Original Article | Iran J Med Microbiol. 2023; 17(3): 301-308

10.30699/ijmm.17.3.301

Iranian Journal of Medical Microbiology | ISSN:2345-4342

Metabolic Parameters and Oral Microbiota in Patients with Atherosclerosis

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ABSTRACT

Background and Aim: Oral infections are common among people of any age and can trigger systemic inflammation. The microbiota is a diverse group of microorganisms that play important roles in metabolism, immune function, and homeostasis. Oral microbiota in human atherosclerotic plaques has been identified using various techniques. Therefore, the focus of this study was to determine the correlation between metabolic parameters and oral microbiota composition in patients with atherosclerosis using Denaturing Gradient Gel Electrophoresis (DGGE) assays.

Materials and Methods: In this case-control study, saliva samples were collected from 139 patients with atherosclerosis and healthy individuals from Imam Ali Cardiovascular Hospital, Kermanshah, Iran. After DNA extraction, PCR products were examined and evaluated using DGGE assays.

Results: The study included 89 (36%) patients with a history of atherosclerosis and 50 (36%) healthy individuals. There was a significant relationship between the mean total cholesterol, Low-Density Lipoprotein (LDL), Fasting Blood Sugar (FBS), and Blood Urea Nitrogen (BUN) in the two groups. However, there was no significant difference in the mean high-density lipoprotein (HDL) and Triglyceride levels between the study groups.

Conclusion: Our results showed a relationship between metabolic parameters and oral microbiota composition in patients with atherosclerosis. Additionally, our results indicated that the DGGE assay is a useful method for diagnosing and comparing the oral microbiota of people with atherosclerosis and healthy individuals. Therefore, further examination of the oral microbiota is necessary to determine its potential as a biomarker for atherosclerosis.

Keywords: Metabolic Parameters, Atherosclerosis, Oral microbiota, Denaturing Gradient Gel Electrophoresis

Received: 2023/01/07; **Accepted**: 2023/03/11; **Published Online:** 2023/06/26

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Nazari Z, Abiri R, Heidary Moghadam R, Chehri G, Alvandi A, Mohajerani H R. Metabolic Parameters and Oral Microbiota in Patients with Atherosclerosis. Iran J Med Microbiol. 2023; 17(3):301-8.

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1. Introduction

Cardiovascular disease (CVD) is a complex human disease (1). Atherosclerosis is a systemic disorder that causes high mortality and morbidity worldwide (2). Atherosclerotic plaques are composed of macrophages (Macs), cholesterol ,dendritic cells (DCs), and fatty acids (3). The relationship between atherosclerosis and levels of Low-Density Lipoprotein (LDL), high-density lipoprotein (HDL) and cholesterol has well been well established (4). Plasma levels of lipids have been well shown to be strong predictors of

CVD (5). High levels of cholesterol, diabetes, and hypertension increase the risk of CVD (6, 7). Additionally, Dysbiosis, or abnormal changes in the composition or diversity of the oral microbiota, has been linked to a variety of diseases, including, Alzheimer's disease, atherosclerosis, and inflammatory bowel disease (1, 8, 9). Unhealthy diet, abnormal cholesterol concentrations and dysbiosis in the gut microbiota have also been associated with the development of atherosclerosis (1, 9). Oral microorganisms can enter the bloodstream by crossing disturbed oral mucosa in periodontal diseases (10). Macrophages are the major inflammatory cells in atherosclerotic lesions (11, 12). Some studies confirm that the oral microbiota, leading to the development of metabolic syndrome (MetS) (13), which is characterized by high levels of triglycerides (TGs), and an increased risk of atherosclerosis (14). The presence oral bacteria in atherosclerotic plaques has been demonstrated by using various methods. Denaturing Gradient Gel Electrophoresis (DGGE) is an assay used to identify unculturable bacteria. In this assay, amplified DNA fragments with the same length but different sequences are separated based on their electrical charges. Eventually, a band pattern is produced, with each band representing a unique molecular sequence related to a single species (11, 15). Therefore, the focus of this study was to determine the relationship between metabolic parameters and oral microbiota composition in patients with atherosclerosis using DGGE assays.

2. Materials and Methods

Clinical Sampling

In this case-control study, written consent was obtained from all participant before saliva was collected from atherosclerosis patients and healthy individuals using sterile falcons for molecular assays from Imam Ali Cardiovascular Hospital, Kermanshah, Iran between April and October 2021. All atherosclerotic patients were approved by a cardiologist during sampling. This research was approved by the ethics committees with code 1398.1077 at Kermanshah University of Medical Sciences.

DNA Extraction

Saliva DNA was extracted according to the manufacturer's instructions (Yekta tajhiz azma, Iran), and the quality of extracted DNA was assessed by spectrophotometry equipment, the Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, DE, USA).

PCR Assay

The PCR reaction was performed using S1000™ Thermal Cycler (BioRad, Singapore) as follows: denaturation at 95˚C for 2 min followed by 35 cycles, denaturation at 94˚C for 45 seconds, annealing at 55˚C for 45sec, extension at 72˚C for 1 min and final extension for 7 min. The PCR mixture included 2X master mix (Yekta Tajhiz Azma), 10 pmol/ µl of each primer, and 2 µl (50ng) DNA (16). The primers used are shown in Table 1. In the following, PCR products were separated by DGGE electrophoresis.

Table 1. List of Primers for DGGE Assay

Name	Sequences (5' -> 3)'	Position	References
I-34IfGC	GC clamp connected to the 5 end of I-341f	341–356	(16)
I-533r	TIACCGIIICTICTGGCAC	515–533	

I, inosine.

DGGE Assay

PCR products were loaded on the polyacrylamide gel and DGGE was performed for 17h at 60V and 60˚C, according to previously published papers [16, 18].

Sequencing

The eligible bands were cut with a scalpel and PCR product was extracted using a gel extraction kit. The sequencing process was carried out by Pishgam Company (Iran).

Statistical analysis

After data collection, the data were analyzed using SPSS version 19 (Chicago, IL, USA). The Chi-square test was used to determine the correlation between variables, and the t-test was used to determine logistic regression and odds ratio. The significance level of statistical tests was set sat less than 0.05.

3. Results

Patients

Out of 139 Saliva samples collected, 89 (64%) were from atherosclerosis patients and 50 (36%) were from healthy individuals. The mean age of atherosclerosis patients was

62.71 years, while that of healthy individuals was 57.88 years. Also, 46.6% (90) of the participants were male and 25.4% (49) were female. Body Mass Index (BMI) of 47.1% of atherosclerosis patients and 54% of healthy people in this study was 20-25. There were no significant differences in sex, age, and BMI among the two study groups (Table 2).

Table 2. Characteristics of Atherosclerosis patients and healthy people

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

Comparison of biochemical parameters in atherosclerosis patients and healthy people

Significant differences were observed between the mean of total cholesterol, Low-Density Lipoprotein (LDL), Fasting Blood Sugar (FBS), and Blood Urea Nitrogen (BUN) in the two groups of control and atherosclerosis patients. However, there was no any significant relationship in the mean levels of Triglyceride and High-Density Lipoprotein (HDL) between the two groups (Table 3). The DGGE of the PCR product sample in both study groups was shown in Figures 1, and 2.

Table 3. Comparison of Biochemical data in both study groups.

Biochemical Parameters	Atherosclerosis	Healthier	p-value
FBS(mg/dl)	89(143.34)	50(114.60)	>0.05
TG(mg/dl)	89(131.03)	50(128.14)	-
Total cholesterol (mg/dl)	89(149.69)	50(138.64)	>0.05
LDL cholesterol(mg/dl)	89(109.83)	50(83.86)	>0.05
HDL cholesterol(mg/dl)	89(37.03)	50(38.54)	-
BUN(mg/dl)	89(39.54)	50(33.08)	>0.05

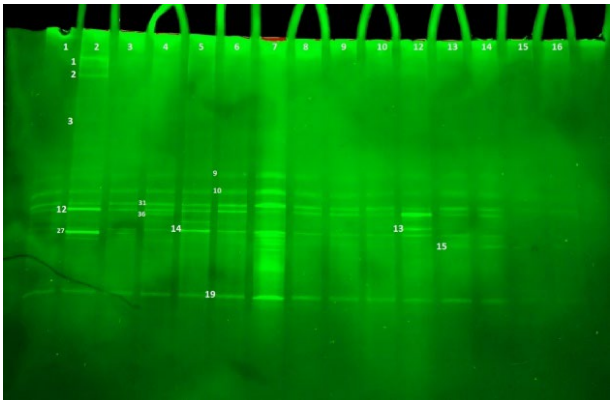

Figure 1. DGGE of PCR product sample of healthy and Atherosclerosis, Ladder: well No. 7

Wells No. 1, 3, 5, 8, 10, 12, (Healthy Samples), Wells No. 13, 2, 4, 6,9,11, (Atherosclerosis samples), 9: *Bifido bacterium* 10: *Staphylococcus* 12: *Peptosterptococcus* 13: *Actinomyces* 14: *Lactobacillus* 15: NC8 19: *Prevotella* 25: *Nc2* 27: *Neisseria mucosa* 36: *NC3* NC: Not Culturable

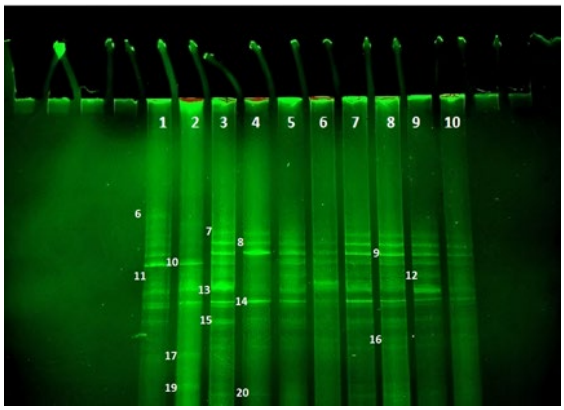

Figure 2. DGGE of PCR product sample of healthy and Atherosclerosis, Ladder: well No. 3

Wells, 1, 2, 4, (healthy samples), Wells No. 5-10 (Atherosclerosis samples), 6: NC6 7: Nc8 8: Nc5 9: *Bifido bacterium* 10: *Staphylococcus* 11*: Enterococcus* 12: *Peptosterptococcus* 13: *Actinomyces* 14: *Lactobacillus 16: Streptococcus 17: Micrococcus 19: Prevotella 20: Porphyromonas*, NC: Not Culturable

LDL and HDL in atherosclerosis patients and healthy

The LDL levels in the two groups with and without Streptococcus salivarius was significantly different (P-value <0.05). This means that people who have the *S. salivarius* in their mouth have a low and normal average LDL, whereas people who do not have this bacterium in their mouth have a high LDL. However, there was no significant difference in the HDL between the two groups.

FBS in atherosclerosis patients and healthy

Significant differences were observed between the FBS levels in the two groups with *Actinomyces* and *Bacterium culaenoe* (P-value <0.05). This means that people who have Actinomyces and *B. culaenoe* in their mouth have a low and normal average FBS, whereas people who do not have these bacteria in their mouth have a higher average FBS.

Cholesterol and TG in atherosclerosis patients and healthy

The Cholesterol levels in the two groups with and without *Actinomyces*, *Neisseria perflava* were significantly different (P-value <0.05). This means that people who have *Actinomyces*, and *N. perflava* in their mouths have average or low cholesterol, whereas people who do not have these bacteria in their mouths have higher cholesterol averages. However, there was no significant difference in the mean levels of Triglyceride between the two groups.

BUN in atherosclerosis patients and healthy

The BUN in the two groups with and without *N. perflava* were significantly different (P-value <0.05). This means that people who have *N. perflava* in their mouth have a lower and normal average BUN, but, people who do not have this bacterium in their mouth have a higher average BUN. The oral microbiota composition and biochemical data in the two groups was shown in Table 4.

Table 4. Oral microbiota composition and biochemical data in two groups

Bacteria	Bacteria LDL (mg/dL)	No bacteria LDL (mg/dL)	Bacteria Cholesterol (mg/dL)	No bacteria Cholesterol (mg/dL)	Bacteria BUN (mg/dL)	No bacteria BUN (mg/dL)	Bacteria FBS (mg/dL)	No bacteria FBS (mg/dL)
Bacteroides	93(96.47)	46(108.61)	93(145.58))	46(145.89)	93(15.520)	46(13.960)	93(132.39)	46(127.50)
Bifidobacterium	138(100.59)	1(87.00)	138(145.64)	1(156.00)	138(37.28)	1(29.00)	138(131.10)	1(85.00)
Staphylococcus areus	85(96.94)	54(106.07)	85(148.74)	54(140.94)	85(38.16)	54(35.72)	85(125.44)	54(139.17)
Enterococcus fecalis	33(108.82)	106(97.90)	33(149.48)	106(144.54)	33(35.30)	106(37.81)	33(125.85)	106(132.30)
Peptostreptococcus	129(101.71)	10(84.70)	129(145.43)	10(149.40)	129(37.07)	10(39.10)	129(131.43)	10(122.30)
Streptococcus	139(100.49)	-	139(145.71)	-	139(37.22)	-	139(130.77)	0
Actinomycois	117(96.96)	22(119.27)	117(143.11)	22(159.55)	117(37.45)	22(35.95)	117(126.39)	22(154.05)
Lactobacillusfermentum	102(100.57)	37(100.27)	102(145.49)	37(146.32)	102(36.73)	37(38.57)	102(126.58)	37(142.32)
Micrococcus	61(100.34)	78(100.60)	61(148.61)	78(143.45)	61(39.84)	78(35.17)	61(127.49)	78(133.33)
Prevotella	66(101.86)	73(99.25)	66(146.95)	73(144.59)	66(39.02)	73(35.59)	66(135.05)	73(126.90)
Porphyromonas	138(100.51)	1(98.00)	138(130.4)	1(74.00)	138(37.18)	1(4200)	138(130.82)	1(124.00)
Nc4	19(88.32)	120(102.42)	19(136.74)	120(147.13)	19(40.79)	120(36.65)	19(111.89)	120(133.76)
NC8	36(90.72)	102(103.88)	36(139.94)	103(147.73)	36(39.11)	103(36.55)	36(139.33)	103(127.87)
Actinomyces oris	22(112.00)	117(98.32)	22(155.77)	117(143.82)	22(38.18)	117(37.03)	22(124.32)	117(131.98)
Neisserria perflava	44(95.52)	95(102.79)	44(134.86)	95(150.74)	44(42.73)	95(34.66)	44(141.43)	95(125.83)
Bacterium strain sulresv	19(85.32)	120(102.89)	19(133.16)	120(147.70)	19(34.00)	120(37.72)	19(113.58)	120(133.49)
Bacterium culaenoe	21(86.67)	118(102.95)	21(143.52)	118(146.10)	21(37.57)	118(37.15)	21(106.90)	118(135.02)
Nc3	29(92.93)	110(102.48)	29(144.17)	110(146.12)	29(38.69)	110(36.83)	29(131.07)	110(130.29)
Neisserria mucosa	29(103.21)	110(99.77)	29(150.17)	110(144.54)	29(39.62)	110(36.58)	29(136.34)	110(129.30)
NC2	19(94.74)	120(101.40)	19(147.68)	120(145.40)	19(36.63)	120(37.31)	19(119.68)	120(132.52)
Streptococcus salivarius	18(138.33)	121(94.86)	18(141.22)	121(146.38)	18(43.28)	121(36.31)	18(120.67)	121(132.27)
NC7	25(95.56)	114(101.57)	25(146.04)	114(145.64)	25(41.52)	114(36.27)	25(123.20)	114(132.43)

Bacteria	Bacteria LDL (mg/dL)	No bacteria LDL (mg/dL)	Bacteria Cholesterol (mg/dL)	No bacteria Cholesterol (mg/dL)	Bacteria BUN (mg/dL)	No bacteria BUN (mg/dL)	Bacteria FBS (mg/dL)	No bacteria FBS (mg/dL)
NC6	25(102.96)	114(99.95)	25(150.16)	114(144.74)	25(34.92)	114(37.72)	25(137.76)	114(129.24)
NC5	25(109.68)	114(98.47)	25(143.00)	114(146.31)	25(34.92)	114(37.72)	25(116.44)	114(133.91)
Enterococcus mundtii	38(102.63)	101(99.68)	38(145.95)	101(145.62)	38(39.82)	101(36.24)	38(136.76)	101(128.51)

NC: Not Culturable

4. Discussion

It has been shown that oral microbiota can enter the bloodstream and localize in atherosclerotic lesions **(17, 18)**. Several studies confirm the relationship between metabolic parameters and increased risk of atherosclerosis **(19, 20)**. In our study, we detected bacterial DNA in atherosclerotic plaques in 64% of the samples, possibly due to the method used for bacteria detection **(21, 22)**. We developed DGGE assay to the investigate relationship between metabolic parameters and oral microbiota composition in patients with atherosclerosis. DGGE assays have been used to identify multiple different single-base substitutions in a variety of sequences **(23, 24)**.

Out of 139 saliva samples collected, 89 people (64%) had arteriosclerosis and 50 people (36%) were healthy. The mean age of patients with atherosclerosis was 62.71 years and healthy individuals was 57.88 years. Also, 46.6% **(90)** of the participants were male and 25.4% were female. The BMI of 47.1% of atherosclerosis patients and 54% of healthy people in this study was 20-25. These results disagree with other studies, which could be due to the low sample size, different geographical areas, and lifestyles. There are considerable differences between the mean levels of total cholesterol, LDL, FBS, and BUN in the two groups of control and atherosclerosis patients. However, there was no significant relationship in the mean levels of Triglyceride and HDL between the two groups, which is consistent with other studies by Kron et al., Xian et al. and Abbate et al. **(22, 25, 26)**.

DGGE assay was used to study the profile of bacteria in the oral cavity, which showed that The LDL levels in the two groups with and without *S. salivarius* was significantly different (P-value <0.05). This means that people who have the *S. salivarius* in their mouth have a lower and normal average LDL, whereas people who do not have this bacterium in their mouth have a higher LDL. This is consistent with Calandrini et al. study **(27)**. There was also no significant difference in HDL levels between the two groups, which disagreed with another study, possibly due to the small sample size (139 samples) compared to other studies. Moreover, significant differences exist between the FBS in the two groups with *Actinomyces* and *B. culaenoe* (P-value <0.05). This means that people who have *Actinomyces* and *B. culaenoe* in their mouth have a lower and normal average FBS, whereas people who do not have these bacteria in their mouth have a higher average FBS. Similar results were reported by Grau et al **(28)**. Our result shown that cholesterol levels in the two groups with and without *Actinomyces* and *N. perflava* are significantly different. This means that people who have *Actinomyces*, and *N. perflava* in their mouths have average to low cholesterol, whereas other people have higher cholesterol averages. These results were agreement to Niemi et al., Leishman et al., and Calandrini et al. **(27, 29, 30)** Additionally, similar to a study by Kato-Kogoe N's et al **(31)**, there was no significant relationship between the mean triglycerides levels of the two groups. On the other hand, BUN levels in the two groups with and without *N. perflava* was significantly different (P-value <0.05). This means that people who have *N. perflava* in their mouth have a lower and normal average BUN, whereas people who do not have this bacterium in their mouth have a higher average BUN, which is consistent with the findings of Bouzid et al. study **(32)**. According to our result and several studies, these bacteria or their products probably caused the decrease in FBS, cholesterol, BUN, etc **(33)**, which is due to a favorable condition for growth of oral cavity bacteria such as *Veillonella atypica*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Streptococcus faecalis* **(34, 35)**. Many non-culturable organisms which are only identifiable by molecular assay, reside in the oral cavity **(36)**.

One of the limitations of this study is the low sample size. The DGGE assay can be used as an effectual screening assay to investigate the relationship between metabolic parameters and oral microbiota composition in patients with atherosclerosis.

5. Conclusion

Our data shown DGGE assay is a good way to diagnose and compare the oral microbiota of people with atherosclerosis and healthy. Thus, the oral microbiota should be further examined to determine its potential as a biomarker for atherosclerosis.

Acknowledgment

The authors would like to thank the staff of the Department of Microbiology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.

Funding

None.

Conflict of Interest

The authors declare no conflict of interest.

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