Early detection of oral bacteria causing gum infections and dental caries in children

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ABSTRACT

Background and Objectives: Periodontal diseases are resulted from gum infections and dental plaques, which are mainly caused by the bacterial agents. Since dental monitoring includes important prognostic roles, the aim of this study was to detect the most common periodontal pathogenic bacteria in children.

Materials and Methods: A total of 200 clinical samples were collected from dental plaques and gingival grooves. Target-specific primers were designed for *hbpA* in *Aggregatibacter actinomycetemcomitans*, *fimA* in *Porphyromonas gingivalis* and 16S rRNA in *Prevotella intermedia, Tannerella forsythia* and *Treponema denticola*. Then, a multiplex polymerase chain reaction method was optimized for the highlighted bacterial agents.

Results: In general, the highest and the lowest bacterial prevalence rates belonged to *Tannerella forsythia* (88%) and *Porphyromonas gingivalis* (13%), respectively. Furthermore, prevalence rates of *Aggregatibacter actinomycetemcomitans, Prevotella intermedia* and *Treponema denticola* were 25, 21 and 45% in samples, respectively.

Conclusion: There were significant associations between dental/oral health and microbial community. Metabolism of the oral bacteria, including biofilm formation, can affect gums and develop dental plaques and hence dental caries, especially in children. Early diagnosis of dental caries in children via rapid, accurate molecular methods can increase the diagnostic capacity in clinical cases and therefore prevent periodontal infections in adulthood.

Keywords: Periodontal diseases; Bacterial pathogens; Dental plaques; Dental caries; Children

INTRODUCTION

The oral cavity is the primary surface for the microbial colonization due to its physiological conditions (1). Opportunistic activity and metabolic products of the oral microbiota can lead to infectious lesions (2). Acid production by the bacteria induces dental plaque fermentation (3). Dental plaque is a dense and complex biofilm structure that accumulates on the hard tissues such as teeth and soft tissues including gingival sulcus in the oral cavity (4). Supporting tissues around the teeth can be affected by the plaques. Without regular teeth screening, patients may not be aware of the infection progression on dental support-

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ing tissue. This issue results in severe damages to the gums and jawbones and therefore tooth loss cannot be inevitable (5). Gingivitis is the first symptom of the infected surrounding teeth tissue, leading to chronic periodontitis (6). Periodontitis is an inflammatory disease of the periodontium and a significant sign of the gum infections. Periodontal infections are the most common infections and significant causes of tooth loss. Developing periodontal infections can affect heart and brain as well (7, 8). During pregnancy, periodontal infections can lead to low-birth-weight babies (9). Patients with chronic infections such as gum infections are at high risks for coronary heart diseases such as atherosclerosis (8). Several oral bacteria cause periodontal diseases with inflammatory processes involved in growth and formation of cholesterol plaques in walls of arteries. Recent studies have shown increases in heart diseases and stroke in people with gum infections (10).

Majorly, bacterial species of periodontal infections include *Aggregatibacter actinomycetemcomitans, Bacteroides gingivalis, B. intermedius, Eikenella corrodens, Wolinella recta, Fusobacterium nucleatum, Prevotella intermedia, P. nigrescens, Treponema denticola, Tannerella forsythia, Campylobacter rectus, Micromonas micros* and *Dialister pneumosintes* as well as enteric rods (9, 11-14). Periodontal pathogenic bacteria play significant roles in induction and progress of periodontal diseases. Early detection can help screen children, who need further effective oral health procedures to protect them from risks of periodontal diseases and hence decrease health care costs (15). Oral pathogenic bacteria can develop infections during mechanical treatments of the gums and teeth. Therefore, it is critical to detect these bacterial agents and provide appropriate antibiotic therapy regimens to decrease or eliminate the bacterial loads (16). Relatively, traditional culture-based methods have been used to detect the oral pathogenic bacteria. However, due to the slow growth, time-consuming and costly characteristics of the conventional diagnostic methods, it is necessary to develop molecular diagnostic methods such as polymerase chain reaction (PCR) for the faster and better diagnosis of the oral microbial agents (17, 18). Culture and fluorescence microscopy methods are further used for the clinical specimens; however, these methods are relatively time-consuming and expensive. In addition, these methods cannot detect *T. denticola* and *B. forsythus*. Technically, 16S rRNA

genes, as housekeeping genetic markers, can be used for the detection of pathogenic bacteria. Moreover, other conserved genetic regions can be used in various PCR methods (19, 20). Therefore, the major aim of the current study was to assess prevalence rates of most common periodontal pathogenic bacteria in spaces between the children's gums and dental plaques as a prognosis factor.

MATERIALS AND METHODS

Patient selection. The current study was approved by the Ethical Committee of Tehran University of Medical Sciences (ethical code: IR.TUMS.SPH. REC.1400.135). An expert dentist assessed the patients based on clinical gingival index (GI) and plaque index (PI). In this study, inclusion criteria were patients under 15 years old with dental plaques or gingivitis and exclusion criteria were patients, who received antibiotics within the last two weeks.

Sample collection. In this study, sampling from the patients was carried out thrice a week. For the sample collection, supragingival spots were dried and then sampling was carried out to minimize cross-contaminations. Dentist applied a detector to the tooth surfaces for plaque sampling, except for the occlusal surface. In this technique, discoloration was seen after 5 min and samples were collected from the dental surfaces using sterile endodontic paper points (ISO40). Samples were also collected from a depth of 1-3 mm in the gums. These were immediately transferred to the microbiology laboratory under cold conditions using tubes containing 1 ml of DNAase-free deionized water. Samples were stored at -20°C until use.

Primer design. In this study, primers were designed using AlleleID software, regarding dynamic factors such as primer melting temperature (Tm), G/C count, loops and dimer production. Primer sequences and characteristics of the bacterial gene targets are shown in (Table 1).

DNA extraction. Based on the manufacturer's instructions, extraction was carried out using Cinna Pure extraction kit (Sinaclon, Iran). Then, concentration and purity of the extracted DNA were assessed at 260/280 nm using NanoDrop spectrophotometers (Thermo Fisher Scientific, USA). Moreover, DNA

Table 1. Primer sequences and their gene targets used in this study

Bacteria	Gene	Primer	Primer sequence	Tm (°C)	(bp)
Aggregatibacter	*hbpA*	F-Aggrega-*hbpA*	ACAGACCCAATGCAAAAGTAAC	50	397
actinomycetemcomitans		R-Aggrega-*hbpA*	TTTGTGAGATTATATACTCCGGC	50	
Porphyromonas gingivalis	*fimA*	F-Porphy-*fimA*	CTACTCAGCTAACGGTGGGAC	50	292
		R-Porphy-*fimA*	CTCTGTGATAGGATTCTCGGG	50	
Prevotella intermedia	16s rRNA	F-Pre 16	TGAGTATCGCGTATCCAACC	50	617
		R-Pre 16	CTTCGCAATCGGAGTTCG	50	
Tannerella forsythensis	16s rRNA	F-Tan-16	GCGTAGGTGGGCTGTTAAG	50	188
		R-Tan-16	TCGTGCTTCAGTGTCAGTTATAC	50	
Treponema denticola	16s rRNA	F-Tre-16	GGAGCATGTGGTTTAATTCG	50	484
		R-Tre-16	CCTCGACTCGGATGGTGT	50	

samples were electrophoresed on agarose gels. Samples were stored at -20°C until use.

Multiplex PCR and DNA sequencing. Multiplex PCR was carried out for the bacterial gene targets using designed primers. The PCR was carried out in a volume of 50 µl using sterile microtubes of 0.5 ml and thermal cycler (PeqLab, Germany). Master mix included 10× buffer (5 µl), 2.5 mM of MgCl$_2$ (3 µl), Taq polymerase (2.5 µl), 200 mM of dNTP (4 µl), each primer with a concentration of 0.2 µM (1 µl) and the DNA template with a concentration of 50 ng/µl (2 µl). Sufficient sterile water was added to the reaction make a total volume of 50 µl. The PCR amplicons were electrophoresed on 1% agarose gels. Then, Vivantis gel extraction kit (Vivantis, USA) was used to extract the favorite DNA bands from the gels based according to the manufacturer's instructions. After purification, PCR amplicons were sequenced (Life Bioscience, UK) and then the raw sequences were edited using CLC Sequence Viewer v.6.5 and Finch TV software v.1.4.0. Furthermore, sequences were compared with the original sequences using NCBI BLAST database.

RESULTS

Patients. Out of total 200 patients, 102 patients were female and 98 patients were male. The mean age of the patients was 4-12 years.

Multiplex PCR. In this study, multiplex PCR was carried out for the 16S rRNA gene of *P. intermedia, T. forsythia* and *T. denticola*. Furthermore, *fimA* gene of *P. gingivalis* and *hbpA* gene of *A. actinomycetemcom-*

itans were amplified using similar conditions (Fig. 1). The highest and the lowest prevalence rates belonged to *T. forsythia* (88%) and *P. gingivalis* (13%). Moreover, prevalence rates of *P. intermedia, A. actinomycetemcomitans* and *T. denticola* were 21, 25 and 45%, respectively (Fig. 2). Results showed that *T. forsythia* was most prevalent in 40% of male and 48% of the female patients. However, *P. gingivalis* was the lowest prevalent bacterial agent within the groups.

DISCUSSION

As oral germs propagate, dense, complex masses form on the surface of the teeth called dental plaques.
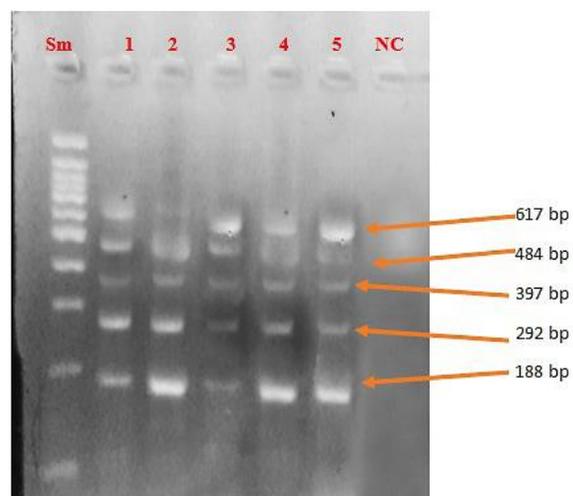

Fig. 1. Multiplex polymerase chain reaction of Lanes 1-5, *Prevotella intermedia* (617 bp), *Treponema denticola* (484 bp), *Aggregatibacter actinomycetemcomitans* (397 bp), *Porphyromonas gingivalis* (292 bp) and *Tannerella forsythia* (188). Sm, 100 base-pair DNA ladder. NC, negative control
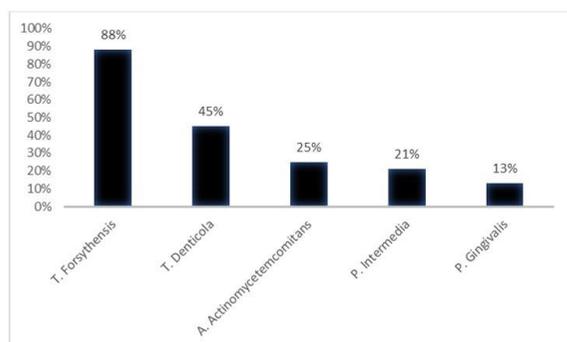
Fig. 2. Total prevalence rates of the target bacteria

Plaques occur in the cavities and grooves of the masticatory surfaces, gingival sulcus and periodontal pockets (21, 22). Recent studies have shown increases in heart diseases and stroke in patients with gum infections. The leading cause of dental and periodontal diseases is microbial plaque accumulating on the teeth. The oral cavity includes more than 1,300 strains of microorganisms that could develop plaques and dental caries due to damages or poor oral hygiene (23).

Nucleic acid-based methods for the analysis of bacterial diversity in the oral cavity have majorly identified a primary list of pathogen-associated diseases (24). Diverse groups of bacteria that colonize oral cavity need their own physicochemical and nutritional situations for appropriate growth. Previous studies have indicated that most of these bacteria cannot not be cultured in the laboratory. Introduction of non-cultured nucleic acid methods has resulted in identification of further comprehensive ranges of oral bacteria (25). In summary, data from culture and molecular studies showed that 700 species of microorganisms such as bacteria could live in the human oral cavity (23).

However, some species are limited to areas of the mouth. Most species act selectively for a particular location. This possibility may play critical roles in oral health and disease (23). Molecular analyses have reported that a broad range of bacteria are detected in the oral cavity, belonging to various phyla and orders such as *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Fusobacteria, Spirochaetes, Saccharibacteria* (TM7), *Chloroflexi, Cyanobacteria, Deinococcus* and *Acidobacteria*. Furthermore, recent studies have suggested that *Aquatica-a, Nitrospira, Planctomycetes* and *Thermomicrobia* members may be represented in the mouth (26, 27). The high-throughput molecular techniques such as next generation sequencing (NGS) platforms analyze microbial communities in saliva and dental plaques, targeting V6 region of the 16sRNA gene (28). The estimated number of the oral microflora in the current studies is much higher than that in previous culture studies. Acid nucleic methods have verified that diversity of dental cavities is more significant than that previously predicted. Reliable fast methods of identifying microorganisms help investigate possible associations between the oral bacteria and periodontal diseases. Traditional diagnostic techniques include culture and immunological methods. Routine culture methods are time-consuming and expensive and may fail to propagate fastidious microorganisms. Additionally, immunological diagnostic methods need specific antibodies that may not be available. These methods may also lead to false-positive results by cross-reacting with non-target microorganisms (29). Recently, quantitative real-time PCR system using TaqMan have been used to detect a broad spectrum of microorganisms, including bacteria (30, 31).

In a study, Ashimoto et al. used 16S rRNA gene-based PCR to investigate prevalence of bacteria associated with periodontal diseases. Prevalence of *A. actinomycetemcomitans, B. forsythus, C. rectus, E. corrodens, Porphyromonas gingivalis, P. intermedia, P. nigrescens* and *T. denticola* were investigated in 50 patients with pneumonia and minor gingivitis. Study demonstrated advantages of 16S rRNA-based PCR for the detection of significant oral microorganisms. Furthermore, results showed significant associations between the microbial species and periodontal diseases (32). Asai et al. used species-specific PCR methods to detect oral bacteria such as *T. denticola, T. vincentii* and *T. medium* in human subgingival plaque specimens. They assessed numbers of the microorganisms using quantitative real-time PCR (33). Use of PCR to identify periodontal pathogenic agents in clinical specimens verified that this method could be a promising method for the clinical samples of children (34). Comparing culture and real-time PCR methods to diagnose *P. gingivalis* in subgingival plaque samples, they concluded that not only real-time PCR verified quantitative culture results, it included further advantages such as specificity and sensitivity in detecting the bacteria (20, 35). Current studies have designed multiplex PCR methods that detect *Actinomycetemcomitans* and *P. gingivalis* (36, 37). Takeuchi et al. reported that *T. socranskii, T. denticola* and *P. gingivalis* were frequently isolated in periodontitis patients using PCR. They also

showed that the isolates were associated to the severity of periodontal tissue destruction (38). In a study on relationships between the age and periodontal diseases, Rodenberg et al. reported that age is a risk factor for *P. gingivalis* infection (27). Another study by Al-Qadami et al. assessed periodontal-causing pathogenic bacteria in high-school children. Their clinical study included gingival and periodontal support tissue measurements, including loss of strength and gingival pocket depth. Samples of the bacterial microflora were assessed using multiplex PCR. Results showed that *A. actinomycetemcomitans* was present in 21.7% and *P. gingivalis* in 21.3% of the cases. In addition, *T. forsythia* was detected in 10.1%, *T. denticola* in 34.7% and *P. intermedia* in 12.3% of the patients. The red complex bacteria were also observed in 2.9% of the patients (39).

A recent study reported significant associations of *Scardovia wiggsiae* in severe early childhood caries (40). Use of additional methods could detect other bacterial agents. Chen et al. reported that *Helicobacter pylori* colonization in dental plaque was associated with *H. pylori* gastritis. They concluded that follow-up of dental plaques using DNA-biosensors was a noninvasive diagnostic method (41). Bashirian et al. demonstrated that dental caries background and plaque formation in elementary school children in Hamadan, Iran, were high affected by the children's socioeconomic situations (42). Although dental plaques cause gingival inflammation, but not all the inflammatory sites are developed to periodontitis. However, antibiotic therapy is critical to control attachment and destruction of periodontal tissues during gingivitis (43). In general, further studies are necessary to better understand mechanisms of biofilm formation by the oral bacterial pathogens (44).

CONCLUSION

In this study, a multiplex-PCR method was designed to achieve fast, accurate, sensitive detection of the most critical oral pathogenic bacteria. Based on the findings, higher proportions of gingival and periodontal damaging bacteria were detected in patients of older ages. No significant differences were reported between the sex and periodontitis. Follow-up and routine periodontal screening in childhood can decrease risks of developing periodontal infections in adulthood.

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