

IDENTIFICATION OF PERIOPATHOGENES FROM DENTAL PLAQUE IN PERIODONTAL PATIENTS WITH PCR TECHNIQUE AND THEIR ASSOCIATION WITH COMPOSITE INTERLEUKIN-1 GENOTYPE

Aneta Atanasovska Stojanovska¹, Saska Todoroska²,
Mirjana Popovska¹, Ilijana Muratovska¹, Linda Zendeli Bedzeti²

¹ Faculty of Dentistry – Ss. Cyril and Methodius University - Skopje, University Dental Clinic “St. Pantelejmon”, Skopje

² University Dental Clinic “St. Pantelejmon”, Skopje

Corresponding author: Prof. PhD Aneta Atanasovska Stojanovska, Faculty of Dentistry – Ss. Cyril and Methodius University - Skopje, University Dental Clinic “St. Pantelejmon”, Skopje, Majka Tereza 43 Skopje, R. Macedonia, E-mail: anetaatanasovska@yahoo.com

ABSTRACT

Introduction: The present study aimed to assess the presence of main types of microorganisms involved in the aetiopathogenesis of chronic periodontitis with PCR technique and determinates the presence of composite IL-1 genotype and their associations with founded bacteria.

Material and method: The examined group was consisted from 20 subjects with diagnosed chronic periodontitis and 20 healthy control without periodontitis. Clinical parameters like gingival index (GI), plaque index (PI), bleeding on probing (BOP), periodontal pocket depth (PPD) and clinical attachment lost (CAL) were determinates. Subgingival dental plaque was collected using a sterilized paper point. We used Parodontose Plus test, reverse hybridization kit, for the detection of periodontal marker bacteria, as well as for the detection of composite Interleukin -1 Genotype

Results: The most present bacterial species detected from subgingival dental plaque was *Treponema denticola* and *Porphyromonas gingivalis* which was present in 65% of examined patients. In relation to the presence of positive genotype in patients, there was no significant difference between the test and control group for $p > 0.05$ ($p = 1.00$). For $\chi^2=8,17$ ($p=0,06$, $p<0,05$) there is an association between *Prevotella intermedia*, and composite genotype .

Between positive genotype and analyzed bacterial species *A. actinomycetem comitans* for $p > 0.05$ ($p = 1.00$), *P. gingivalis* for $p > 0.05$ ($p = 0.16$), *T. Forsythia* for $p > 0.05$ ($p = 0.20$), *T. Denticola* for $p > 0.05$ ($p = 0.64$) no association was found.

Conclusion. This investigations confirmed the strong association of these five examined periopathogenes with periodontitis.

Keywords: periodontitis, periopathogenes, composite genotype

INTRODUCTION

Periodontitis, an inflammatory disease of the supportive dental tissues is among the main reasons for the tooth loss [1]. Increased number of gram negative strict anaerobes as the cause of the destructive

process directly reflect the onset and progression of the periodontitis [2].

Recent studies suggest composite IL-1 genotype IL-1A Allele2. (-889)/IL-1B Allele2 (+3954) presence in patients with periodontal disease [3-7].

Scientific evidence point to the periopathogenic microorganisms and their strong influence on certain health conditions and diseases [8].

There is no adequate clinical or radiological indicator that shows periodontitis true activity and it was noted that there is a great delay between the onset of the disease and the time at which changes can be detected clinically and with x-ray radiography [9].

It is therefore reasonable to track and define specific bacteria who are indicators of the initial phase and periods of progression and disease activity [10]. Sub-gingival plaque is a biofilm that consists of more than 300 different bacterial species who were identified from the samples collected from periodontal pockets in different individuals [11]. Periodontitis and complications with placed implants (peri-implantitis) are associated with specific microorganisms: *Actinobacillus actinomycetemcomitans*, *B. Forsitus*, *Campilobacter rectus*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Prevotella intermedia* entitled as periodontopathic bacteria. These bacteria cause various immune response reactions in the host and induce periodontal breakdown [12,13].

Molecular biology techniques have been developed and used for identification of the microbial organisms [14-16]. Polymerase chain reaction (PCR) is a simple technique which performs replication of a fragment from DNA and/or RNA and it has found its use in the detection of periodontopathic bacteria [17-19]. PCR doesn't require living cells for identification of the microorganisms and offers significantly higher sensitivity than bacterial culture [20, 21].

Cutting edge method for quantification and classification of bacterial species recommended for monitoring of the bacterial micro flora [22-24]. PCR technique enable specific and sensitive detection of periopathogen microorganisms in sub-gingival plaque samples [25-30]. The aim in our study was to use PCR technique to assess the presence of the main types of microorganisms involved in the aetiopathogenesis of the chronic periodontitis: *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella Intermedia*, *Tannerella forsythia* and *Treponema denticola*, and to determine the presence of composite IL-1 genotype [IL-1A Allele2 (-889)/IL-1B Allele2 (+3954)], and their associations with the detected bacteria.

MATERIAL AND METHOD

Twenty subjects diagnosed for generalized chronic periodontitis (according to criteria of American Academy of Periodontal Disease (AAP, 1999)) consisted the test group. None of the subjects in the test group have received periodontal treatment at least 6 months before the enrollment in the study or any antimicrobial medications at least three months before. All participants in the test group were with good oral health. Twenty healthy subjects without periodontitis or gingivitis in similar age with the participants in the tested group consisted the control group. Informed consent was obtained from each participant in the study.

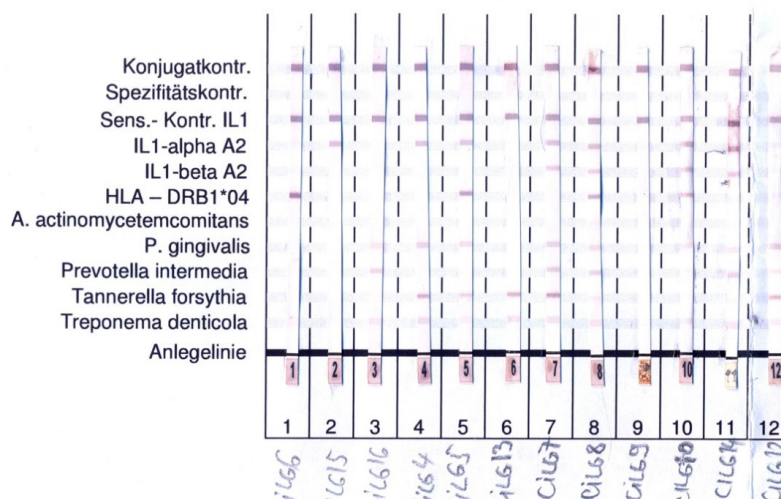
Clinical parameters: gingival index (GI Löe and Silness), plaque index (PI-Silness-Loe), , periodontal pocket depth (PD) According to the Community Periodontal Index for Treatment Needs (CPITN) for PPD the following definition for periodontitis was used: PD 0-3 mm as no/mild periodontitis, at least one pocket ≥ 4 mm and < 6 mm as moderate and with at least one pocket ≥ 6 mm as severe periodontitis.) 31, bleeding on probing (BOP) 32 and clinical attachment level (CAL) were detected in each patient and all measurements were performed by the same trained examiner.

Polymerase chain reaction (PCR) is a method that uses biological material (dental plaque, inflamed gingival tissue, gingival fluid or saliva) which contain DNA of the infecting microorganisms for determination of the most prevalent periopathogenic bacteria.

Using sterilized paper points, sub-gingival dental plaque was collected from the mesial and distal side around 6 teeth in the mouth: -16, -21, -24, -36, -41 and tooth -44.

We have performed PCR using Parodontose plus test reverse hybridization kit (Geni ID GmbH, Straßberg-Germany) (Ph. 1) for the detection of periodontal marker bacteria: *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* and *Treponema denticola*, with a detection limit of 10^3 as well as for detection of composite Interleukin -1 Genotype.

Auswertung: RDB2040 Parodontose Plus



Ph. I Interpretation of the results of ParodontosePlus test

Statistical analysis was done using IBM SPSS Statistics 23. Pearson Chi-Square, Fisher's Exact Test and Multiple linear regression were methods used in the statistical analysis in this study.

Statistical significance was determined for p value less than 0.05 (<0.05).

RESULTS

Analyzed clinical parameters in patients with periodontitis and descriptive statistics are showed in Table 1. The age of our patients varies in the interval $48,80 \pm 6,63$; value of dental plaque index (PI) varies in the interval $2,10 \pm 0,39$; value of gingival inflammation index (GI) varies in the interval $2,35 \pm 0,42$; values for bleeding on probing score (BOP) varies in the interval $0,81 \pm 0,22$; periodontal pocket depth (PPD) value varies in the interval $5,37 \pm 0,80$ mm, and clinical attachment level (CAL) value varies in the interval $5,52 \pm 0,78$ mm.

Table 1. Basic clinical parameters of the patients with chronic periodontitis

Parameter	Valid N	Mean	Confidence -95,00%	Confidence +95,00%	Minimum	Maximum	Std.Dev.
Age	20	48,80	45,70	51,90	37,00	60,00	6,63
PI	20	2,10	1,92	2,28	1,50	3,00	0,39
GI	20	2,35	2,15	2,55	1,80	3,00	0,42
BOP	20	0,81	0,71	0,91	0,20	1,00	0,22
PPD	20	5,37	4,99	5,74	4,00	7,00	0,80
CAL	20	5,52	5,16	5,88	4,20	7,00	0,78

Differences between the test and the control group in terms of presence of certain bacterial types are demonstrated in Table 2. There is no significant difference between the test and control group for $p > 0,05$ referring to *A. actinomycetemcomitans* ($p=0,11$). *P. gingivalis* $p < 0,001$ ($p=0,000$), *P. intermedia* $p < 0,01$ ($p=0,001$), *T. forsythia* $p < 0,001$ ($p=0,000$) and *T. denticola* $p < 0,01$ ($p=0,004$) are significantly more present in the test group.

We haven't found significant difference between the test and control group for $p > 0,05$ ($p=1,00$) for the presence of positive genotype in our patients.

Positive genotype and its association with the type of bacteria present in periodontal pockets are showed in Table 4. For $p > 0,05$ we didn't found association between positive genotype and the analyzed types of bacteria: *A. actinomycetemcomitans* ($p=1,00$), *P. gingivalis* ($p=0,16$), *T. Forsythia* ($p=0,20$), *T. Denticola* ($p=0,64$). For $\chi^2=8,17$ ($p=0,06$, $p < 0,05$) there is an association between *Prevotella intermedia*, and composite genotype.

Table 2. *Presence of certain bacterial types in patients*

Variable	Total number	Positive result (%)	p value
A.actinomycetemcomitans:			
Experimental group	20	4(20, 0%)	0, 11**
Control group	20	0(0, 0%)	
P.gingivalis:			
Experimental group	20	13(65, 0%)	0,000*
Control group	20	0(0, 0%)	
P. intermedia:			
Experimental group	20	9(45, 0%)	0,001**
Control group	20	0(0, 0%)	
T. forsythia:			
Experimental group	20	11(55, 0%)	0,000*
Control group	20	0(0, 0%)	
T. denticola:			
Experimental group	20	13(65, 0%)	0,004*
Control group	20	4(20, 0%)	

Pearson Chi-Square* Fisher s Exact Test**

Table 3. *Presence of positive (composite) genotype in patients*

Variable	Total number	Positive findings (%)	p values
Positive genotype:			
Experimental group	20	8(40, 0%)	1, 00*
Control group	20	8(40, 0%)	

Pearson Chi-Square*

Table 4. *Positive (composite) genotype and its association with bacteria types*

Variable	Positive genotype	Positive finding (%)	p value
A.actinomycetemcomitans:			
There is	8	2(25, 0%)	1, 00**
There is not		12 2(16, 7%)	
P.gingivalis:			
There is	8	7(87, 5%)	0, 16**
There is not	12	6(50, 0%)	
P. intermedia:			
There is	8	6(75, 0%)	0, 06**
There is not	12	3(25, 0%)	
T. forsythia:			
There is	8	6(75, 0%)	0, 20**
There is not	12	5(41, 7%)	
T. denticola:			
There is	8	6(75, 0%)	0, 64**
There is not	12	7(58, 3%)	

Fisher s Exact Te

Our findings for the type of bacteria present in the patients from the test group are presented in Table 5.

Bacteria: 2, 3, 4 and 5 were present in the largest percent of patients = 5 (25, 00%); bacteria:

2, 4, 5 were present in 3 patients (15, 00%); findings of other bacteria were at individual level (5, 00%); findings were negative in 5 patients (15, 00%).

Table 5. *Finding of bacterial types / Experimental group*

Finding	Count	Cumulative Count	Percent	Cumulative percent
Negative	5	5	25,00	25,00
2	1	6	5,00	30,00
1; 2; 3	1	7	5,00	35,00
1; 2; 3; 4; 5	1	8	5,00	40,00
2; 4; 5	3	11	15,00	55,00
2; 3; 4; 5	5	16	25,00	80,00
4; 5	1	17	5,00	85,00
2; 3; 5	1	18	5,00	90,00
1; 2; 3; 5	1	19	5,00	95,00
1; 4; 5	1	20	5,00	100,00
Missing	0	20	0,00	100,00

Legend: 1. *A.actinomycetemcomitans*; 2. *P.gingivalis*; 3. *Prevotella intermedia*; 4. *Tannerella forsythia*; 5. *Treponema denticola*.

DISCUSSION

Periodontitis is a chronic bacterial infection that starts when bacteria from dental biofilm cause gingival inflammation [1]. Periodontitis is among the leading causes of the tooth loss; aggressive form attacks younger population and causes serious aesthetic and functional problems such as distorted speech particularly difficulty in chewing food [2].

Composition of the microbial ecosystem and its equilibrium is a deciding factor for periodontal tissue destruction [8]. Over 1000 different microorganisms are colonising the oral cavity during a lifespan; normal balance among these microorganisms is the key factor for the oral health [6].

Most often these bacteria are settled in the oral mucosa, on hard dental surfaces, in the saliva and in the gingival fluid. These bacteria also form the dental biofilm which represents adherent, complex structure that functions as a multi-cellular organism containing over 100 different types of microorganisms per cubic millimetre [7, 8].

Approximately 300 species of bacteria have been identified in samples collected from the periodontal pocket of different individuals. Species such as: *A.actinomycetemcomitans*, *B. Forsytus*, *Campylobacter rectus*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and

Prevotella intermedia are considered periodontopathic bacteria [18]. These bacteria cause various immune responses in the host, inducing periodontal breakdown [1]. In the recent years, molecular biology techniques have been developed and PCR diagnostic techniques are an important aspect in the treatment of periodontitis [12]. Specific and sensitive detection of periopathogens in the sub-gingival plaque allow us quick identification of the high-risk patients and offer us a vital information of which type of therapeutic option we should choose for best results from the treatment [6, 11, 12]. This method is also of value during the course of periodontal treatment and it can be used to document the success of therapy during and after the treatment. During the control visits in the maintaining phase to support and continue previously achieved results from the periodontal treatment this method helps us in the early detection of the diseases repeated activation and inflammation of the dental supportive tissues [13]. It is important to note that the test should be conducted before each major prosthetic reconstruction in the oral cavity, in order to assess any possible risk posed by the placed implants. Finally, the results from PCR identification of the microorganisms can increase patient's motivation to maintain good oral hygiene. Unlike the bacterial cultivation method, PCR technique is carried out at the nucleic acid level and it doesn't depend on living bacteria presence [19, 24]. This means simple collection and transport of the sample.

The result from our study showed presence of all five periopathogen microorganisms among which the most present were *Treponema denticola* and *Porphyromonas gingivalis*, while *A.actinomycetemcomitans* was presented in four patients only (test group) which suggested that it is not characteristic pathogen for the chronic periodontitis. In the control group we only found *Treponema denticola* in four samples that clearly points that aetiology factor for periodontitis are microorganisms from the dental biofilm. The absence of the periopathogen microorganisms can prove that a certain quantum of periopathogen microorganisms in the dental biofilm is necessary for the start of the destructive processes in the supportive dental tissues [33, 34, 35, 36]. Pro-inflammatory cytokine interleukin-1 plays important role in periodontal tissue destruction by stimulating the bone destruction and participating in the production of proteases and ara-

chidonic acid, i.e. in activities that are directly connected to periodontitis.

Composite genotype was pointed by some authors as a susceptible for the periodontal disease and for that reason it is included in such a test who would have a goal to detect genetic susceptibility of a person to the periodontal disease and to allow its early detection which is important for right time and suitable therapy that will be able to prevent tooth loss [37, 38].

In our study for composite genotype presence in patients with periodontitis in Macedonian population no significant association of IL1 composite genotype (IL1 -889A:IL1B +3962) with periodontitis was found. The association was found to be significant in genotype IL1B -511/C: T, haplotype TTTCT, and haplotype TCTTT, but without significant association in IL1 composite genotype. This finding is in favor of our research where significant association between the presence of composite genotype in the participants in the test group wasn't found on the other side composite genotype was registered at 8 of the patients with periodontitis in the control group. Also we didn't found significant association between the presence of the composite genotype and any of the tested periopathogen microorganism that only additionally confirms the inability to determine the association between the composite genotype and the microorganisms that cause periodontitis [39].

The microbial community in the subgingival plaque is complex, and bacterial interactions, whether positive (synergism or commensalism) or negative (antagonism), are likely to play an important role in the development and maintenance of the members in this community and thereby influence and the severity of periodontal disease. Alexander proposed that the ability to maintain homeostasis within a microbial community increases with the species diversity [40]. Previous in vitro studies have investigated potential antagonistic microbial interactions. Bostanci et al. demonstrated that *P. gingivalis* was able to antagonize the ability of other bacterial species (including periodontal pathogens) to induce production of the proinflammatory cytokine interleukin-1 (IL-1) [41]. Other studies have shown that *P. gingivalis* was able to antagonize the ability of *Campylobacter rectus* to induce production of IL-6 and IL-8. Johansson et al. demonstrated that several periodontal bacteria are able to inhibit the activity of the *A. actinomycetemcomitans* leukotoxin; in-

deed, *P. gingivalis* exhibited the strongest inhibition of *A. actinomycetemcomitans* leukotoxicity [42]. These previous studies have demonstrated that both cytokine production and the activity of virulence determinants can be reduced when the producer organisms are part of a mixed microbial community. If polymicrobial infections are able to moderate the immune response, then it is possible that a reduction in cytokine levels combined with an inhibition of virulence factors may reduce the severity of the disease. Conversely, if a patient's microbiota is less diverse, the species present may not be subjected to the same degree of antagonistic interactions and may therefore be able to promote a greater inflammatory response leading to more severe clinical outcome. These data suggest that a reduction in the number of species present may be important in moderating the severity of periodontal diseases. Further studies are required to investigate if other microbial combinations also affect the clinical severity of periodontal diseases and also to determine the precise nature of the antagonistic interactions involved.

CONCLUSION

1. The most present bacterial species detected from subgingival dental plaque was *Treponema denticola* and *Porphyromonas gingivalis* which was present in 65% of examined patients.

2. We found that most of patients, 40% had presence of 3 types of periopathogens at the same time, 35% had 4 types of periopathogenes, and only 5 % had all five periopathogenes.

3. In relation to the presence of positive genotype in patients, there was no significant difference between the test and control group for $p > 0.05$ ($p = 1.00$)

4. For $\chi^2=8,17$ ($p=0,06$, $p<0,05$) there is an association between *Prevotella intermedia*, and composite genotype.

5. Between positive genotype and analyzed bacterial species *A.actinomycetem comitans* for $p > 0.05$ ($p = 1.00$), *P.gingivalis* for $p > 0.05$ ($p = 0.16$), *T. Forsythia* for $p > 0.05$ ($p = 0.20$), *T. Denticola* for $p > 0.05$ ($p = 0.64$) no association was found.

6. This investigations confirmed the strong association of these five examined periopathogenes with periodontitis.

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Резиме**ИДЕНТИФИКАЦИЈА НА ПЕРИОПАТОГЕНИ ОД ЗАБЕН ПЛАК
КАЈ ПАРОДОНТАЛНИ ПАЦИЕНТИ СО PCR ТЕХНИКА
И НИВНА ПОВРЗАНОСТ СО КОМПОЗИТНИОТ ГЕНОТИП ИНТЕРЛЕУКИН-1**

Анета Атанасовска Стојановска¹, Сашка Тодороска²,
Мирјана Поповска¹, Илијана Муратовска¹, Линда Зендели Беџети²

¹ Стоматолошки факултет - Универзитет Св. „Кирил и Методиј“ – Скопје

² Универзитетска стоматолошка клиника „Св. Пантелејмон“ – Скопје

Вовед: Оваа студија имаше цел да го процени присуството на главните видови микроорганизми што се вклучени во етиопатогенезата на хроничната пародонтопатија со ПБР-техника, а воедно да се одреди и присуството на композитен ИЛ-1 генотип и неговата асоцијација со детектираните бактерии.

Материјал и метод: Испитаната група се состоеше од 20 лица со дијагностицирана хронична пародонтопатија и 20 здрави, односно пациенти без пародонтопатија како контролна група. Утврдени се клинички параметри како гингивален индекс (GI), индекс на плак (PI), крвавење при сондирање (BOR), длабочина на пародонталниот џеб (PPD) и клиничко губење припој (CAL). Субгингивален дентален плак беше собран со употреба на стерилни хартиени гутаперки. Ние го користевме тестот Parodontose Plus, комплет за реверзна хибридизација, со кој се детектираат пет вида периопатогени бактерии, како и за откривање композитен генотип Interleukin -1.

Резултати: Најзастапени бактериски видови откриени во субгингивалниот плак беа *Treponema denticola* и *Porphyromonas gingivalis*, кој беше присутен кај 65 % од испитаните пациенти. Во однос на присуството на позитивен генотип кај пациентите, немаше значајна разлика меѓу испитуваната и контролната група за $p > 0,05$ ($p = 1,00$). За $\chi^2 = 8,17$ ($p = 0,06$, $p < 0,05$) постои асоцијација меѓу *Prevotella intermedia* и композитниот генотип.

Меѓу композитниот генотип и анализираниите бактериски видови *A. actinomycetem comitans* за $p > 0,05$ ($p = 1,00$), *P. gingivalis* за $p > 0,05$ ($p = 0,16$), *T. Forsythia* за $p > 0,05$ ($p = 0,20$), *T. Denticola* за $p > 0,05$ ($p = 0,64$) не е пронајдена асоцијација.

Заклучок: Овие истражувања ја потврдија силната поврзаност на овие пет испитувани периопатогени со пародонтопатијата.

Клучни зборови: пародонтопатија, периопатогени, композитен генотип