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Original Article

Comparison of the oral microbiota of patients with atherosclerosis and healthy controls by denaturing gradient gel electrophoresis

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Abstract

Oral infections can activate local and systemic inflammation. The inflammatory response plays a main role in atherosclerosis. several studies have reported a relationship between oral pathogen infection and Atherosclerosis. Recently it was indicated that some oral microbiome has a significant role in triggering atherosclerosis. Denaturing Gradient Gel Electrophoresis (DGGE) is an acceptable assay for identification of uncultivable bacteria. Therefore, we compared the bacterial population diversity in the oral microbiota between atherosclerosis patients and healthy people. Oral microbiota profiling was performed for 139 individuals including 89 patients with CAD and 50 healthy individuals. After DNA was extracted from saliva, PCR products were examined and evaluated using DGGE assay. We found that significant relationship between the increased risk of atherosclerosis and the presence of Actinomyces oris, Enterococcus faecalis, Bacterium strain sulresv, Bacterium Culaenoe, NC4, NC7, and NC5 in atherosclerosis patients and healthy individuals. There was also a significant relationship between reducing the risk of atherosclerosis in the presence of NC3 and Entreococcus munotii in atherosclerosis patients and healthy individuals. In conclusion, presence of some oral microbiota increases the risk of atherosclerosis and the presence of some oral microbiota reduces the risk, so the oral microbiota should be further examined to determine its potential as a biomarker for atherosclerosis.

Keywords: Atherosclerosis, Denaturing gradient gel electrophoresis, Oral microbiota.

1. Introduction

The mouth is a common entry point for bacteria into the human body. After the gut microbiome, the oral cavity contains unique communities of microorganisms that are present in saliva (1). Recent research has emphasized the growing importance of the oral microbiome in human health and disease. Several studies have shown that the oral microbiome is involved in systemic diseases such as rheumatoid arthritis, diabetes, and atherosclerosis, which is a form of cardiovascular disease characterized by the buildup of plaques in the arteries due to inflammation and cholesterol accumulation (2). In Iran, there has been an increase in mortality rates related to atherosclerosis in recent decades (3). Evidence suggests that the host's microbiota, including oral bacteria, may play a significant role in triggering atherosclerosis (4, 5). Oral bacteria and their byproducts can enter the bloodstream through inflamed oral tissues or via saliva and cause systemic inflammatory and immune responses (6). This suggests that oral bacteria associated with infectious diseases like caries and periodontitis could initiate the formation of atherosclerotic plaques through an inflammatory stimulus (7). However, some studies have found no significant qualitative changes in the oral microbiota between individuals with atherosclerosis and healthy controls (8). Denaturing Gradient Gel Electrophoresis (DGGE) is a DNA-based method that allows for the detection of small mutations and SNP analysis with a high detection rate and less time and labor compared to other techniques (9). Therefore, this study aimed to compare the relationship between the indicator bacteria of the oral microbiota in patients with atherosclerosis and healthy people with the DGGE method.

2. Materials and Methods

2.1. Study population

Eighty-nine patients with atherosclerosis were enrolled in the study from patients of the Imam Ali Cardiovascular Hospital, Kermanshah, Iran. Between April 2020 to October 2021. Also, 50 healthy subjects were included in the study. All participants answered an anonymized questionnaire containing personal information (age, sex, smoking, etc.), medical history, and nutritional habits. Written consent was obtained from all participants. Samples col-

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lection for oral microbiome profiling, saliva samples were collected from all recruited subjects in sterile tubes under fasting conditions to avoid the effects of diet on the oral microbiota. Each sample was placed in an ice bag immediately and delivered to the laboratory.

2.2. DNA extraction

DNA was extracted on fresh saliva samples upon arrival to the laboratory using YektaTajhizAzma DNA Extraction Kit (Iran), following the manufacturer's instructions and the concentration of DNA that measured using NanoDrop (Thermo Fisher Scientific, USA). stored at −20◦ until analysis.

2.3. PCR amplification of 16S rRNA genes

The PCR mixture contained 2X master mix YektaTajhizAzma (Iran), 10pmol/ µl of each primer and 2 µl (50ng) DNA template. Primers are shown in Table 1. The PCR reaction was performed using S1000™ Thermal Cycler (BioRad, Singapore) as follows: Cycle conditions were 95◦C (2 min), then 35 cycles of 94◦C (45 s), 55◦C (45 s), 72◦C (1 min), then a final extension of 72◦C (7 min). The amplified products were checked by agarose gel electrophoresis. Next, PCR products were separated by DGGE Electrophoresis.

2.4. Denaturing Gel Gradient Electrophoresis (DGGE)

General bacterial 16S rDNA gene profiles of oral bacteria in atherosclerosis groups and control group were generated using the DGGE (Bio-Rad Laboratories, Inc., USA). Electrophoresis of PCR products was carried out on polyacrylamide gels as described by Muyzer *et al* (10, 11). PCR products were loaded on polyacrylamide gel 10 (wt/vol) in 1X TAE (1X TAE is 0.04MTris base, 0.02Macetic acid, and 1.0 mM EDTA [pH 7.5]). The denaturing gradient included 20% to 70% denaturants. Electrophoresis was performed for 17h at 60V and 60˚C.

2.5. Sequencing

A sample of any distinct band was sequenced and aligned in standard databases in order to detect any band on gel. For this purpose, the desired bands were cut with sterile scalpel and were placed in 50 µl 1X TAE. This mixture was stored at 4˚C for 24h and PCR product was extracted with gel extraction kit. The sequencing process was carried out in Pishgam Company (Iran).

2.6. Statistical analysis

Data were compiled and analyzed using statistical software SPSS version 19 (Chicago, IL, USA). In order to determine the relationship between the variables, Chi-square test, and then t-test will be used to determine logistic regression and Odds Ratio. Also, the significance level of statistical tests is less than 0.05.

3. Results

3.1. Characteristics of study participants

A total of 89 atherosclerosis patients (age: 62.71 years, 19 females and 70 males), and 50 controls (age: 57.88 years, 15 females and 35 males) not suffering from coronary syndromes were enrolled. Clinical and laboratory data and the most documented medications are presented in Table 2.

Compared with healthy controls, the BMI was not significantly different between atherosclerosis patient group and healthy subjects and obesity was observed only for 28% of atherosclerosis patients and 16% of controls. We surveyed salivary bacterial communities of 89 patients with atherosclerosis and 50 controls. The bacterial 16S rRNA gene was PCR amplified. Following PCR amplification separated by DGGE Electrophoresis, no amplicon bands were observed in negative controls confirming the absence of contaminations (Figure 1).

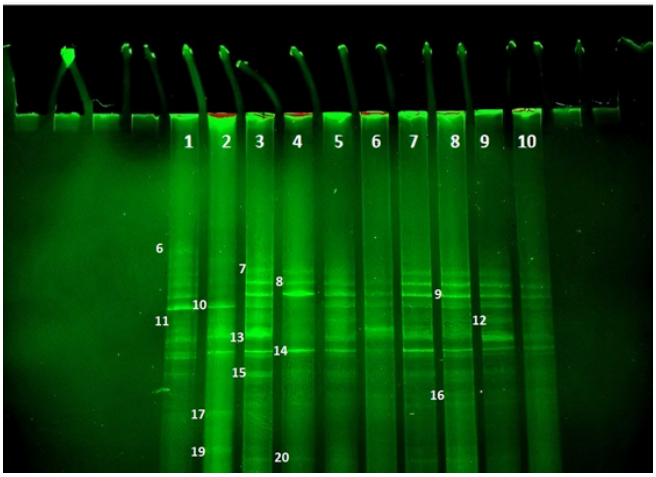

Fig. 1. DGGE of PCR product; any band shows any bacterial genus or species. This method identified a greater number of bacteria than the cultural method. Bacteria numbers: 6(NC6), 7(Nc5), 8(Nc8), 9(*Bifidobacterium*), 10 (*Staphylococcus*), 11(*Enerococcus*), 12(*Peptosterptococcus*), 13(*Actinomyces*), 14(*Lactobacillu*), 15(NC8), 16 (*Streptococcus*), 17(*Micrococcus*), 19(*Prevotella*), 20(*Porphyromonas*), wells 6,7,8,9 were patient samples. 1, 2, and 4 were healthy control. NC: Uncultivable Bacteria.

Table 1. List of primer sequences used in this study.

Name	Sequences (5' -> 3')
I-34IfGC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGIGGCIGCA
I-533r	TIACCGIIICTICTGGCAC

Table 2. Characteristics of atherosclerosis patients and controls .

Characteristics	(%)Atherosclerosis Patients	(%)Controls
Family history of atherosclerosis	68(76.4%)	26(52%)
History of antibiotic use	28(27%)	18(36%)
History of heart disease	89(100%)	18(9%)
Tobacco consumption	64(71.9%)	41(82%)
Alcohol consumption	84(94.4%)	46(92%)

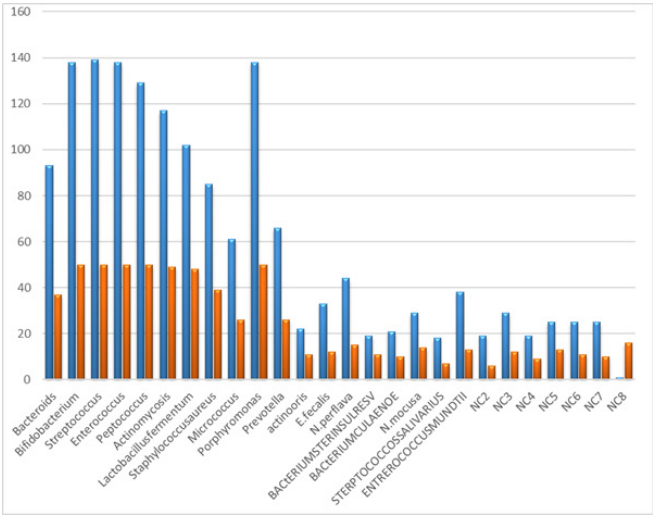

Fig. 2. Frequency of bacteria isolated from people with Atherosclerosis (blue) and healthy (red). NC: Uncultivable Bacteria.

The frequency of bacteria isolated from atherosclerotic patients and healthy subjects is shown in Figure 2.

3.2. Comparison of oral microbiota between control and cases

There was a significant relationship between atherosclerosis and the presence of *Actinomyces oris*, *Bacterium strain sulresv*, *Staphylococcus aureus*, *Lactobacillus fermentun*, in atherosclerosis and healthy individuals (Odds Ratio15.132) (Odds Ratio2.85) Odds Ratio 3.314 (Odds Ratio 15.556), were present. More than healthy people. The result is shown in Table 3.

4. Discussion

In this study, a total of 89 atherosclerosis patients (age: 62.71 years, 19 females and 70 males), and 50 controls (age: 57.88 years, 15 females and 35 males) not suffering from coronary syndromes and metabolic diseases (e.g. diabetes mellitus) were enrolled. Compared with healthy controls, the BMI was not significantly different between atherosclerosis patient group and healthy subjects and obesity was observed only for 28% of atherosclerosis patients and 16% of controls. We surveyed salivary bacterial communities of 89 patients with atherosclerosis and 50 controls. Following PCR amplification separated by DGGE Electrophoresis. There was a significant relationship between atherosclerosis and the presence of *Actinomyces oris*, (Odds Ratio15.132) ($P < 0.05$), *Bacterium strain sulresv*, ($P < 0.05$) (Odds Ratio2.85), *Staphylococcus aureus*, Odds Ratio 3.314 ($P < 0.05$), *Lactobacillus fermentum*, ($P < 0.01$) (Odds Ratio 15.556) in atherosclerosis and healthy individuals, the presence of these bacteria in the mouth of atherosclerotic patients was more than healthy people. A previous study showed that bacteria from the oral cavity and the gut can be recovered from atherosclerotic plaque (12). Atherosclerotic vascular disease is determined by genetic and environmental factors such as diet and lifestyle, and recent evidence suggests that the host microbiota may be considered an environmental factor that contributes to the stability of atherosclerotic plaques (13). Hyvarinen *et al*. showed that the periodontal pathogen *Aggregatibacter actinomycetemcomitans* was increased in the saliva of patients with both symptomatic and asymptomatic coronary disease as compared to healthy individuals (14).

A. actinomycetemcomitans has been shown to affect progression of plaques in mouse models (15). However, as only 11% of the patients were positive for the bacterium, like our study *Actinomyces* family had significant relationship with atherosclerosis (14). On the other hand, our study showed that *Staphylococcus aureus* in oral cavity is one of periodontal pathogens in atherosclerosis patients, also, Zhao *et al*. reported that *Staphylococcus aureus* superantigen toxic shock syndrome toxin-1 exposure accelerates the progression of atherosclerosis in rabbits (16). Thus, identifying the appropriate strains is essential to the therapeutic potential of *Lactobacilli* as an anti-atherosclerotic agent. On the other hand, DGGE assay is simple and useful to detect these strains Limitations of the present study include the number of patients investigated, as our results indicate that the study may have been underpowered to detect subtle changes in the oral microbiota.

5. Conclusion

Our findings indicate that the presence of some oral microbiota increases the risk of atherosclerosis and the presence of some oral microbiota decreases the risk. Also, our results show that DGGE is a suitable method for diagnosing and comparing the oral microbiota of people with atherosclerosis and healthy people, Furthermore, the association between oral bacteria and cardiovascular risk factors needs further investigation.

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Conflict of interest

The authors have no conflicts of interest to declare.

Availability of data and materials

All generated or analyzed data during this study are included in this published article. For other data, these may be requested through the corresponding author.

Authors' contributions

ZN, RA, HM: wrote original draft, methodology; ZN, RA: wrote original draft, perform; molecular method, prepared figures; ZN, RA, and HM: wrote original draft, wrote a text discussion, editing; RA (Corresponding author): methodology, project administration, wrote original draft, editing

Ethics approval and consent to participate

The present study was ethically approved by the Kermanshah University of Medical Sciences, Institutional Review Board (IR.KUMS.REC.1398.1077).

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Table 3. Bacteria in patient and healthy groups.

Bacteria		Patient Frequency (%)	Health Frequency (%)	Odds Ratio	P value	Bacteria		Patient Frequency (%))	Health Frequency (%)	Odds Ratio	P value
Bacteroides	Yes	56(62.9%)	37(74%)	-	-	*Neisserria perflava*	Yes	29(32.5%)	15(30%)	-	-
	No	33(37.1%)	13(26%)				No	60(67.4%)	35(70%)		
Bifidobacterium	Yes	88(98.9%)	50(100%)	-	-	*Bacterium strain sulresv*	Yes	8(9%)	11(22%)	2.856	<0.05
	No	1(1.1%)	-				No	81(91%)	39(78%)		
Staphylococcus areuse	Yes	46(51.7%)	39(78%)	3.314	<0.05	*Bacterium culaenoe*	Yes	11(12.4%)	10(20%)	-	-
	No	43(48.3%)	11(22.0%)				No	78(87.6%)	40(80.0%)		
Peptostreptococcus	Yes	79(88.7%)	50(100%)	-	-	*Nc4*	Yes	10(11.2)	9(18%)	-	-
	No	10(11.2%)	-				No	79(88.8%)	41(82%)		
Streptococcus	Yes	89(100%)	50(100%)	-	-	*Nc3*	Yes	17(19.1%)	12(24%)	-	-
	No	-	-				No	72(80.9%)	38(76.0%)		
Actinomyces	Yes	68(76.4%)	49(98%)	15.132	0.05<	*Nc2*	Yes	13(14.6%)	6(12%)	-	-
	No	21(23.6%)	1(2.0%)				No	76(85.4%)	44(88.0%)		
Lactobacillusfermentun	Yes	54(60.7%)	48(96%)	15.556	<0.01	*Streptococcus salivarius*	Yes	11(12.4%)	7(14%)	-	-
	No	35(39.3%)	2(4.0%)				No	78(87.6%)	43(86.0%)		
Micrococcus	Yes	35(39.3%)	26(52%)	-	-	*Nc7*	Yes	15(16.9%)	10(20%)	-	-
	No	54(60%)_	24(48%)				No	74(83.1%)	40(80.0%)		
Prevotella	Yes	40(44.9%)	26(52%)	-	-	*Nc6*	Yes	14(15.7%)	11(22%)	-	-
	No	49(55.1%)	24(48%)				No	75(84.3%)	39(78.0%)		
Porphyromonas	Yes	88(98.9%)	50(100%)	-	-	*Nc5*	Yes	12(13.5%)	13(26%)	-	-
	No	1(1.1%)	-				No	77(86.5%)	37(74.0%)		

Nc8	Yes	21(23.5%)	15(30%)	-	-	*Enterococcus mundtii*	Yes	25(28%)	13(26%)	-	-
	No	68(76.4%)	34(68.0%)				No	64(71.9%)	37(74.0%)		
Actinomyces oris	Yes	11(12.4%)	11(22%)	15.132	<0.05	*Enterococcus. fecalis*	Yes	21(23.6%)	12(24%)	-	-
	No	78(87.6%)	39(87%)				No	68(76.4%)	38(76%)		
Neisserria mucosa	Yes	15(16.9%)	14(28%)	-	-						
	No	74(83.1%)	36(72.2%)								

NC: Uncultivable Bacteria.

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