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Original Article

جداسازی و شناسایی فلور باکتریایی قابل کشت نمونه‌های ضایعات دندانی از یک دندانپزشکی

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چکیده

سابقه و هدف: پلاک دندان از نظر ساختاری و عملکردی یک بیوفیلم می‌باشد که در اثر برهم خوردن هموستازی میکروبی ممکن است به پوسیدگی و عفونت ریشه منجر شود. هدف از مطالعه حاضر، بررسی مولکولی فلور باکتریایی دندانی بیماران شرق تهران بود.

مواد و روش‌ها: در این مطالعه مقطعی، تعداد ٩ نمونه پلاک دندان، پوسیدگی و کانال ریشه از پنج بیمار که به طور تصادفی در سال ١٣٩٦ انتخاب شده بودند، در شرایط استریل جمع‌آوری شد. باکتری‌ها، با استفاده از محیط کشت استاندارد BHI broth کشت و خالص‌سازی و از پرایمرهای عمومی ژن 16S rRNA برای شناسایی مولکولی باکتری‌ها و بررسی روابط فیلوژنیک آن‌ها استفاده شد. مشخصات دموگرافیک افراد مورد مطالعه نیز بررسی شد.

یافته‌ها: تعداد ١٣ جدایه باکتری از نمونه‌های کلینیکی پلاک و پوسیدگی دندان شناسایی شدند. باکتری‌های جدا شده متعلق به سه شاخه، پنج خانواده، شش جنس Arthrobacter، Brevundimonas، Granulicatella، Kocuria، Neisseria و Streptococcus و هفت گونه بودند. فراوان‌ترین باکتری‌های جدا شده N. perflava (n=5) و S. salivarius (n=3) بودند. باکتری‌های شناسایی شده در چهار شاخه از درخت فیلوژنی مرتب شدند. ارتباطی بین باکتری‌ها و مشخصات دموگرافیک یافت نشد.

نتیجه‌گیری: شناسایی عوامل دخیل در عفونت‌های دندان رویکرد موثری برای پیشگیری محسوب می‌شود. در این مطالعه، ١١ جدایه باکتری از پلاک و ٢ جدایه باکتری از پوسیدگی دندان شناسایی شد و هیچ سویه‌ای از نمونه ریشه شناسایی نشد. عدم مشابهت باکتری‌های پلاک و پوسیدگی می‌تواند به دلیل حجم کم نمونه‌های مورد بررسی و روش‌های میکروب شناسی به کار رفته باشد.

واژگان کلیدی: بیماری‌های دندان، فلور باکتریایی، پلاک دندان، پوسیدگی دندان.

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Original Article

Isolation and identification of culturable bacterial flora of dental lesions in a clinic

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Abstract

Background & Objectives: Dental plaque is structurally and functionally a biofilm that may lead to caries and root infection due to disruption of microbial homeostasis. The present research aimed to investigate the molecular characteristics of dental flora of patients in East Tehran.

Materials & Methods: In this cross-sectional study, 9 samples of dental plaque, caries, and root canal from five patients who were randomly selected in 2017 were collected under sterile conditions. Bacteria were cultured using the standard BHI broth culture medium. General primers of the 16S rRNA gene were used for molecular identification of bacteria and investigation of their phylogenetic relationships. Demographic characteristics of the subjects were also examined.

Results: Thirteen bacterial isolates were identified from clinical specimens of plaque and tooth decay. The isolated bacteria belonged to three phyla, five families, six genera Arthrobacter, Brevundimonas, Granulicatella, Kocuria, Neisseria, and Streptococcus, as well as seven species. The most abundant isolates were N. perflava (n=5) and S. salivarius (n=3). The identified bacteria were arranged in four branches of a phylogenetic tree. No association was found between bacteria and demographic characteristics.

Conclusion: Identifying the factors involved in dental infections is an effective approach to prevention. In this study, 11 bacterial isolates from dental plaque and 2 bacterial isolates from tooth decay were identified and no strains from the root specimens were identified. The discrepancy between plaque bacteria and caries may be due to the small sample size and microbiological methods used.

Keywords: Tooth Diseases, Microbiota, Dental Plaque, Dental Caries.

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Introduction

A large number of commensal microbial species are found in the human oral cavity that are essential for maintaining the homeostasis of the oral microbial ecosystem (1). These microbial communities, known as oral microbiota, are found in saliva, gingival epithelium, and other internal surfaces of the oral cavity (2). Many oral diseases (including tooth decay, periodontitis, and bad breath) are

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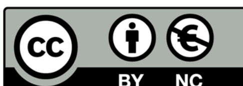

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attributed to imbalances in the microbial communities (3).

There are many factors such as tooth structure, a specific dietary habit, the level of oral hygiene, mechanical function of saliva to wash away food debris and microbes, and saliva enzymatic function contributing tooth decay development (4-5).

Carbohydrate-rich substrates specifically provide favorable habitat conditions for a number of bacterial species by lactic acid production and consequently by lowering the pH value of the oral cavity, thereby playing an important role in the development of tooth decay (4).

Dental plaque biofilms are organized by a collection of oral bacteria, some of which can cause tooth decay and gingivitis, thereby increasing periodontal disease (6). Bacteria on the plaques are in physical contact with the tooth, and this can enhance possible interactions, some of which can give rise to tooth degeneration and decay (7). Medical research has demonstrated that caries is associated with an increase in the proportion of acidogenic bacteria and uric acid bacteria, such as Lactobacillus and Streptococcus, which can destroy the tooth enamel (7). In many studies, Streptococcus mutans and S. sobrinus have been introduced as the leading causes of caries (5). Although evidence suggests that S. mutans play an important role in caries, a combination of bacterial species is actually engaged in caries development. Gram-negative cocci of salivary flora (e.g. Neisseria and Branhamella), which are in contact with teeth, are among the other invasive microorganisms (8).

Most studies have shown the oral microbiome plays a key role in the pathogenesis and progression of several oral and systemic disorders (9). The migration of oral pathogens into the bloodstream may occur in cases, including trauma, flossing, dental and medical treatments, or even chewing food, and consequently introduce bacteria to the systemic bloodstream (10). Evidence suggests that oral diseases are associated with many non-oral and systemic diseases, including cancer, cardiovascular diseases, type 2 diabetes, respiratory tract infections, adverse pregnancy outcomes, and neurological disorders (10).

In recent decades, several studies have been carried out on the isolation and identification of uncultured bacteria found in oral microbial communities using metagenomics and the 16S rRNA gene sequencing methods (11).

Nonetheless, dead and live bacterial cells are indistinguishable by molecular biology techniques alone, and hence the pathogenicity of living bacterial cells still remains undetermined (12).

Numerous studies have shown that oral microbiota vary in different geographical areas (13) and ethnicities (14). Therefore, due to the significant effect of oral microbiota diversity on human health, more investigations are needed to better understand the diversity and composition of oral bacterial populations (4). The aim of this study was to isolate and sequence 16S rRNA gene of culturable bacteria associated with dental plaque, caries and root canals in patients referred to a dental clinic in East Tehran during spring 2017. The results of the present study can be helpful in explaining the relationship between microbial flora of plaque and dental caries as well as the relationship between oral bacterial flora and demographic characteristics of patients.

Materials and method

Sample collection: In this cross-sectional study, patients were selected from those who

referred to a dental clinic in East Tehran during May 2017. Patients with symptoms of dental problems at the roots, caries, and dental plaques were included in the study with the following criteria: lack of any systemic disease, not taking antibiotics in the last 3 months, and no use of mouthwash in the last 3 weeks. Patients were randomly selected and a checklist containing demographic information; including age, sex, and health status, was completed for each patient. A total of nine clinical specimens including four, two, and three specimens of plaque, caries, and root canal, respectively, were obtained from five patients (three females and two males) aged 14-50 years, were included in the study in agreement with the specified criteria.

Dental plaques were sampled by a sterile curette from the upper gingival plaque in the anterior regions of the patient's mandible. To sample decayed dentin tissue, initially the infected tooth enamel and then the decayed tooth tissue was removed by a turbine and an angle, respectively, under aseptic conditions. For root canal sampling, the teeth were completely isolated and their crowns were disinfected with 70% ethanol. The root canal was then sampled by a sterile barbroch inserted into the canal at about the same length as the root. All samples were transferred to microtubes containing 500 µl phosphate buffer saline (PBS) next to the flame. The specimens were then transported to the Microbiology Laboratory.

Bacteriolgical methods: The specimens inside the PBS were homogenized and then incubated in a test tubes with screw tops containing 5 cc Brain Heart Infusion (BHI) BHI broth medium at 37 °C for 48 h. BHI medium is a non-selective nutrient-rich medium and can therefore be used to a variety of bacteria, including fastidious and non-fastidious bacteria, aerobic and facultative anaerobic bacteria, from a variety of clinical and nonclinical materials (13). To obtain individual pure colonies, 200 µl of the suspension was then transferred to a BHI agar medium and incubated aerobically at 37°C overnight. Colonies with different phenotype and morphology (form, elevation, margin, surface, opacity, and chromogenesis) were isolated and sub-cultured successively. Pure isolates were partially preserved and partially used for molecular studies.

Molecular identification: Each purified colony was subjected to genomic DNA extraction using a commercial DNA extraction kit (MBST, Iran) following the manufacturer's instructions. The universal primers 16suF 5'-GAG TTT GAT CCT GGC TCA G-3' and 16suR 5'-GTT ACC TTG TTA CGA CTT-3' were used to amplify a 1.5 kilo base (kb) partial sequence of the 16S rRNA gene. The PCR amplification was done using Maxime PCR PreMix Kit (i-Taq) Cat. No. 25026 in 20 µl reaction mixtures containing 1 µl of 10 µM both primers and 1–2 µl (~0.1 µg) of bacterial genomic DNA. PBS and BHI agar medium were applied as DNA extraction and PCR negative controls. The following thermal conditions was set for the PCR amplifications: initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 57.5 °C for 40 sec, and extension at 72 °C for 30 sec, and final extension at 72 °C for 8 min. 5 µl of the amplified samples were visualized by gel electrophoresis on 1% agarose gel with 1 kb marker size at 100 volts for one hour. Finally, the PCR product was purified by a PCR Purification Kit (Bioneer, Korea), followed by the sequencing in Genomin company (Iran).

The MEGA7 software was used for tree construction. Phylogenetic analyses and

lineage position confirmations were respectively done using neighbor joining method and boot strap analyses with 1000 replicates. The sequences were deposited in GenBank database.

Results

Microbiological results showed that six out of the nine clinical samples were contained bacteria (Figure 1). Based on the morphology of colonies, 13 unique bacterial colonies including 11 colonies from dental plaque and 2 colonies from caries samples were grown on the BHI agar medium. No colonies were formed from the three samples obtained from root canals (Table 1). DNAs from dental bacteria generated ~ 1450 bp amplicons, while negative controls yielded no PCR product (Figure 1).

Molecular identification was performed according to the 16S rRNA gene sequence similarity rates between the amplified specimens and the available data in GenBank (Table 1). The analysis of sequence similarity revealed bacteria identity with high similarity scores (99-100%) to the closest relative species. The sequences data were deposited in GenBank under the accession numbers of MF374812-24 (Table 1).

The identified bacteria belonged to three phyla, five families, six genera Arthrobacter, Brevundimonas, Granulicatella, Kocuria, Neisseria, and Streptococcus, as well as seven species. Most of isolates were N. perflava (38.46%) and S. salivarius (23%). Detailed information on identified bacteria are presented in Table 1.

Phylogenetic relationships of the bacteria species are shown in a diagrammatic representation in Figure 2. The generated tree based on neighbor joining method classified all bacteria in four lineages.

No association was found between bacteria and demographic characteristics of the patients studied (Table 2).

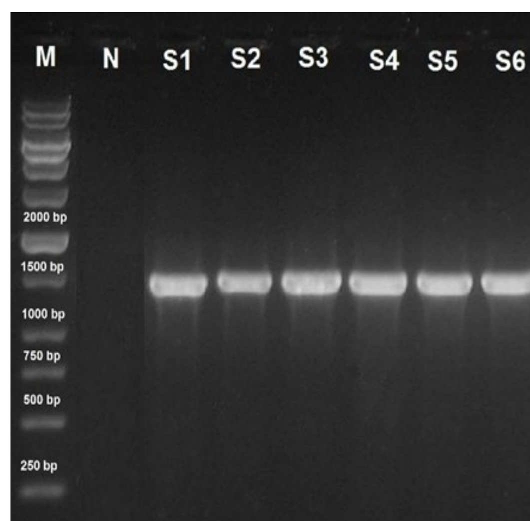

Figure 1. Agarose gel electrophoresis analysis of 16S rRNA genes amplified from dental associated bacteria. PCR amplified products were run on 1% agarose gel; Lane M, the 1 kb DNA ladder; Lane N, negative control; Lanes S1-S6, 16S rRNA gene fragments of the bacterial isolates.

Table 1. Details of the isolated bacteria from dental plaque and tooth decay based on 16S rRNA sequences.

Sample code	Isolation source	Classification (Family)	The closest relative species	The highest similarity score %	GenBank accession number
D10	Decay	Streptococcaceae	*Streptococcus oralis*	99%	MF374812
P17	Plaque	Micrococcaceae	*Arthrobacter crystallopoietes*	100%	MF374813
P16	Plaque	Neisseriaceae	*Neisseria perflava*	99%	MF374814
D12	Decay	Micrococcaceae	*Kocuria palustris*	100%	MF374815
P16	Plaque	Neisseriaceae	*Neisseria perflava*	99%	MF374816
P17	Plaque	Streptococcaceae	*Streptococcus salivarius*	100%	MF374817
P18	Plaque	Caulobacteraceae	*Brevundimonas aurantiaca*	100%	MF374818
P16	Plaque	Streptococcaceae	*Streptococcus salivarius*	99%	MF374819
P15	Plaque	Streptococcaceae	*Streptococcus salivarius*	100%	MF374820
P15	Plaque	Neisseriaceae	*Neisseria perflava*	99%	MF374821
P17	Plaque	Carnobacteriaceae	*Granulicatella elegans*	100%	MF374822
P17	Plaque	Neisseriaceae	*Neisseria perflava*	99%	MF374823
P17	Plaque	Neisseriaceae	*Neisseria perflava*	99%	MF374824

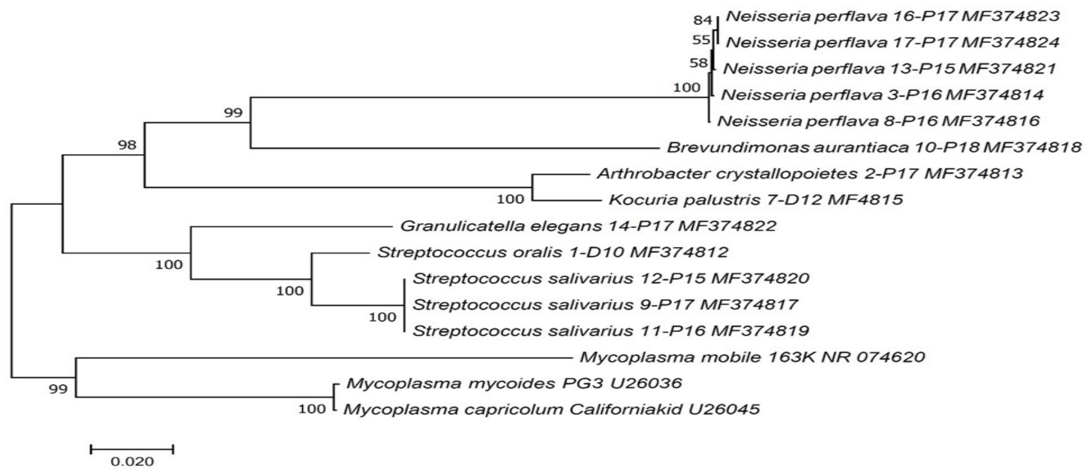

Figure 2. Neighbor-Joining tree inferred from 1400 bp of 16S rRNA gene sequences of 13 bacterial species obtained in this study. The bar indicates substitutions per site. Three Mycoplasma spp. (NR 074620, U26036, and U26045) were set as outgroups.

Table 2. Summary of demographic information and nutritional-health status of the studied patients.

	Patient No.1	**Patient No.2**	**Patient No.3**	**Patient No.4**	**Patient No.5**
Sample code	P16	D12 –E5- P18	E3- *D10	*E4-P17	*P15
Age	14	31	30	23	45
Gender	Female	Male	Female	Male	Female
Reason for referral	Pain	Check-up	Pain	Pain	Pain
Number of consultations/visits with a dentist per year	0	1	0	0	1
Brush times a day	1	1	1	0	3
Brush time in minutes	1	5-10	2	0	14
Flossing	No	No	Yes	No	No
Taking antibiotics in the last three months	No	No	No	No	No
Consumption of mouthwash in the past month	No	No	No	No	No
Number of teeth extracted	2	2	1	3	7
Number of filled teeth	0	3	3	0	2
Daily milk consumption	Yes	Yes (high)	No	No	Yes
Smoking or drug abuse	No	No	No	Heavy tobacco use	No
Bowel movements status	Healthy	Healthy	Healthy	Healthy	Constipation
Mouth odor	Normal	Normal	Unpleasant	Very unpleasant	Unpleasant
Sugar consumption	High	Medium	Low	High	High
Chocolate consumption	High	Medium	Low	High	High
Brushing after consuming sweet sugary substances	No	No	No	No	Yes
Fast food consumption per week	Low	Low	Low	High	Low

* Abbreviations: P stands for plaque, D for caries samples and E for root canal clinical samples.

Discussion

Identifying the factors involving in dental infections is an effective approach to prevention. A wide range of studies have been conducted on the oral cavity bacteria in the of healthy and diseased individuals, but the actual structure of the dental microbiota remains to be determined. Findings of the current study demonstrated the presence of 11 bacterial isolates from dental plaque, 2 bacterial isolates from tooth decay, and no strains from the root specimens were identified. The discrepancy between plaque bacteria and caries may be due to the small sample size and microbiological methods used. In a study on 31samples from dental plaque, caries, and root canal, distinct bacterial communities were reported. In fact, the difference in the co-occurrence pattern of the species present among dental plaque, caries, and root canal specimens is further in favor of the complexity of the dental caries process (11).

The culture medium used in this study was one of the nutrient rich environments but the three samples taken from the root canal did not form any colony. Actually, the main reason for uncultured microbiota is that the conditions required for their growth, such as unknown nutritional needs, temperature, pH, and interaction with other microbes of their group (9). The culture of the other six specimens resulted in 13 bacterial colonies, with N. perflava (n =5) and S. salivarius (n =3) being the most abundant isolates, both of which separated from dental plaque samples. Although many species of Neisseria found in the human mouth and nasopharynx are harmless microbial flora, their presence and frequencies have been associated with the onset and progression of many diseases. Neisseria species are found in abundance in the human oral cavity. Although there are several species of Neisseria in both healthy and decayed dental specimens, the increasing abundance of N. perflava is significant. Because the microbial community changes and acid is secreted, which leads to erosion of the enamel (14). Studies have demonstrated that this opportunistic species is still preserved in various geographical areas, ethnicities, and lifestyles (15). In a study the presence of four species of Neisseria, N. meningitidis, N. gonorrhoeae, N. sicca, and N subflava have been demonstrated by a metagenomic method in calcified dental plaque samples from four adults with periodontal disease in Dahlheim, Germany (16). In our previous study, N. perflava was also isolated from carious dentin sample of one patients who underwent for dental treatment (11).

Streptococci are the most frequent acid producers in caries and occur as part of a complex microbial community in dental biofilms (17). These species can play a crucial role in developing caries since they function effectively to preserve the integrity of dental biofilms (17). Some streptococcal species, including S. mutans and S. anginosus, which play a role in tooth decay and periodontitis (18), were not found in the present study However, S. salivarius, which was found in this study and is generally considered as a commensal bacterium in the human oral cavity, have also been isolated from a deep sample of proximal caries, may confirm the association of the bacterium with dental caries (20). According to the results of experiments of Chen et al., S. salivarius has shown a minor role in the pathogenesis of caries (21). The bacterium was also isolated from tooth decay samples with a medium biofilm (22). Similarly, some findings suggest that S. oralis is likely to be associated with oral health (23), but in the present study and our

previous study (11) it was isolated from caries specimens. Yet, it is too early to categorize these bacteria as cariogenic microorganisms.

In this study, Granulicatella elegans was isolated from a patient who had the unpleasant mouth odor and a history of heavy tobacco use. Granulicatella are streptococci with complex nutritional requirements. G. elegans is a part of the normal microbiota in the oral cavity, urogenital, and intestinal tract. It may also participate in periodontitis, caries, and endodontic infections (19). G. elegans is considered as the most fastidious bacteria and a suitable source for enter to the bloodstream via tooth-tissue as a seldom cause sepsis associated with oral flora (20). G. elegans is also considered as a bacterium involved in developing halitosis, since hydrogen sulfide is a volatile organic compound involved in halitosis (21), the unpleasant mouth odor of patient No. 4 can probably be attributed to the presence of this bacterium. However, there is a possibility of the involvement of other bacteria that are not detectable by culture-dependent methods.

The unusual Arthrobacter and Kocuria genera belong to the Actinobacteria. In the present study, it is interesting that A. crystallopoietes was isolated from dental plaque samples. Arthrobacter species are a group of multiform bacteria, that are isolated from soil and water with effective roles in agriculture (22). Nonetheless, in a microbiome analysis study of equine peripheral dental caries, Arthrobacter was one of the most common identified genera in the peripheral dental caries (23). As far as we know this genus has not yet been found in human dental specimens. Conversely, K. palustris isolates have been reported from soil, clinical specimens, and fermented foods, as well as from human mouth and skin (24).

Brevundimonas spp., are members of a genus of non-fermenting Gram-negative bacteria considered of minor clinical importance. In line with our findings, B. aurantiaca was also isolated in an African study, where the presence of methanogens in the oral cavity was examined using culture-dependent techniques (25).

A systematic understanding of the microbial diversity of plaque biofilms is essential for the development of effective strategies for the prevention and treatment of dental caries. Careful evaluation of microbiota in cases of smoking, aging, sex hormone changes, genetic disorders and metabolic diseases, including diabetes, is also crucial to understand more about dental diseases (31). Due to the disagreement of dentists as well as patients, and difficulties in sterile sampling and high costs of molecular methods, this study was performed with a small sample size. However, in order to understand the role of bacteria in the occurrence of dental lesions and their relationship with personal and environmental characteristics, it is recommended to repeat the study with a larger sample size.

Acknowledgment

The Ethical Approval Code (IR.IAU.K.REC.1396.043) was obtained by the research ethics committee of Islamic Azad University, Karaj Branch. The written informed consent forms were received from all participants who were fully explained about this study.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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