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A Fast and Sensitive Molecular Detection of *Streptococcus mutans* and *Actinomyces viscosus* from Dental Plaques

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Abstract

Polymerase chain reaction was used in this study in comparison with conventional method for the detection of cariogenic bacteria (Streptococcus mutans and Actinomyces viscosus) in dental plaque samples taken from two different teeth sites, a crowned tooth (tooth with crown restoration) and a natural tooth. Nested Polymerase chain reaction (N- PCR) was performed on genomic DNA which was isolated directly from plaque samples and it revealed the presence of Streptococcus bacteria and Streptococcus mutans species using two sets of 16s rDNA specific primers. Actinomyces viscosus isolates were detected also by using conventional PCR. The plaque samples were recorded negative for the presence of cariogenic bacteria, depending on conventional microbiological methods. But the same samples were recorded positive for the presence of those bacteria depending on molecular approach. This finding demonstrates that the sensitivity and specifity of the PCR techniques in the detection of the cariogenic bacteria in plaque samples are higher than the conventional culture method.

الملخص

تمت في هذه الدراسة مقارنة تفاعل السلسلة المبلمرة مع الطرق التقليدية في زراعة البكتيريا في الكشف عن وجود البكتيريا المسببة للتسوس: الستربتوكوكس ميوتانس (Streptococcus mutans) والأكتينومايسس فيزكوسس Actinomyces viscosus. حيث تم الكشف عن هذه البكتيريا في عينات البلاك (الصفائح الجرثومية) المأخوذة من أسطح مختلفة من الأسنان: من على سطح تركيبة سنية (تاج) ومن سطح سن طبيعي. تم استخدام طريقة تفاعل السلسلة المبلمرة المتشابكة (N-PCR) للمادة الوراثية المعزولة مباشرة من عينات البلاك وأظهر التفاعل وجود بكتيريا الستربتوكوكس (Streptococcus) و الستربتوكوكس ميوتانس (Streptococcus mutans). باستخدام زوجين من البوادئ (primers) ، الزوج الأول مطابق للسلسلة النيوكلوتيدية لجين الحمض الرايبوسومي (rDNA) للجنس الستربتوكوكس والثاني مطابق لجين الحمض الرايبوسومي (rDNA) للنوع ستربتوكوكس ميوتانس (Streptococcus mutans). لقد تم الكشف أيضا عن بكتيريا الأكتينومايسس فيزكوكس Actinomyces viscosus باستخدام تفاعل السلسلة المبلمرة الإعتيادية. أظهرت الطرق الميكروبيولوجية التقليدية في زراعة البكتيريا عدم وجود البكتيريا بينما أظهرت نفس العينات وجود البكتيريا باستخدام الطرق الجزيئية (PCR). تبين هذه النتائج مدى حساسية و خصوصية وسرعة طرائق تفاعلات السلسة المبلمرة في الكشف عن وجود البكتيريا المسببة لتسوس الأسنان في عينات البلاك مقارنة بالطرق التقليدية.

Keywords: Streptococcus mutans. Actinomyces viscosus. Cariogenic bacteria. Nested PCR.

1. Introduction

The most cariogenic bacteria in dental plaque are *Streptococcus mutans* (Samaranayake et al., 1996; Rosan and Lamont, 2000; Hoshino et al., 2003). These bacteria have many properties which enable them to be potentially cariogenic (Melville and Russell, 1960). *Actinomyces* species were found to be associated with root caries, especially *Actinomyces viscosus* and *Actinomyces naslundii* (Bowden, 1989; Nyvad and Kilian, 1990).

Common methods of detection and characterization of pathogenic bacteria from the oral cavity are conventional especially culture methods, which are used to identify bacterial pathogens in dental plaque samples. Culture techniques are limited in their sensitivity and specificity, and they consume much time. In addition, it was found that 50% of oral micro-flora does not grow on culture media in the laboratory (Paster et al., 2001; Munson et al., 2004).

Molecular biology methods have been developed to overcome culture problems. Polymerase chain reaction (PCR) is now used in bacterial identification in environmental and clinical specimens. PCR methods are more sensitive and specific, and faster than conventional methods in bacterial determination. They allow the detection of viable and nonviable microorganisms, and consume less time and effort than conventional methods. A developed PCR method, which is called "Nested PCR' allows more sensitive detection of pathogenic bacteria. This method consists of first-step amplification with universal primers. The amplified products are used as

template in second-step amplification, where species specific primers are used (Sato et al., 2003).

This study is the first to be done in Jordan using molecular methods for the detection of the most cariogenic bacteria in the oral cavity obtained from dental plaque from different teeth sites.

The aim of this study is to detect the most cariogenic bacteria in plaque samples taken from different teeth sites by using polymerase chain reaction (PCR) method. PCR was performed for specific detection *Actinomyces viscosus*. Nested PCR was used to specifically detect *Streptococcus mutans species.* The approache of this study can be used in future research for detecting the effect of certain dental restorations, or materials, on the bacterial composition of dental plaque.

2. Materials and Methods

2.1. Subjects and Plaque Sampling

The study consisted of four plaque samples taken from one person who had a metal ceramic crown restoration and a natural tooth in the closet proximity to the crown site. The study protocol was approved by the Committee of Search on Human at Jordan University of Science and Technology. The subject was a patient of Dental Teaching Center.

Dental plaque samples were collected by using sterile curettes (Gracey Curettes). Supragingival plaque was taken first from the crown site, and then was taken from the subgingival plaque. The same procedure was applied to the plaque samples obtained from the natural tooth. The plaque samples were suspended in 1 ml of sterile Phosphate buffer saline PBS (0.12 M NaCl, 0.01 M Na_2HPO_4, 5mM KH_2PO_4 [pH 7.5]). The samples were transported on icebox to the laboratory.

2.2. Detection of Cariogenic Bacteria by Cultivation Methods

Plaque samples were dispersed by vortexing for 30s with glass beads (diameter 4 mm), and samples were diluted into different decimal serial dilutions in phosphate buffer saline (pH 7.5). The appropriate dilutions of each sample was plated on the following selective media: Mitis salivarius agar (MSA) supplemented with 0.2 U/ml bacitracin and 5% sucrose and Cadmium Flouride Acriflavine Tellurite (CFAT) medium supplemented with 5% human blood for the culture of *Streptococcus mutans* and *Actinomyces species* respectively. The plates were incubated at 37 °C for three days in an anaerobic jar with CO_2 gas generating kit. To confirm the presence of *Streptococcus mutan*s, the bacterial isolates grown on MSBS were subjected to certain biochemical tests (Sneath et al., 1986). For the identification of the bacterial isolates, which were grown on CFAT media, the RapID ANA II kit (Remel Compny. USA) biochemical kit was used according to the manufacture's instructions.

2.3. Extraction of The Genomic DNA from Streptococcus Isolates

Extraction of genomic DNA from bacterial colonies (local isolates in our laboratory), grown on MSA supplemented with bacitracin and sucrose was done as following: one colony from each pure bacterial isolate was inoculated into 10 ml of Trypticase soya broth and incubated for 24 hours at 37°C. 1 ml from each of the trypticase broth of the 24 hours broth was transferred to a new sterile eppendorf tube and the bacterial cells were harvested by centrifugation at 8000 xg for 15 minutes. The resulted pellet was digested by addition of 500 µl of lysis buffer (50 mM tris buffer, 1 mM EDTA, 0.5% Tween 20, and proteinase K (200µg/ml). pH 8) and then incubation at 55°C for 2 hours. Heating at 90 °C for 5 minutes was done for the inactivation of proteinase K. The DNA was precipitated by addition of an equal volume of cold isopropanol and incubated in freezer for 20 minutes. The pellet was washed with 70% ethanol and then rehydrated by addition of 35-50µl TE buffer (10 mM Tris- HCl, and 1 mM EDTA) (pH 8). This DNA was subjected to N-PCR reaction for amplification of 16s rDNA specific to the genus Streptococcus and species Streptococcus mutans.

2.4. Extraction of Total Genomic DNA Directly from Plaque Samples.

The total genomic DNA was isolated from plaque samples according to Paster *et. al.* (2001) with few modifications as follows: 100 µl of plaque sample was transferred to a new sterile eppendorf tube where 100 µl of the lysis buffer was added (50 mM tris buffer, 1 mM EDTA, 0.5% Tween 20, and proteinase K (200µg/ml). pH 8), then incubation at 55-60 ºC was done for 1.5 hour. Inactivation of proteinase K was done at 90 ºC for 5 minutes. After that, samples were cooled on ice for few minutes and the DNA was precipitated by the addition of an equal volume of ice-cold isopropanol and incubated at refrigerator overnight. The DNA pellet was washed with 70% ethanol, and was then rehydrated in 20- 30 µl TE buffer (10 mM Tris- HCl, and 1 mM EDTA) (pH 8).

2.5. Polymerase Chain Reaction (PCR)

Genomic DNA of 4 plaque samples that belong to one person was subjected to PCR reactions in order to detect the presence of *Actinomyces viscosus*. A Nested PCR (N-PCR) reaction was performed to detect the presence of mutans Streptococci. The first (N- PCR) was conducted to detect genus *Streptococci*, and the second (N- PCR) detected *Streptococcus mutans*.

For each PCR reaction, a negative control reaction was performed where no DNA template was added. All PCR reactions were performed in a Perkin Elmer DNA thermal cycler (Perkin Elmer 480). All PCR products were kept at 4 ºC until analyzed.

2.6. Detection of The Presence of Streptococcus Mutans Using Nested PCR Polymerase Chain Reaction, using primer pair specific to 16S rDNA specific to Streptococcus, was performed according to Sato et al. (2003) to detect Streptococcus. The target sequence of 16S rDNA was amplified by using PCR mixture (total volume 25 µl) containing 3mM $MgCl_2$, 0.4 mM dNTPs, 5U of Taq DNA polymerase, 1µl of each primer (5uM) (Table 1), 2.5 µl of 10x PCR buffer, and 1 µl of template DNA (20 ng). The PCR program consisted of initial denaturation at 95 ºC for 15 min, and 35 cycles involving denaturation at 94 ºC for 1 min, and annealing at 55 ºC for 1 min, and

extension at 72 ºC for 1.5 min followed by a final extension at 72 ºC for 10 min. All reaction mixtures were held at 4°C.

Table 1: Primers that were used for the amplification of oral bacteria

Primer set		Sequence 5'→3'	bp[a]	Reference
Streptococci species	8UA (F)	AGA GTT TGA TCM TGG CTC AG	1505	Sato et al. 2003
	1492 (R)	TAC GGY TAC CTT GTT ACG ACT T		
Streptococcus mutans	Sm1 (F)	GGTCAGGAAAG TCTGGAGTAAA AGGCT A	282	
	Sm2 (R)	GCG GTA GCT CCG GCA CTA		
Actinomyces viscosuss	A.vis (F)	ATG TGG GTC TGA CCT GCT	96	Suzuki et al. 2004
	A.vis (R)	CAA AGT CGA TCA CGC TCC G		

[a]: Size of the amplified product bp

The second nested PCR reaction was done for the detection of *Streptococcus mutans* by using species specific primers based on the 16S rDNA, Sato. et al. (2003). Briefly: The target sequence of 16S rDNA was amplified by using PCR mixture (total volume 25 µl) containing: 3mM $MgCl_2$, 0.4 mM dNTPs, 5U of Taq DNA polymerase, 1µl of each primer (5µM) (Table 1), 2.5 µl of 10x PCR buffer, and 1 µl of template DNA (20 ng). The PCR program is isentical to the one used in the detection of *Streptococcus* genus in the first reaction.

2.7. PCR Detection of The Presence of Actinomyces Viscosus by Using Primer Pair Specific to The Species

Polymerase Chain Reaction for *Actinomyces viscosus* using species specific primers to each species was performed according to Suzuki et al. (2004). The target sequence of 16S rDNA was amplified using PCR mixture (total volume 25 µl) containing: 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 5U of Taq DNA polymerase, and each of the primer added at a concentration of 0.2µM (Table 1), 2.5 µl of 10x PCR buffer, and 1 µl of template DNA (20 ng). The PCR program had initial denaturation at 95 ºC for 15 min, and 35 cycles involving denaturation at 94 ºC for 1 min, and annealing at 55 ºC for 1 min, with extension at 72 ºC for 1.5 min followed by a final extension at 72 ºC for 10 min. All PCR products were held at 4°C.

2.8. Gel Electrophoresis and Photography

The PCR amplified products were separated in ultra-pure agarose gels dissolved in 1X TBE buffer (0.89 M Tris Base, 0.89 M Boric Acid, 20 mM EDTA) (pH 8.3). 1.5 % w/v gel was used for separation of amplified PCR products for *Streptococcus* detection. And 3% w/v gel was used for the separation of PCR bands of *Actinomyces* amplification products. 5 µl of PCR product was mixed with 2 µl of 6x loading dye and then loaded into the well of the gel. 1 Kilo base pair (Kbp) and 100 base pair (bp) markers were included in the gel. PCR products were separated through the gel at an electric current of 90 V for 1 hour by using horizontal gel electrophoresis apparatus (Sigma Chemicals Co. USA). Gels were stained with ethidium bromide (0.5µg/ml) and visualized on a UV transilluminator by using BioDocAnalyze (Biometra, Germany).

3. Results

3.1. Detection of Cariogenic Bacteria by Cultivation Methods

All cultivated plaque samples were recorded 'negative' (no bacterial growth) regarding the detection of Streptococcus mutans and Actinomyces species.

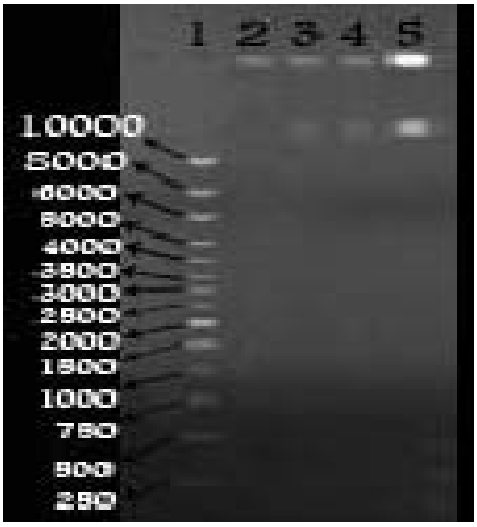

Figure 1: Agarose gel electrophoresis of the total genomic DNA isolated from four plaque samples (Lane 2 to Lane 5). Lane 1; molecular weight marker (1K bp ladder). Lane 2; the supragingival plaque of natural site, Lane 3; the supgingival plaque of the natural site, Lane 4; the supragingival plaque from crown site, Lane 5; the subgingival plaque from the crown site.

3.2. Total Genomic DNA Extraction Directly from Plaque Samples

The total Genomic DNA was isolated as described previously in materials and methods from four plaque samples (Figure 1), and revealed good quality and quantity of genomic DNA.

3.3. Detection of the presence of Streptococci mutans using Nested PCR

Total genomic DNA, which was isolated directly from plaque samples, was subjected to N-PCR reactions. The detection of the genus *Streptococci* was performed by using primer pair specific to 16s rDNA of the genus *Streptococci*. The positive amplified PCR product (1505 bp size), representing *Streptococcus* genus, was detected in all the 4 samples (Fig. 2). In the second reaction, the detection of the *Streptococci mutans* was done by using 16s rDNA primer pair specific to the species *Streptococcus mutans*. The species *Streptococcus mutans* was detected in all, four, samples. (Fig. 3) represents the presence of the amplified PCR product (282 bp).

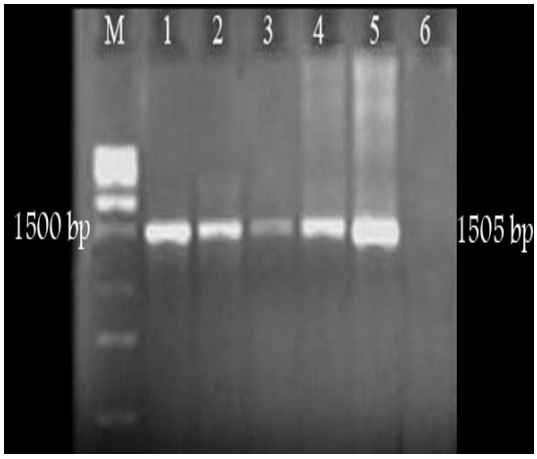

Figure 2: Agarose gel electrophoresis of PCR amplified products for 16s rDNA specific to Streptococcus genus for four plaque samples. The amplified band is 1505 bp. Lane M; molecular weight marker (1K bp ladder). Lane 1: positive control (PCR amplified product from genomic DNA isolated from the bacterial species Streptococcus mutans), (Lanes 2 and 3: represents plaques from natural tooth, (Lanes 4 and 5): represents plaques from crown tooth. (Lanes 2 and 4): represents the supragingival plaque, (Lanes 3 and 5): represents the subgingival plaque. Lane 6: negative control.

3.4. PCR detection of the presence of Actinomyces viscosus by using primer pair specific to these species

PCR detection of the presence of species *Actinomyces viscosus* using primer pair specific for this species (Table 1) resulted in an amplified product of 96 bp which was detected in all four samples (Fig. 4). All these samples were recorded negative of *Actinomyces* species by using the cultivation on CFAT medium.

4. Discussion

In this study four plaque samples, which belong to one person, were included in the study. The plaque samples, which were obtained from crown site, have zero count of *Streptococcus mutans* and of *Actinomyces viscosus*. However, PCR methodology was able to positively detect *Streptococcus mutans* and *Actinomyces viscosus* in all these samples. Wade (2002) indicated that many bacteria escaped the conventional culture techniques for detection either because they are unproductive, or because there are not distinguishable from similar species by observable phenotypic characteristics. In this study, *Streptococcus mutans* and *Actinomyces viscosus* were not detected in previous samples based on the conventional culture method. This could be attributable to their presence in low proportions in the samples, and/or the culture medium was not sensitive enough for the detection of the low levels of those bacterial species. This confirms and proves that PCR is more sensitive than conventional culture method.

The results of this study confirm the disadvantages of conventional method such as poor specificity and sensitivity; detection of only viable culturable bacteria - and that they are time consuming and laborious.

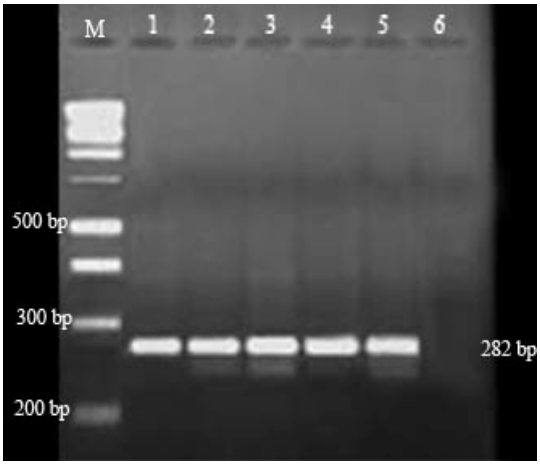

Figure 3: PCR detection of the species *Streptococcus mutans* in four plaque samples. Lane M; molecular weight marker (100 bp ladder), Lane 1: positive control, (Lanes 2 and 3): represents plaque samples from natural tooth, (Lanes 4 and 5): represents plaque samples from crown tooth, (Lanes 2 and 4): represents supragingival plaque samples, (Lanes 3 and 5): represents subgingival plaque samples. The size of amplified product is 282 bp. Lane 6: negative control.

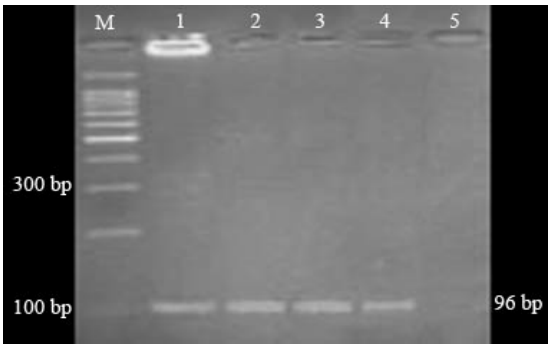

Figure 4: PCR detection of the species *Actinomyces viscosus* in four plaque samples. Lane M; molecular weight marker (100 bp ladder), (Lanes 1 and 2): represents plaque samples from natural tooth, (Lanes 3 and 4): represents plaque samples from crown tooth, (Lanes 1 and 3): represents supragingival plaque samples, (Lanes 2 and 4): represents subgingival plaque samples. The size of amplified product is 282 bp. Lane 5: negative control.

On the other hand, PCR methodology provides a more sensitive mean of detection of putative bacterial species even non-culturable bacteria if compared with conventional culture techniques. Also it is able to detect low numbers of bacterial species, being quick and relatively simple to perform. Moreover, a PCR assay has been found to be suitable for the specific detection and identification of human cariogenic bacteria like *Streptococcus mutans* (Sato et al., 2003). This study detected both *Streptococcus mutans* and *Actinomyces* species to be the most serious human cariogenic bacteria.

This research provides protocols that are potentially considered a cornerstone of the oral microbiological research that can be conducted in order to investigate the composition of dental plaque with many stressful factors, for instance, presence of dental materials.

5. Conclusion

With the methods of this study, the following conclusions could be drawn: PCR molecular approach was very sensitive, and it was specific and rapid in detecting and identifying the presences of cariogenic bacteria if compared to conventional culture approach. So it is recommended, for further research, to utilize PCR technique as a sensitive and specific method for the detection and identification of the human cariogenic bacteria.

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