Metagenomic Analysis of Bacterial Microflora in Dental and Atherosclerotic Plaques of Patients With Internal Carotid Artery Stenosis

Clinical Medicine Insights: Cardiology
Volume 18: 1–9

DOI: 10.1177/11795468231225852

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ABSTRACT

BACKGROUND: Internal carotid artery stenosis is primarily attributed to atherosclerosis in the carotid artery bifurcation. Previous studies have detected oral bacteria in atherosclerotic lesions, suggesting an association between oral bacteria and atherosclerosis. In this study, we compared the bacterial flora of the atherosclerotic plaque in the carotid artery and dental plaque of patients with internal carotid artery stenosis using 16S ribosomal RNA (16S rRNA) metagenomic sequencing.

METHODS: Fifty-four patients who underwent internal carotid endarterectomy for internal carotid artery stenosis at the Showa University Hospital between April 2016 and February 2018 were included. Polymerase chain reaction targeting the 16S rRNA gene detected bacterial DNA in the carotid plaques of 11 cases, of which only 5 could be further analyzed. Thereafter, DNA extracted from the carotid and oral plaques of these 5 cases were analyzed using metagenomic sequencing targeting 16S rRNA. In addition, their general condition and oral conditions were evaluated. The patients were classified into symptomatic and asymptomatic groups based on the presence or absence of symptoms of transient ischemic attack, and their bacterial flora was evaluated.

RESULTS: The results demonstrated that the microflora of carotid plaques (n = 5) contained bacterial species from 55 families and 78 genera. In addition, 86.5% of the bacteria detected in the carotid plaques were also detected in oral plaques. Cariogenic and periodontopathic bacteria accounted for 27.7% and 4.7% of the bacteria in the carotid plaques, respectively.

CONCLUSIONS: These results suggest that oral bacteria are directly or indirectly involved in the pathogenesis of atherosclerosis. More extensive studies of oral commensal bacteria detected in extra-oral lesions are warranted to comprehensively investigate the role of oral bacteria in the pathogenesis of systemic diseases.

KEYWORDS: Carotid artery stenosis, metagenomic 16S rRNA analysis, oral bacterium, iatrogenic infections, polymicrobial interactions

RECEIVED: June 7, 2023. **ACCEPTED:** December 22, 2023.

TYPE: Original Research

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by JSPS KAKENHI Grant Numbers JP17K17381 and JP20K18834. The funders had no role in the study design, data collection, decision to publish, or manuscript preparation.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Background

Internal carotid artery stenosis is caused by atherosclerotic plaques in the carotid artery bifurcation. Hypertension, dyslipidemia, diabetes mellitus, and smoking are the most common risk factors for atherosclerosis in approximately half of all cases, while the cause is unknown in the remaining cases.[1] In 1999, Ross posited that atherosclerosis is a chronic inflammatory disease and the augmented presence of macrophages in the blood, due to inflammation, combined with the activation of these macrophages when they infiltrate the inner linings of vascular walls—stimulated by inflammation—lead to the formation of atherosclerotic lesions. Moreover, he listed infection as a potential underlying cause.[2,3] Viruses and bacteria such as *Chlamydia pneumoniae*, Cytomegalovirus, and Herpesvirus have been reported to be detected in atherosclerotic lesions. Many studies have been conducted on the relationship between oral bacteria and atherosclerosis, with Wu et al[4] reporting that the relative risk of developing cerebrovascular disease for individuals with is 1.66 times that of healthy individuals, and Jönsson et al[5] reporting that the risk of developing acute coronary syndrome

is 2.8 times higher for individuals with severe periodontal disease. Furthermore, oral bacteria, including periodontopathogenic bacteria, were detected in the atheromatous lesions.[6-10] These epidemiological and pathological reports suggest that there is a relationship between oral bacteria, particularly periodontal pathogens, and atherosclerosis.[4,5]

In the oral cavity, numerous species of pathogenic and non-pathogenic bacteria are involved in the development of dental caries and periodontal disease.[10] Evidence also suggests that conditions, such as dental caries and periodontal disease, are triggered by dysbiosis of the oral bacterial flora.[11] In atherosclerosis as well, multiple species have been observed, rather than a single pathogen.[3,10,12,13] Comprehensive analysis of the bacterial flora in atherosclerotic lesions is necessary to understand the relationship between the pathogenesis of atherosclerosis and bacterial flora. Conventional culture methods are not sufficient to elucidate the composition and pathogenicity of the complex commensal flora.[13-15] In the past, the majority of the oral anaerobic bacteria was not culturable; however, metagenomic sequencing methods have allowed understanding of the whole picture with the advent of next-generation sequencing.[14,15] Therefore, it is now possible to analyze the interactions between bacteria and the constituent species of the microflora by comprehensively analyzing genetic information. In this study, we analyzed the bacterial flora of carotid artery plaques and oral plaques of patients with internal carotid artery stenosis using 16S ribosomal RNA (16S rRNA) metagenomic sequencing to investigate whether oral bacterial flora contributes to the pathogenesis of internal carotid artery stenosis.

Methods

Study participants

This study included 54 patients who underwent carotid endarterectomy (CEA) for internal carotid artery stenosis at Showa University Hospital, Department of Neurosurgery, between April 2016 and February 2018. This study was conducted following the ethical standards of the Declaration of Helsinki and approved by the Showa University Institutional Review Board (approval number: 2015-010). Written informed consent was obtained from all eligible patients.

Sample collection

Atherosclerotic plaques from the stenosed sites of the internal carotid artery and dental plaques from the teeth of the same patient were collected from each participant. Carotid plaques were surgically collected from the intima of the stenosed internal carotid artery at the time of CEA. The dental plaque samples were collected during preoperative oral cleaning (the day before surgery) at the Department of Dentistry and Oral Surgery, Showa University Hospital. Two dentists specializing in perioperative management at Showa Hospital collected the dental samples from supragingival plaque using a sterile explorer. Samples were frozen at −20°C immediately after collection and stored at the Department of Oral Microbiology, Showa University School of Dentistry.

Assessment of systemic and oral conditions

Data on age, sex, medical history, carotid plaque stenosis rate, histopathology examination, oral examination, and dental history were collected from the patients' medical records. Patients were classified into 2 groups, symptomatic and asymptomatic, based on the presence or absence of symptoms of transient ischemic attack (TIA) in the medical record entries. TIA is an episode of focal brain, retinal, or spinal cord dysfunction lasting <24 h, which is of a non-traumatic, vascular origin.[16] All specimens in the symptomatic group were obtained 2 weeks after the last attack.

DNA extraction from the samples

DNA was extracted from the carotid and dental plaque samples according to the instructions stated on the DNA extraction kit (QIAamp UCP Pathogen Mini, Qiagen, Hilden, Germany) after pretreatment and mechanical disruption of the bacterial cell wall. For pretreatment and bacterial cell wall disruption, the Pathogen Lysis Tube Kit (Pathogen Lysis Tubes S, Qiagen) was used. The carotid plaques were shredded for pretreatment using a scalpel suspended in Reagent DX with Proteinase K and Buffer ATL (Qiagen), and protein lysis treatment (56°C, 1 h) was performed. The suspensions were then subjected to a grinding procedure (Disruptor Genie, Scientific Industries, Inc., NY, USA).

Detection of bacterial 16S rRNA

The extracted DNA was used as a template for polymerase chain reaction (PCR) amplification using Go TaqR Master Mix (Promega, Madison, WI, USA) and Universal primers based on specific 16SrRNA (515F: 5′-GTGCCAGCMG CCGCGGTAA_3′ 806R: 5′-GGGACTACHVGGGGT WTCTAAT-3′). The PCR process included the following sequential steps: (1) initial denaturation at 94°C for 5 minutes; (2) 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds; and (3) a final extension at 72°C for 5 minutes followed by a temperature hold at 4°C. The PCR products were then analyzed by electrophoresis on 1.2% agarose gel, and the presence or absence of amplified products was used to confirm the presence of bacteria.

Next-generation sequencing analysis

Carotid and dental plaque samples, in which the presence of bacteria was confirmed in the first PCR amplification, were subjected to comprehensive next-generation sequencing by amplifying the 16S rRNA V3-4 region. Next-generation

sequencing was performed by Hokkaido System Science Co., Ltd. (Sapporo, Japan). PCR amplicons were pooled and sequenced on an Illumina MiSeq with 300 bp pair-end sequencing by Hokkaido System Science Co. Ltd. Sequencing data were analyzed using the standard Quantitative Insights into Microbial Ecology pipeline, and low-quality reads and adaptor contaminants were eliminated. To evaluate the alpha diversity of bacteria, richness, and number of operational taxonomic units were employed.

Statistical analysis

Statistical analysis was performed using the statistical software JMP Pro 16.0.0 (SAS, Tokyo, Japan). For comparisons of continuous variables such as age and BMI, the normality of the data distribution was assessed by the Shapiro–Wilk test. If normality was confirmed, the *t*-test was used; otherwise, the Mann–Whitney *U* test was used. For comparisons of categorical variables such as the presence of diabetes, hypertension, and symptomatic status, Fisher's exact test were used. The statistical significance level was set at less than 5%.

Results

Detection of oral bacterial 16S rRNA genes in carotid plaques

PCR amplification confirmed the presence of bacteria in 11 (20.4%) of the 54 carotid plaques collected. Next-generation sequencing analysis of DNA extracted from the carotid and dental plaques of these 11 patients identified multiple bacterial 16S rRNA sequences in 5 cases (9.3%). Among the remaining 6 cases, 3 were not suitable for next-generation sequencing analysis due to the limited amount of DNA extracted, and in the other 3 cases, the results of next-generation sequencing indicated that the genus *Staphylococcus* or other (unclassified sequences) constituted more than 90% of the sequence of samples; therefore, all 6 cases were excluded from further analysis.

Characteristics of the subject and oral cavity

Based on the results of next-generation sequencing analysis, the group in which oral bacteria were detected was categorized as the bacterial DNA (bDNA) (+) group (n = 5) and the other group was termed the bDNA (–) group (n = 43). No significant differences were found between the bDNA (+) and bDNA (–) groups in the following parameters: age, sex, smoking habits, hypertension, dyslipidemia, diabetes mellitus, body mass index, symptomatic classification, internal carotid artery stenosis rate, and histopathology.

There were 15 (31.3%) symptomatic patients with subjective symptoms, such as transient ischemic attacks, and 33 (68.7%) asymptomatic patients. Among those with symptomatic internal carotid artery stenosis, bacterial DNA was only detected in the plaque samples of 3 patients (60%). Among asymptomatic patients, bacterial DNA was detected in only 2 patients (40%); the remaining 31 (72.1%) were in the bDNA (–) group. Oral examination showed no significant differences (⩽5%) between the bDNA (+) and bDNA (–) groups. There were, however, significant differences in age, with the (symptomatic: median 78 [interquartile range: 70-80] years, asymptomatic: median 72 [interquartile range: 70-80] years, $P < .03$), smoking habits (odds ratio [OR] = 4.24, 95% confidence interval [CI]: 1.1-15.7, $P < .04$), and histopathological findings of ulceration (OR = 5.1, 95% CI: 1.3-19.7, $P < .02$) between the symptomatic and asymptomatic groups (Table 1).

Oral examination showed no significant differences (⩽5%) between the bDNA (+) and bDNA (–) groups. There were also no significant differences (⩽5%) between the symptomatic and asymptomatic groups (Table 2).

Next-generation sequencing analysis of carotid and dental plaques

The bacterial flora of carotid and dental plaques obtained from 5 patients with internal carotid artery stenosis were analyzed by next-generation sequencing. These carotid plaques contained bacteria from 55 families and 78 genera (mean 48.0, standard deviation ± 10.5), while the oral plaque samples contained bacteria from 39 families and 57 genera (mean 45.0, standard deviation ± 12.3) (Figure 1A and B).

At the family level, *Actinomycetaceae, Streptococcaceae, Corynebacteriaceae, Neisseriaceae, Burkholderiaceae*, and *Micrococcaceae* families were predominant in the oral plaques, accounting for 65.6% of the total microflora. In carotid plaques, *Streptococcaceae, Actinomycetaceae, Lactobacillaceae*, and *Corynebacteriaceae* were the predominant families in descending order. The 6 families that were predominant in the oral cavity accounted for 44.8% of the bacterial microflora in carotid plaques. The families *Lactobacillaceae, Enterobacteriaceae, Pseudomonadaceae, Bacillaceae*, and *Oxalobacillaceae* were detected only in the carotid plaques (Figure 1A).

The results of the genus-level analysis are shown in Figure 1B. In both the carotid and oral plaques, *Streptococcus, Actinomyces*, and *Corynebacterium* were predominant at the family level. *Neisseria* and *Haemophilus* were the second most common families in the carotid plaques. These 5 genera accounted for 46% of the bacteria detected in the carotid plaques. Additionally, 86.5% of the bacteria detected in the carotid plaques were found in oral plaques. Intestinal bacteria, such as *Lachnospiraceae* and *Lautropia*, and infectious endocarditis-causing bacteria, such as the HACEK group (*Haemophilus, Cardiobacterium*, and *Kingella*), and other bacteria groups (*Granulicatella* and *Abiotrophia*) were detected in the carotid artery plaques. The *Neisseriaceae* family was significantly more frequently detected in oral plaques than in carotid plaques ($P = .01$). There were no significant differences in the detection rates of other bacterial groups in carotid and oral plaques.

Table 1. Characteristics of patients with internal carotid artery stenosis.

RISK FACTOR (PERCENTAGE OF TOTAL PATIENTS)	NUMBER OF PATIENTS	INITIAL POLYMERASE CHAIN REACTION FINDINGS			CLINICAL PRESENTATION		
		DNA OF ORAL BACTERIA PRESENT [BDNA (+)]	DNA OF ORAL BACTERIA ABSENT [BDNA (–)]		SYMPTOMATIC	ASYMPTOMATIC	
	N = 48	N = 5	N = 43	*P*-VALUE	N = 15	N = 33	*P*-VALUE
Age (y)	72.5 (78.8-65.3)	73 (62.5-78)	72 (66-79)	.78[b]	78 (70-80)	72 (62.5-75)	**<.03**[b]
Sex (Male/Female)	40/48	4/5	36/43	1.00[c]	11/15	29/33	.23[c]
Smokers (%)	33 (68.8)	4 (80)	29 (67.4)	1.00[c]	7 (46.7)	26 (78.8)	**<.04**[c]
Hypertension (%)	39 (81.3)	5 (100)	34 (79.1)	.56[c]	10 (66.7)	29 (87.9)	.11[c]
Diabetes (%)	22 (45.8)	3 (60)	19 (44.2)	.65[c]	5 (33.3)	17 (51.5)	.35[c]
Dyslipidemia (%)	29 (60.4)	0	19 (44.2)	.14[c]	6 (40.0)	13 (39.4)	1.00[c]
BMI (kg/m²)	23.2 (25.2-21.1)	23.6 (21.3-25.2)	23.1 (21.1-25.2)	.75[†]	23.2 (21.2-26.7)	23.1 (20.6-24.9)	.60[a]
Clinical presentation							
Symptomatic (%)	15 (31.3)	3 (60)	12 (27.9)	.31[c]			
Asymptomatic (%)	33 (68.7)	2 (40)	31 (72.1)				
Carotid plaque findings							
Degree of stenosis (ECST)	73 (77.8-66.3)	68 (60.5-80)	73 (67-78)	.54[b]	77 (66-83)	72 (66-75.5)	.36[b]
Histological findings of calcification							
Calcification	40 (83.3)	4 (80)	36 (83.3)	1.00[c]	13 (86.7)	27 (81.8)	1.00[c]
Ulceration	14 (29.2)	1 (20)	13 (30.2)	1.00[c]	8 (53.3)	6 (18.1)	**<.02**[c]
Hemorrhage	24 (50)	2 (40)	22 (51.2)	1.00[c]	9 (60)	15 (45.4)	.53[c]

Abbreviations: BMI, body mass index; ECST, European Carotid Surgery Trial.
Data represent the median (interquartile range) for age, BMI, ECST, and number (percentage) for others.
Included patients were those diagnosed with a transient ischemic attack or cerebral infarction of the internal carotid artery within 6 months. Statistical analyses were performed to compare groups in which bacterial DNA were and were not detected. *P* values with significant differences are shown in bold.
[a]Mann–Whitney *U* test.
[b]*t*-Test.
[c]Fisher's exact test.

The composition of the bacterial flora in the carotid plaque (Figure 2A) and dental plaque specimens (Figure 2B) from symptomatic and asymptomatic patients are shown at the genus level in Figure 2. We found 50 genera of bacteria in the symptomatic group and 40 genera in the asymptomatic group. In our results, 50 genera of bacteria were found in the symptomatic group and 40 genera in the asymptomatic group, the bacterial diversity was evaluated using the Shannon Index with the mean value of the symptomatic group of 3 cases being 5.13 (#1: 5.34, #2: 5.36, and #3: 4.70), while that of the asymptomatic group of 2 cases was 4.46 (#4: 4.27 and #5: 4.52), indicating a decrease in the bacterial diversity in the asymptomatic group. In the carotid plaques of symptomatic patients, the genera *Corynebacterium, Actinomyces, Selenomonas*, and others accounted for approximately half of the total microflora. A comparison between the bacterial composition of the carotid plaques of symptomatic and asymptomatic patients showed an increase in the number of sequencing counts for *Streptococcus* spp. ($P = .1489$) and *Haemophilus* spp. ($P = .1489$) in the asymptomatic group; however, the sample size (n = 2) was small, and we therefore could not compute statistical significance. No significant differences were found in the bacterial composition of the dental plaque from asymptomatic and symptomatic patients (Figure 2B).

Frequency of detection of cariogenic and periodontopathic bacteria at the genus level

Bacteria detected in dental and carotid plaques were categorized as per the oral diseases they caused (3 genera of cariogenic bacteria and 6 genera of periodontopathic bacteria), and the

Table 2. Dental findings of patients with internal carotid artery stenosis.

DENTAL FINDINGS	INITIAL POLYMERASE CHAIN REACTION FINDINGS			CLINICAL PRESENTATION		
	DNA OF ORAL BACTERIA PRESENT [BDNA (+)]	DNA OF ORAL BACTERIA ABSENT [BDNA (–)]		SYMPTOMATIC	ASYMPTOMATIC	
	N = 5	N = 43	*P*-VALUE	N = 15	N = 33	*P*-VALUE
Number of residual teeth	25 (21-29)	25 (14-28)	.66[a]	24 (14-27)	26 (17-28)	.24[a]
Number of periodontal pockets (>4 mm)	0 (0-2.5)	2 (0-4.8)	.16[a]	1.5 (0.7-9.5)	2 (0-4)	.85[a]
Number of bleedings on probing	6 (0-7)	6 (0-11)	.37[a]	6 (0-14)	6 (0-10)	.43[a]
Number of mobile teeth	0	9 (20.9)	.57[b]	2 (13.3)	7 (21.2)	.70*
Number of treated teeth	9 (3-10)	7 (3-11)	.93[a]	9 (3-12)	7 (2.5-10)	.48
Length of dental consultation (month)	1 (0.5-6.5)	6 (1-24)	.22[a]	7(1-24)	3 (1-18)	.54[a]

Data represent the median (interquartile range) for the number of residual teeth, periodontal pockets, bleeding on probing, treated teeth, and number (percentage) of mobile teeth.
[a]Mann–Whitney *U* test.
[b]Fisher's exact test.

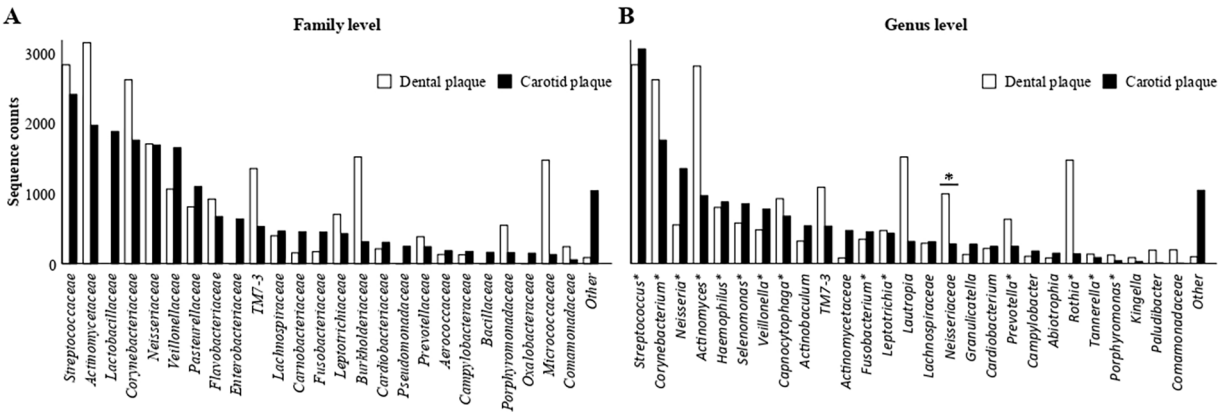

Figure 1. Bacteria flora composition of the dental and carotid plaques: (A) the bacterial flora composition is shown at the genus level, centering on the 28 bacterial groups detected at a high rate in the data obtained from the 16S rDNA sequences and (B) the bacterial flora composition is shown at the family level, centering on the 26 bacterial groups detected at a high rate in the data obtained from the 16S rDNA sequences. The average values of the results obtained from 5 participants in each group are shown.
*Oral flora.

frequency of detection in the samples is shown in Table 3. Both categories of bacteria were detected in both groups regardless of the symptomatic condition. Cariogenic and periodontopathic bacteria were detected in 27.7% and 4.7% of the carotid plaques, respectively. These frequencies were similar to the percentages of cariogenic and periodontopathic bacteria found in oral plaques (29.9% and 6.4%, respectively). Cariogenic bacteria, such as *Streptococcus, Actinomyces*, and *Veillonella*, were detected more predominantly than periodontopathic bacteria, such as *Porphyromonas, Prevotella*, and *Fusobacterium*, in carotid plaques.

Discussion

Although skin, lung, and respiratory commensal bacteria and intestinal bacteria have been detected in atheroma lesions, several studies have reported that more than half of the bacteria found in atheroma lesion sites were oral commensal bacteria.[12,15,17] Many studies have attempted to detect specific periodontopathogenic bacteria from atheromatous lesions based on the characteristics of periodontopathogenic bacteria, but reports of detection of cariogenic bacteria in greater numbers than periodontopathogenic bacteria are rare compared to previous reportsl.[8,18-20] The human oral cavity is inhabited by a number of bacteria, such as viridans streptococci, and even in the oral cavity of healthy individuals, the formation of complex microflora, referred to as biofilms, is inevitably comprised of multiple bacteria. Recently, it has been highlighted that these oral microflora can cause ectopic infections in other parts of the body.[21] Furthermore, "polymicrobial infections" in which the oral microflora are infected as a group are considered to be

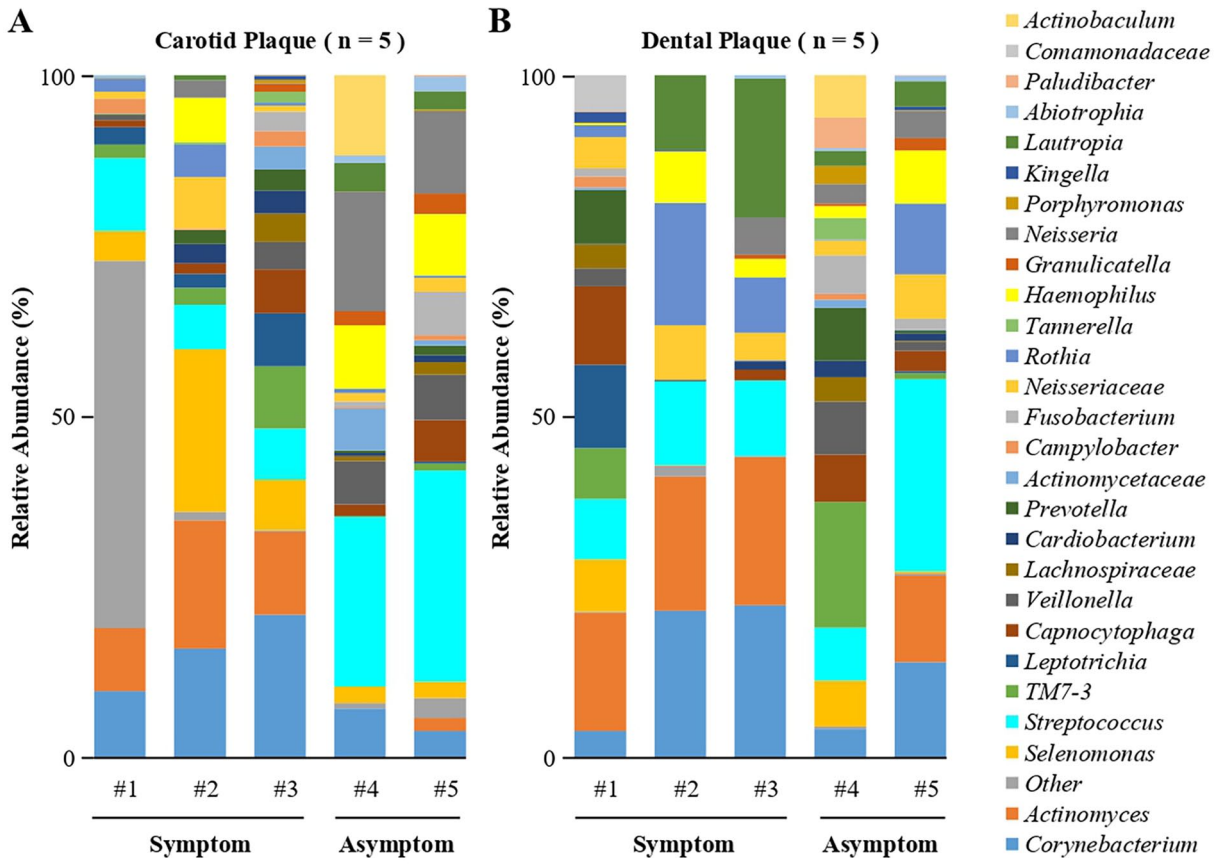

Figure 2. Bacteria flora composition detected among symptomatic and asymptomatic patients. The bacterial flora composition was observed at the genus level. The 16S rDNA sequencing revealed that 28 bacterial groups were detected at a high rate. The bacterial composition of carotid plaques was compared between symptomatic and asymptomatic groups: (A) carotid plaques were classified based on whether the participant from whom the sample was collected was symptomatic or asymptomatic, and the difference in proportion was evaluated and (B) dental plaques were also classified based on whether the participant providing the sample had symptoms of internal carotid artery stenosis, and the difference in proportion was evaluated.

Table 3. Presence of cariogenicity and periodontopathogenicity of bacteria detected in samples.

CATEGORY OF BACTERIA	GENUS	CAROTID PLAQUE N=5	DENTAL PLAQUE N=5
Cariogenic bacteria	*Streptococcus*	3108 (17.6)	2878 (13.8)
	Actinomyces	982 (5.6)	2859 (13.7)
	Veillonella	788 (4.5)	487 (2.4)
	Total sequence reads (%)	4878 (27.7)	6224 (29.9)
Periodontopathic bacteria	*Porphyromonas*	40 (0.2)	119 (0.6)
	Prevotella	246 (1.4)	641 (3.1)
	Aggregatibacter	1 (0)	12 (0.1)
	Tannerella	83 (0.5)	133 (0.6)
	Treponema	8 (0)	53 (0.3)
	Fusobacterium	458 (2.6)	352 (1.7)
	Total sequence reads (%)	836 (4.7)	131 (6.4)

Data represent the number of sequence reads obtained from all patients in whom DNA of oral bacteria were detected in the initial polymerase chain reaction of DNA extracted from oral and carotid plaques.
Percentages were calculated as the number of sequence reads of oral bacteria divided by the number of sequence reads of all bacteria.

significant.[22] The relationship between thrombus formation and "polymicrobial infection" by microflora has not yet been fully elucidated, although in our study, multiple bacteria were detected in both symptomatic and asymptomatic thrombi, suggesting an association between thrombus formation and the presence of multiple bacteria. This suggests an association between thrombus formation and the presence of multiple bacteria.

In the results of a comprehensive survey of the bacterial flora of lesions, as in the present study, the detection rate of periodontopathic bacteria was low.[12,15,20,23] There have been reports of no detection of periodontal pathogens from atheromatous lesions in the same patient despite the fact that many periodontal pathogens were detected in oral samples.[24,25] Even using similar PCR-based techniques with the same specific primers, detection rates varied (0%-32% for *P. gingivalis*).[15] The detection of periodontal pathogens in lesioned tissues suggests that periodontal pathogens, among other factors, may induce the pathogenesis of cardiovascular disease. One is that *P. gingivalis*, a typical periodontopathogenic bacterium, adheres to endothelial cells via the major fimbriae and stimulates the expression of cell surface-associated ICAM-1 (Intercellular adhesion molecule-1), VCAM-1 (Vascular cell adhesion molecule-1), E- and P-selectins.[26] *P. gingivalis* can also express and induce chemokines that modulate leukocyte chemotaxis and migration across the endothelium.[26] Furthermore, *P. gingivalis* may cause platelets to aggregate and form thrombi.[27] This helps form and promote atherosclerosis, which is believed to be caused by the accumulation of blood-borne leukocytes in inflamed tissues in response to antigenic stimuli. In an experimental animal model, Li et al[28] also observed atherosclerotic plaque formation in ApoE-knockout mice exposed to *P. gingivalis*. In addition, the detection of oral bacteria in carotid plaques was rare according to Isoshima et al.[10] In a co-occurrence network analysis, patients with periodontal disease showed a change in the bacterial flora network between *Cutibacterium acnes*, which causes acne and other skin inflammation, and other bacteria as the predominant constituent of the bacterial flora.[10] This study suggests that systemic effects may have been indirectly involved through hematogenous spread of lipopolysaccharides and other bacterial components from the oral cavity and micro chronic inflammation.[10] Others have reported that a greater variety of bacteria was detected in atherosclerotic lesions in a group affected by periodontal disease than in a group not affected by periodontal disease.[15] The difference between detection of periodontal pathogens and cariogenic bacteria is that antigenic periodontal pathogens are easily recognized and eliminated by the immune system,[15] while serotype (k) of *Streptococcus mutans* persists in the blood due to its low antigenicity.[29]

Similar to the present study, previous reports have detected various types of oral bacteria, including *Streptococcus* spp., at sites of atherosclerotic lesions.[15,17,23] *Streptococcus* and *Actinomyces*, which were the most abundant species in the present results, are considered to have the property of adhering to the surfaces of oral mucosa and teeth, forming biofilms, and helping bacteria to invade the tissues.[30] In addition, *Streptococcus spp.* as well as *P. gingivalis* have been found to invade vascular endothelial cells,[31] stimulate the production of inflammatory cytokines,[32] and induce foam cell formation and platelet aggregation.[23,33] Corynebacterium spp. have been implicated in tartar formation and have been implicated in calcification of heart valves in cardiovascular disease.[34] The properties of various oral commensal bacteria, such as Streptococcus and periodontopathogenic bacteria, are involved in inducing or promoting atherosclerosis.

The symptomatic group was defined by the presence or absence of neurological manifestations of TIA.[16] These symptoms may have been caused by the extent of carotid artery stenosis and presence of instability plaques.[16,35] Instability plaques are characterized by a thin or disrupted fibrous cap, large lipid-rich necrotic core, neovascular growth, and intraplaque hemorrhage, with the symptomatic group being more urgent in carotid artery stenosis, with disease progression.[16,35] Thus, blood markers of the inflammatory response are higher in symptomatic than in asymptomatic cases of vascular stenosis.[36] It was difficult to determine whether the composition of the bacterial flora differed between the symptomatic and asymptomatic groups statistically because of the small number of specimens in this study. In addition, all specimens from the symptomatic group were obtained 2 weeks after the last seizure. Because of the specimen collection, a consent form had to be obtained, so scheduled OPE patients were included; specimens could not be collected immediately after the TIA symptoms occurred, so there may be differences from the flora at the time of the TIA. However, in our study, the Shannon Index was higher in the symptomatic group than in the asymptomatic group, and a wide variety of bacterial flora was observed in the symptomatic group. In the present study, inflammation markers were not tested due to the difficulty in setting up the environment because of the specimen storage period, but the leukocyte count and C-reactive protein (CRP) levels in the blood data did not differ between symptomatic and non-symptomatic patients, but the mean CRP level was higher than normal in both cases, indicating inflammation. Furthermore, there have been previous reports of a large number of cores formed by a wide variety of bacteria in atherosclerotic lesions under the electron microscope.[12,15] This, in turn, suggests that a wide variety of bacteria are involved in atherogenesis. It also suggests that bacteria present in lesions may adhere to vessels damaged for some reason and be detected as secondary infection formers.[13] Recent studies have also revealed that Cnm-positive *Streptococcus pyogenes*, which expresses the collagen-binding protein Cnm, normally detected at low frequency in the oral cavity, can infiltrate vascular endothelial cells and induce vascular damage by binding to collagen IV,[37] and that it can also inhibit platelet aggregation and thereby In addition, it has been found to accelerate cerebral hemorrhage by inhibiting platelet aggregation.[31]

The oral commensal bacteria detected in this study are considered symbiotic bacteria and are not normally pathogenic,

but when they invade blood vessels, they are associated with infections such as endocarditis and bacteremia.[38] The involvement of oral commensal bacteria in the formation and progression of atherosclerosis is not fully understood, but the activation of the innate immune system by bacterial components is probably a key element. The small number of specimens in our study did not allow us to obtain definitive statistical results, however, we cannot rule out a direct or indirect relationship between the high number of the same oral bacteria found in the same patient's oral cavity and in samples from the atheroma lesion. We believe that comprehensive research on commensal bacteria, including cariogenic and periodontopathic bacteria, is needed to understand the characteristics of each bacterium and their interrelationships in patients with internal carotid artery stenosis.

In the present study, bacterial DNA was detected in carotid plaques in 5 of 54 patients (9.3%). This result was relatively low compared to the detection rate of bacterial DNA from atherosclerotic lesion sites (52%-95%) reported in previous studies.[8,15] To verify the relationship between bacteria and the formation or promotion of atherosclerosis, it is necessary to comprehensively understand the bacterial flora, including scaffolding bacteria, rather than simply search for the presence or absence of specific pathogenic bacteria or their characteristics. In the future, we would like to increase sample size of patients to examine this issue further. In addition, the oral cavity of the subjects in this study did not show significant periodontal disease progression. It is not known by what route the bacteria entered the blood from the oral cavity. In the urban areas of developed countries, periodontal disease is generally well controlled, and cariogenic bacteria may play a more important role in endogenous infections of oral origin. Thus, we believe it is necessary to not only comprehensively understand the bacterial flora, but also to examine the pathogenesis of the primary disease in a cross-disciplinary manner. In this interdisciplinary study, researchers from the Department of Neurosurgery and Department of Microbiology at the School of Dentistry collaborated to comprehensively evaluate the bacterial flora of carotid and oral plaques to understand the involvement of oral microorganisms in the pathogenesis of carotid plaques. We believe that interdisciplinary collaboration between medical and dental experts has yielded valuable results in this study. A more comprehensive study of oral commensal bacteria that reside in the extra-oral environment is needed to reveal novel properties of bacteria and better understand the symbiotic relationship between bacteria and their hosts.

Study limitations

In this study, 16S rRNA metagenomic sequencing was not performed on the 43 specimens for which bacterial DNA was not detected in the initial screening. As a result, the sample size was reduced, and we recognize that the bias in the number of specimens compared between groups limited the reliability of assessing statistically significant differences.

Metagenomic sequencing also detects genes in bacteria, both living and dead, whose activity and growth are suppressed.[39] We believe that we need to conduct metatranscriptomic studies to analyze the genes that are at work and conduct microbial composition (metagenomics) and overall gene expression.[39] As a future prospect, we would like to evaluate these points in further studies.

Conclusions

Our results showed that carotid plaques showed many types of bacteria, including oral commensal bacteria. Previous studies have also characterized oral commensal bacteria such as cariogenic and periodontopathogenic bacteria. Bacteria found not only in the oral cavity but also in atheromatous lesions are a polymicrobial infection composed of a complex microflora, and their involvement in atheroma formation and promotion should continue to be investigated. We conclude that comprehensive studies of oral commensal bacteria are needed to understand the characteristics of each organism and their interrelationships with the host defense system.

Acknowledgments

We would like to thank Editage (www.editage.com) for assistance with English language editing.

Author Contributions

AS, SM, MO, HF, YM, KS, TM, and HK conceived and designed the study. AS, SA, KS, SM, and MO participated in data acquisition. AS and HK performed the statistical analysis. AS, HF, and HK performed validation and formal analysis, and AS drafted the original draft. AS, MM, YM, KS, and HK wrote, reviewed, and edited the manuscript. MM, YM, KS, and HK provided project management. All authors read and approved the final manuscript.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available in the DDBJ Sequenced Read Archive (DRA) under accession numbers DRR427422-DRR427431, https://ddbj.nig.ac.jp/search.

Ethics Approval and Consent to Participate

This study was conducted following the ethical standards of the Declaration of Helsinki and approved by the Showa University Institutional Review Board (approval number: 2015-010). Informed consent was obtained from all eligible patients.

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REFERENCES

1. Vita JA, Loscalzo J. Shouldering the risk factor burden: infection, atherosclerosis, and the vascular endothelium. *Circulation*. 2002;106:164-166.
2. Ross R. Atherosclerosis–an inflammatory disease. *New Engl J Med*. 1999;340:115-126.
3. Mattila KJ, Valtonen VV, Nieminen MS, Asikainen S. Role of infection as a risk factor for atherosclerosis, myocardial infarction, and stroke. *Clin Infect Dis*. 1998;26:719-734.
4. Wu T, Trevisan M, Genco RJ, et al. Periodontal disease and risk of cerebrovascular disease: the first national health and nutrition examination survey and its follow-up study. *Arch Intern Med*. 2000;160:2749-2755.
5. Jönsson D, Orho-Melander M, Demmer RT, et al. Periodontal disease is associated with carotid plaque area: the Malmö offspring dental study (MODS). *J Intern Med*. 2020;287:301-309.
6. Chiu B. Multiple infections in carotid atherosclerotic plaques. *Am Heart J*. 1999;138:S534-S536.
7. Padilla C, Lobos O, Hubert E, et al. Periodontal pathogens in atheromatous plaques isolated from patients with chronic periodontitis. *J Periodontal Res*. 2006;41:350-353.
8. Toyofuku T, Inoue Y, Kurihara N, et al. Differential detection rate of periodontopathic bacteria in atherosclerosis. *Surg Today*. 2011;41:1395-1400.
9. Calandrini CA, Ribeiro AC, Gonnelli AC, et al. Microbial composition of atherosclerotic plaques. *Oral Dis*. 2014;20:e128-e134.
10. Isoshima D, Yamashiro K, Matsunaga K, et al. Microbiome composition comparison in oral and atherosclerotic plaque from patients with and without periodontitis. *Odontology*. 2021;109:239-249.
11. Sudhakara P, Gupta A, Bhardwaj A, Wilson A. Oral dysbiotic communities and their implications in systemic diseases. *Dent J*. 2018;6:10.
12. Ott SJ, El Mokhtari NE, Musfeldt M, et al. Detection of diverse bacterial signatures in atherosclerotic lesions of patients with coronary heart disease. *Circulation*. 2006;113:929-937.
13. Marques da Silva R, Caugant DA, Eribe ER, et al. Bacterial diversity in aortic aneurysms determined by 16S ribosomal RNA gene analysis. *J Vasc Surg*. 2006;44:1055-1060.
14. Wilson KH, Blitchington RB, Greene RC. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *J Clin Microbiol*. 1990;28:1942-1946.
15. Armingohar Z, Jørgensen JJ, Kristoffersen AK, Abesha-Belay E, Olsen I. Bacteria and bacterial DNA in atherosclerotic plaque and aneurysmal wall biopsies from patients with and without periodontitis. *J Oral Microbiol*. 2014;6:20140515.
16. Naylor R, Rantner B, Ancetti S, et al. Editor's choice – European Society for Vascular Surgery (ESVS) 2023 clinical practice guidelines on the management of atherosclerotic carotid and vertebral artery disease. *Eur J Vasc Endovasc Surg*. 2023;65:7-111.
17. Pessi T, Karhunen V, Karjalainen PP, et al. Bacterial signatures in thrombus aspirates of patients with myocardial infarction. *Circulation*. 2013;127:1219-1228, e1-e6.
18. Ohki T, Itabashi Y, Kohno T, et al. Detection of periodontal bacteria in thrombi of patients with acute myocardial infarction by polymerase chain reaction. *Am Heart J*. 2012;163:164-167.
19. Chen YW, Umeda M, Nagasawa T, et al. Periodontitis may increase the risk of peripheral arterial disease. *Eur J Vasc Endovasc Surg*. 2008;35:153-158.
20. Fiehn N, Larsen T, Christiansen N, Holmstrup P, Schroeder TV. Identification of periodontal pathogens in atherosclerotic vessels. *J Periodontol*. 2005;76:731-736.
21. Atarashi K, Suda W, Luo C, et al. Ectopic colonization of oral bacteria in the intestine drives T(H)1 cell induction and inflammation. *Science*. 2017;358(6361):359-365. doi:10.1126/science.aan4526
22. Brogden KA, Guthmiller JM, Taylor CE. Human polymicrobial infections. *Lancet*. 2005;365(9455):253-255. doi:10.1016/S0140-6736(05)17745-9
23. Nakano K, Inaba H, Nomura R, et al. Detection of cariogenic Streptococcus mutans in extirpated heart valve and atheromatous plaque specimens. *J Clin Microbiol*. 2006;44:3313-3317.
24. Aimetti M, Romano F, Nessi F. Microbiologic analysis of periodontal pockets and carotid atheromatous plaques in advanced chronic periodontitis patients. *J Periodontol*. 2007;78:1718-1723.
25. Romano F, Barbui A, Aimetti M. Periodontal pathogens in periodontal pockets and in carotid atheromatous plaques. *Minerva Stomatol*. 2007;56:169-179.
26. Khlgatian M, Nassar H, Chou HH, et al. Fimbria-dependent activation of cell adhesion molecule expression in Porphyromonas gingivalis-infected endothelial cells. *Infect Immun*. 2002;70:257-267.
27. Imamura T, Travis J, Potempa J. The biphasic virulence activities of gingipains: activation and inactivation of host proteins. *Curr Protein Pept Sci*. 2003;4:443-450.
28. Li L, Messas E, Batista EL, Levine RA, Amar S. *Porphyromonas gingivalis* infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E–deficient murine model. *Circulation*. 2002;105:861-867.
29. Nakano K, Nomura R, Matsumoto M, Ooshima T. Roles of oral bacteria in cardiovascular diseases–from molecular mechanisms to clinical cases: cell-surface structures of novel serotype k *Streptococcus mutans* strains and their correlation to virulence. *J Pharmacol Sci*. 2010;113:120-125.
30. Chhibber-Goel J, Singhal V, Bhowmik D, et al. Linkages between oral commensal bacteria and atherosclerotic plaques in coronary artery disease patients. *NPJ Biofilms Microbiomes*. 2016;**2**:7.
31. Hosoki S, Saito S, Tonomura S, et al. Oral carriage of *Streptococcus mutans* harboring the *cnm* gene relates to an increased incidence of cerebral microbleeds. *Stroke*. 2020;51:3632-3639.
32. Nagata E, de Toledo A, Oho T. Invasion of human aortic endothelial cells by oral viridans group streptococci and induction of inflammatory cytokine production. *Mol Oral Microbiol*. 2011;26:78-88.
33. Kuramitsu HK, Qi M, Kang IC, Chen W. Role for periodontal bacteria in cardiovascular diseases. *Ann Periodontol*. 2001;6:41-47.
34. Cohen DJ, Malave D, Ghidoni JJ, et al. Role of oral bacterial flora in calcific aortic stenosis: an animal model. *Ann Thorac Surg*. 2004;77:537-543.
35. Tapis P, El-Koussy M, Hewer E, Mono ML, Reinert M. Plaque vulnerability in patients with high- and moderate-grade carotid stenosis - comparison of plaque features on MRI with histopathological findings. *Swiss Med Wkly*. 2020;150:w20174. doi:10.4414/smw.2020.20174
36. Sugioka K, Naruko T, Matsumura Y, et al. Neopterin and atherosclerotic plaque instability in coronary and carotid arteries. *J Atheroscler Thromb*. 2010;17:1115-1121.
37. Nomura R, Otsugu M, Hamada M, et al. Potential involvement of Streptococcus mutans possessing collagen binding protein cnm in infective endocarditis. *Sci Rep*. 2020;10:19118.
38. Kobayashi R, Ogawa Y, Hashizume-Takizawa T, Kurita-Ochiai T. Oral bacteria affect the gut microbiome and intestinal immunity. *Pathog Dis*. 2020;78(3):ftaa024. doi:10.1093/femspd/ftaa024
39. Zhang M, Whiteley M, Lewin GR. Polymicrobial interactions of oral microbiota: a historical review and current perspective. *mBio*. 2022;13(3):e0023522. doi:10.1128/mbio.00235-22