also considered hazardous when it is inhaled since it can depress central nervous system and cause death when it is se-

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vere. The DNA extraction procedure used in this study was modified from Saarela *et al.*[12] and it has simple steps to prepare genomic DNA and does not use toxic reagents. The purpose of this study was to investigate the efficiency of the modified DNA extraction method for the preparation of genomic DNA from saliva samples and identification of oral pathogens.

Ⅱ. Materials and methods

1. Bacterial strains and culture condition

S. mutans GS5, UA159, KCTC3065, and KCTC3066 were cultured in brain heart infusion broth (BHI. Difco, Detroit, USA) and incubated at 37℃ for 24 hours. To compare the result of the PCR with the DNA isolated from the culture to the result of the PCR with the DNA isolated directly from the saliva samples, 100 ㎕ of each saliva was inoculated into 5 ㎖ of BHI broth and cultured overnight at 37℃. To extract DNA from the culture, 100 ㎕ of the culture of the saliva samples was transferred to 1.5 ㎖ tube next day. It was centrifuged at 10,000 × g for 3 min. The supernatants were discarded and the pellet resuspended with 100 ㎖ of 50 mM NaOH. The samples were boiled for 1 min at 95℃ followed by adding 10 ㎖ of 100 mM Tris-Cl (pH 6.0). The cell debris was removed by centrifugation at 10,000 × g for 10 min. The supernatant was stored at 4℃ and used for PCR.

2. Saliva sampling

Saliva samples were collected by spitting saliva into a 50 ㎖ sterile tube from 5 patients (aged between 9 and 12 years old) who visited Dental Hospital of Chonbuk National University. The purpose of this study was explained and consent was obtained from their parents. The procedures were approved by the Ethical Committee of the Chonbuk National University Hospital. The samples were kept on ice and used within 24 hours.

3. DNA extraction from the bacterial strains and saliva samples

One hundred microliters of overnight culture of *S. mutans* GS5, UA159, KCTC3065, and KCTC3066, and saliva samples were transferred to a 1.5 ㎖ microcentrifuge tube and centrifuged at 10,000 × g for 3 min. The supernatants were discarded and the pellet resuspended with 100 ㎕ of 50 mM NaOH. The samples were boiled at 95℃ followed by adding 10 ㎕ of 100 mM Tris-Cl (pH 6.0). The cell debris was removed by centrifugation at 10,000 × g for 10 min. The supernatant was stored at 4℃ and -20℃ until used. The genomic DNA containing supernatant was stored at 4℃ and -20℃ until used. The processes for the extraction of DNA from saliva samples were the same. However, they were boiled for 1 min and stored at 4℃.

4. Polymerase chain reaction (PCR)

The primers for the amplification of specific sequence of each bacteria are listed in the Table 1[4,8,9,12,13]. Total volume of PCR reaction mixture was 20 ㎕ that contains 1 ㎕ of DNA, 4 ㎕ of 5 × PCR mixture (ELPIS, Taejeon, Korea), 1 ㎕ of 5 pM of each primer, and 14 ㎕ of dis-

Table 1. PCR primers for the detection of pathogens in saliva samples

Primers	Sequences	Product size
A. actinomycetemcomitans	5-GCTAATACCGCGTAGAGTCGG-3	443
	5-ATTTCACACCTCACTTAAAGGT-3	
T. forsythia	5-GCGTATGTAACCTGCCCGCA-3	641
	5-TGCTTCAGTGTCAGTTATACCT-3	
F. nucleatum	5-AGAGTTTGATCCTGGCTCAG-3	360
	5-GTCATCGTGCACACAGAATTGCTG-3	
P. gingivalis	5-AGGCAGCTTGCCATACTGCG-3	443
	5-ACTGTTAGCAACTACCGATGT-3	
P. intermedia	5-CCTAATACCCGATGTTGTCCACA-3	855
	5-AAGGAGTCAACATCTCTGTATCC-3	
S. sobrinus	5-GATAACTACCTGACAGCTGACT-3	690
	5-AAGCTGCCTTAAGGTAATCACT-3	
S. mutans	5-AGCCATGCGCAATCAACAGGTT-3	415
	5-CGCAACGCGAACATCTTGATCAG-3	

tilled water. The PCR contains denaturation at 95℃ for 1 min, 30 cylcles of denaturation at 95℃ for 10 sec, annealing at 58℃ for 10 sec, and extension at 72℃ for 10 sec, and then extension at 72℃ for 5 min. The PCR products were analysed in 1% agarose gel at 100 V for 20 min. The gel was stained with EtBr.

Ⅲ. Results

S. mutans GS5, UA159, KCTC3065, and KCTC3066 were Gram positive bacteria that has thicker cell wall than Gram negative bacteria and often need more steps to isolate DNA. Therefore, we added sonication procedure before boiling them for 1, 2, 3, 4, 5, or 10 min and compared the PCR results in the presence and absence of the sonication step. The DNA samples of *S. mutans* were well amplified whether they were sonicated or non-sonicated(Fig. 1). We stored the DNA samples separately at 4℃ and -20℃ to investigate whether temperature affected maintenance and degradation of them since storage temperature could influence DNA and make it not sufficient for PCR. However, the result of the PCR with DNA stored at 4℃ in this study showed that temperature does not have much impact on it for PCR for at least 13 months(Fig. 2) and the DNA could be used many times (data not shown). We extracted DNA of oral pathogens such as *A. actinomycetemcomitans, T. forsythia, F. nucleatum, P. gingivalis, P. intermedia, S. sobrinus* and *S. mutans* from saliva samples by using the modified DNA extraction method. The pathogens were well detected by PCR using the specific primers(Fig. 3)

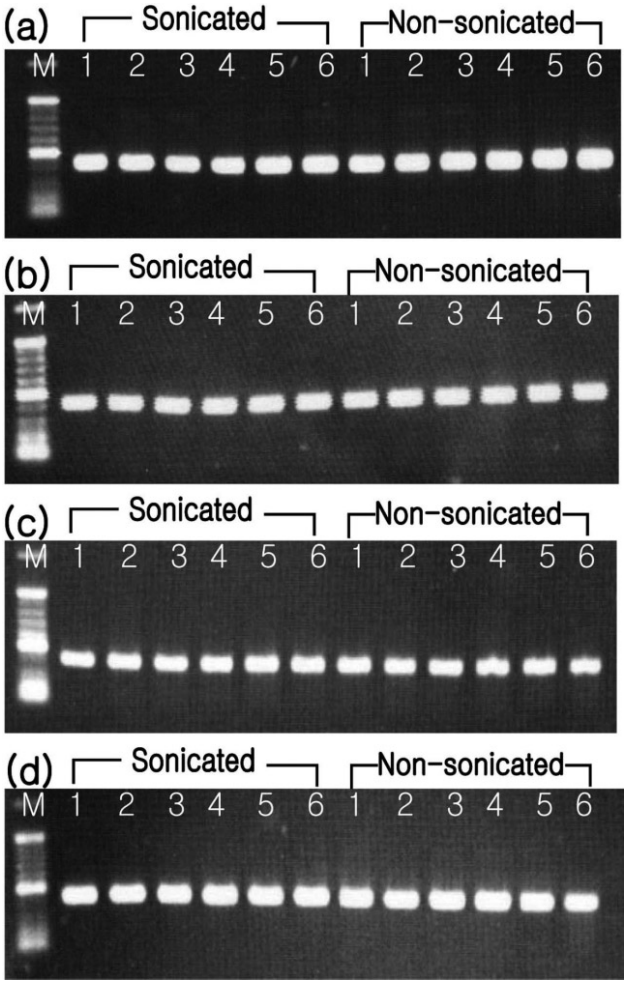

Fig. 1. Gel loading of PCR with DNA extracted from *S. mutans* (a) GS5, (b) UA159, (c) KCTC 3065, and (d) KCTC 3066. M: 100 bp DNA marker. Three ㎕ of DNA was loaded. 1: boiling 1 minute, 2: boiling 2 minutes, 3: boiling 3 minutes, 4: boiling 4 minutes, 5: boiling 5 minutes, and 6: boiling 10 minutes.

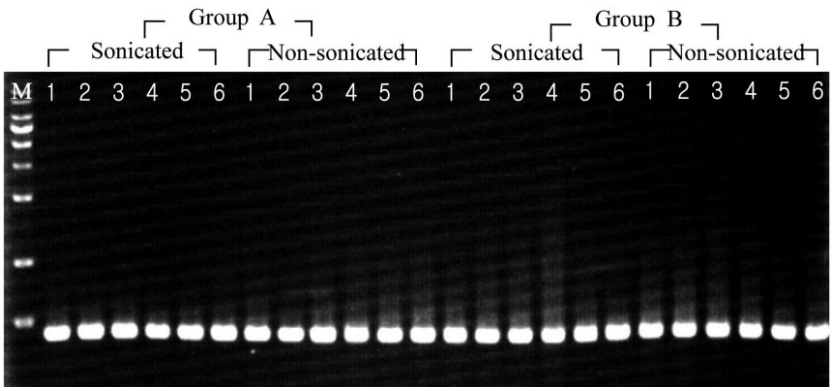

Fig. 2. PCR products from *S. mutans* GS5. PCR was performed with DNA stored at 4℃ (Group A) and -20℃ (Group B) for 13 months. Three ㎕ of PCR products were loaded. M: 1 kb DNA marker. 1: boiling 1 minute, 2: boiling 2 minutes, 3: boiling 3 minutes, 4: boiling 4 minutes, 5: boiling 5 minutes, and 6: boiling 10 minutes.

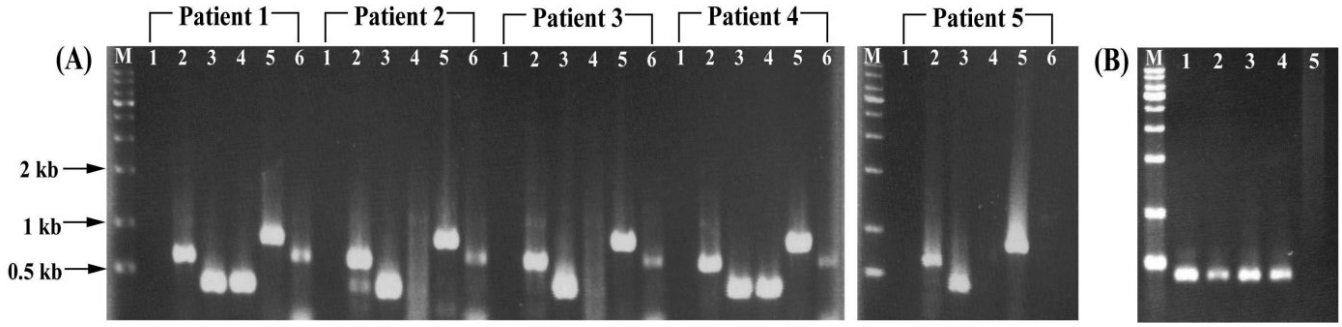

Fig. 3. PCR products from saliva samples. Bacterial DNA was obtained using the modified DNA extraction method. M: 1 kb DNA marker. 1: *A. actinomycetemcomitans*, 2: *T. forsythia*, 3: *F. nucleatum*, 4: *P. gingivalis*, 5: *P. intermedia*, and 6: *S. sobrinus*. (B) PCR products from the saliva samples using *S. mutans* GS5 primers. M: 1 kb DNA marker. 1: patient 1, 2: patient 2, 3: patient 3, 4: patient 4, 5: patient 5.

and the PCR results performed with the DNA maintained for 13 months showed no difference from the first one. It took less than 5 min to prepare genomic DNA from each sample using the modified DNA extraction method and the DNA was well amplified by PCR.

Ⅳ. Discussion

There are bacteria whose prevalence in saliva is implicated in various pathologies. Rapid detection of these pathogenic bacteria would be important and valuable for the diagnosis of the diseases that they cause and studies of them. Conventionally used methods to detect pathogens are PCR and culture methods. However, culture method is difficult to distinguish pathogenic bacteria from samples in a short period of time since it needs at least 48 hr. Furthermore, many oral micro-flora do not grow on culture media. Therefore, PCR assay could be the most prominent method and has been suggested and used for diagnosis of pathogens[2,3,4,14]. DNA isolation for PCR generally needs multiple steps that inhibit to obtain a quick result. A study suggested that the DNA extracted using NaOH was quick but easily degradable and could not be stored more than 1 month at 4℃[11]. However, the DNA we isolated using the modified extraction method in this study was stable and in a good condition for PCR for at least 13 months at 4℃ and it could be maintained for a longer term at -20℃. For the intention of disrupting Gram-positive bacteria more easily, sonication procedure was added to the method. However, it did not affect the DNA preparation and boiling the samples for 1 minute was sufficient for obtaining DNA samples for PCR while the commonly used protocol for DNA extraction contains 10 min boiling time. The DNA extraction protocol allows to obtain DNA from pathogens such as *A. actinomycetemcomitans, T. forsythia, F. nucleatum, P. gingivalis, P. intermedia*, and *S. sobrinus* and *S. mutans* for PCR in a large numbers of saliva samples (data not shown) in a short time safely since the modified DNA extraction method is only two-step procedure while other methods need more than 10 steps and it does not use any hazardous reagents like phenol and chloroform. The DNA extraction method proposed in this manuscript is rapid, simple and economical and therefore, appropriate for PCR to detect pathogens in clinical samples for quick results.

Ⅴ. Conclusion

It took 5 min to extract DNA from one saliva sample and the DNA can be used many times for over 13 months without degradation. Oral pathogens can be detected within 1 hour by PCR using the modified DNA method. In conclusion, the data of this study shows that the modified DNA extraction method used in this study is simple, rapid, and cost-efficient.

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국문초록

PCR을 이용한 치아우식증 및 치주염 연관 병원체의 빠른 검출

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구강 병원체의 검출 방법은 여러 가지가 있지만 그 중 PCR을 이용한 검출이 확실하고 빠른 방법으로 알려져 있다. PCR을 위한 많은 DNA 추출법이 사용되고 있으나 상업적인 DNA 추출 kit들은 일반적으로 가격이 비싸고 절차가 여러 단계로 되어 있으며, 그 외의 방법은 페놀과 클로로포름과 같은 유해한 화학물질을 써야하는 등의 단점이 있다.

이 연구에서 NaOH 용액을 이용한 개선된 DNA 추출 방법은 치아우식증, 치주염과 관련된 병원체를 빠르고 간단하며 비용-효율적으로 검출하였다. 세균으로부터 DNA를 추출하기 위한 boiling은 기존의 10분이 아닌 1분으로 충분하였고 4℃에서 최소 13개월 이상 DNA의 보관이 가능하였으며 sonication 유무에 따른 차이는 없었다.

따라서, 이 방법은 상업적인 kit나 유해한 화학물질을 쓰지 않고서도 타액 표본으로부터 직접적으로 빠른 시간 내에 DNA를 추출하여 병원체의 유무 결과를 확인하는데 매우 적합할 것으로 생각한다.

주요어: DNA 추출, 구강 병원균, 타액, 중합효소연쇄반응(PCR)