

Estimation of total bacteria by real-time PCR in patients with periodontal disease

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SUMMARY

Introduction Periodontal diseases are associated with the presence of elevated levels of bacteria within the gingival crevice.

Objective The aim of this study was to evaluate a total amount of bacteria in subgingival plaque samples in patients with a periodontal disease.

Methods A quantitative evaluation of total bacteria amount using quantitative real-time polymerase chain reaction (qRT-PCR) was performed on 20 samples of patients with ulceronecrotic periodontitis and on 10 samples of healthy subjects. The estimation of total bacterial amount was based on gene copy number for 16S rRNA that was determined by comparing to Ct values / gene copy number of the standard curve.

Results A statistically significant difference between average gene copy number of total bacteria in periodontal patients (2.55×10^7) and healthy control (2.37×10^6) was found ($p=0.01$). Also, a trend of higher numbers of the gene copy in deeper periodontal lesions (>7 mm) was confirmed by a positive value of coefficient of correlation ($r=0.073$).

Conclusion The quantitative estimation of total bacteria based on gene copy number could be an important additional tool in diagnosing periodontitis.

Keywords: periodontitis; qPCR; total bacteria

INTRODUCTION

Periodontitis is a multibacterial infection affecting tissues that surround and support the teeth. A consequence of the host inflammatory reaction in the advanced stage of the disease might be tooth loss and even risk of development of cardiovascular diseases [1]. The microbial etiology of periodontal diseases has been widely studied, and it is generally accepted that periodontitis is associated with a consortium of bacteria participating in the initiation and progression of the disease [2]. Various studies have provided evidence of several hundred recognized species of microorganisms that colonize the soft tissues of the mouth, but only a small group of pathogenic bacteria have an impact on periodontal tissue distraction [2, 3, 4]. To date, various methods have been used to detect periodontal pathogens, including cultivation, direct microscopy, enzyme tests, enzyme-linked immunosorbent assays, and molecular detection – polymerase chain reaction (PCR). As the most sensitive, the real-time PCR detection methods have been introduced in determining specific bacterial species, and their quantitative estimation in biological samples [5-9].

The development of periodontal diseases is attributed to the presence of elevated levels of pathogenic bacteria within the gingival crevice [10, 11]. Also, it has been shown that periodon-

titis patients carry a higher number of disease-associated bacteria than healthy ones [12]. Still, the significance of the relative number of total oral bacteria in the pathogenesis of a periodontal disease remains unclear.

OBJECTIVE

The aim of our study was to evaluate the total amount of bacteria in subgingival plaque samples in patients with a periodontal disease. Also, the relationship between microbiological quantitative data and clinical parameters of the periodontal disease was investigated. To our knowledge, this study is the first report of quantitative estimation of oral bacteria in subgingival plaque samples from Serbian periodontitis patients using quantitative real-time PCR (qRT-PCR).

METHODS

Twenty patients with ulceronecrotic periodontitis (mean age 30 years; 10 females and 10 males) were selected from the Clinic of Periodontology and Oral Medicine, Faculty of Dental Medicine, University of Belgrade. Each patient had at least 20 teeth present and exhibited at least eight sites with probing depths

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>4 mm and attachment loss >3 mm. Ten periodontally healthy subjects (mean age 20 years; five females and five males) constituted the control group. The criteria for excluding subjects from either analyzed group were the following: pregnancy (microbial profile is known to change in pregnancy), systemic disease that might affect immunological response, smoking, and receiving of antibiotics in the previous six months. Clinical measurements were obtained from six sites around each tooth: dichotomous measures of sulcus bleeding index, gingival index, and depth of periodontal pockets (in millimeters). The study was approved by the Ethics Committee of the Faculty of Dental Medicine, University of Belgrade (No 36/8), and all subjects gave informed consent for the collection of plaque samples and periodontal status assessment.

In both analyzed groups, the bacterial samples were collected using paper points placed in the deepest periodontal pockets in patients, or in sulcus in healthy subjects, for 30 seconds, and then transferred to tubes containing 1 ml of reduced transfer fluid (RTF). Two paper points collected from each subject were pooled in the same tube, and stored at -72°C until performing the extraction. Following the protocol, total bacterial DNA (gram-positive and gram-negative bacteria) was isolated from the samples, using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA concentrations were evaluated spectrophotometrically.

The estimation of quantity of total bacteria in the analyzed samples was done by qRT-PCR. Primers for highly conserved regions of 16S rDNA that were used in reaction of amplification were as follows: forward, EuF-5'TCCTACGGGAGCACAGT3' and reverse, EuR-5'GGACTACCAGGGTATCTAATCCTGTT3'. Amplified product of 466 base pairs (bp) was a part of 16S rDNA gene to position 331-797 in *E. coli* [13]. The amplification of target region for 16S rRNA was performed using SYBR Green PCR Master Mix, Maxima (RTC, Vilnius, Lithuania), and monitored in Line-Gene PCR (Hangzhou Bioer Technology Co., Ltd, Shanghai, China). PCR reaction mix in final volume of 25 µl contained 12.5 µl of SYBR Green master mix, 0.2 µM of each primer, and 50 ng of bacterial DNA. The temperature conditions were as follows: initial denaturation at 94°C for 10 min.; 40 cycles at 94°C (1 min.), 60°C (1 min.), 72°C (1.5 min.); and final elongation at 72°C (5 min.). To assess the specificity of amplifications, a melting curve analysis was performed. Each sample was analyzed twice.

To perform quantification by qRT-PCR, two parameters are required: the size of bacterial genome and the copy number of 16S rDNA within a cell [13, 14]. In our study, *Prevotella melaninogenica* (ATCC 25845) was used as reference strain, for obtaining a standard curve. According to genome data of *P. melaninogenica* (<http://www.ncbi.nlm.nih.gov/genome>), genome size of 3.17 Mb was converted to 3.24 fg (one genome copy). This relation was achieved using the following formula: DNA weight (pg) = genome size (bp)×1.023×10⁹ [15]. Assuming that 3.24 fg of DNA represent one cell (one genome copy), and that *Prevotella* species has a maximum of two rDNA-operons, DNA in standard samples was diluted from 32.4 fg DNA (corresponding to 20 operons of rDNA) to 32.4 ng DNA (cor-

responding to 2×10⁷ operons of rDNA). For estimation of rDNA gene copy number in tested samples, in each run standard samples were included, with a series of 10-fold dilutions of DNA from *P. melaninogenica*. Data analysis was done using Line Gene K software (Hangzhou Bioer Technology Co., Ltd, Shanghai, China). The gene copy number in each analyzed sample was determined by comparing to Ct values / gene copy number of standard curve. Statistical analyses were done using SPSS v. 17.1 software.

RESULTS

In an attempt to determine bacterial amount in multi-species population within periodontal lesions, quantification method based on DNA level was applied. The unique target sequence used for detecting all bacterial species was located within gene for rRNA. It is well known that different bacteria species possess a wide range of linked rRNA gene operon numbers (from one to 15), therefore a strict correlation between rRNA genes and a number of bacterial cells in multi-species population is impossible [13, 14, 16]. However, in the present study, measurement of total bacterial load was done by determining gene copy number for 16S rRNA.

The calculation of rDNA gene copy number in analyzed samples was based on the standard, *P. melaninogenica*, due to known fact that its genome has two rRNA operons. The results of total bacterial amount estimation performed using qRT-PCR method are shown in Table 1.

Using Mann-Whitney test we noted a statistically significant difference between average gene copy number of total bacteria in periodontal patients (2.55×10⁷) and healthy control subjects (2.37×10⁶) (p=0.01).

Table 1. Distribution of average number of total rDNA gene copies in patients with a periodontal disease

N	Total gene copy	DPT (mm)	GI	SBI
1	6.14×10 ⁵	6.3	2.1	2.1
2	4.19×10 ⁶	6.6	2.5	4.4
3	2.74×10 ⁶	6.8	2.1	5
4	1.84×10 ⁵	8.0	2.5	2.7
5	2.08×10 ⁴	8.5	2.3	3.3
6	5.46×10 ⁷	6.7	3	4.4
7	6.10×10 ⁷	9.7	3	4.1
8	4.08×10 ⁷	4.2	1.5	1.5
9	5.23×10 ⁷	5.8	2.2	1.2
10	6.34×10 ⁶	8.9	2.9	3.3
11	1.66×10 ⁸	9.3	2.4	2.3
12	2.85×10 ⁵	10.5	2.9	2.7
13	3.91×10 ⁷	11.2	3	4.6
14	4.77×10 ⁷	9.9	2.1	4.9
15	1.33×10 ⁷	8.7	2.7	5
16	6.29×10 ⁶	9.3	2.3	5
17	7.15×10 ⁶	9.8	2.6	4.5
18	3.18×10 ⁵	9.7	2.2	4.9
19	2.70×10 ⁶	8.9	3	4
20	5.37×10 ⁶	5.3	1.9	1.9

N – patient number; DPT – depth of periodontal pockets; GI – gingival index; SBI – sulcus bleeding index

Moreover, correlation between rDNA gene copy and depth of periodontal pocket showed an increased rate of average gene copy number of total bacteria in patients with the pocket deeper than 7 mm, but without statistical significance ($p=0.805$, t-test). A trend of higher numbers of gene copy in deeper periodontal lesions was confirmed by a positive value of coefficient of correlation ($r=0.073$). At the same time, two additional clinical periodontal parameters, plaque index and sulcus bleeding index, showed no association with higher gene copy number (Table 1).

DISCUSSION

Most of oral pathogens could be identified by applying the cultivation method, but some of anaerobic bacteria, such as spirochetes or *Tannerella forsythia*, fail to grow and form colony-forming unit (CFU) [16]. Applying classical PCR method, a significant subgingival prevalence of certain anaerobes at periodontal patients in our population was found [17]. Also, establishing correlation between CFU and one-cell origin is not necessary, and consequently cultivation technique leads to underestimation of the number of viable cells. In our study, we estimated the total bacterial quantity by qRT-PCR method due to its ability to detect a wide range of oral bacteria, especially anaerobic species, and to provide data on the presence of DNA in viable, as well as nonviable cells [18]. In any case, an estimation based on DNA level leads to an evaluation with a higher number of bacteria than in the cultivation method. In contrast, gene copy number of 16S rDNA in biological samples is not in strict correlation to real cell number (one cell \neq one rDNA gene), since complex multi-species population may have a wide range of rDNA linked genes (operons) (from one to 15) [14]. Therefore, quantification of total number of bacterial cells according to gene copy number of rDNA is not possible, but higher rate of these genes might be a valuable tool in assessment of their potential to multiply [19]. In summary, the advantage of defining rDNA gene copies in samples compared to actual cell number lies in their role to predict the capacity to extend microbe population.

A growing number of published clinical studies demonstrate the utility of qRT-PCR for detecting microbial pathogens [5-9, 20]. The qRT-PCR method has been proved to be helpful in the diagnosis of infectious diseases by allowing the amplification of minimum quantities of bacterial nucleic acid in patient specimens. In addition to high sensitivity and specificity, qRT-PCR provides quantitative measurements of the bacterial nucleic acids in diagnostic samples.

The results of our study showed that amount of total gene copy for rRNA (total microorganisms) in subgingival dental plaque was statistically higher in patients with a periodontal disease compared to periodontally healthy subjects. From a clinical point of view, our results might be valuable due to clear correlation between increased rDNA gene copy number and expected oral disease symptoms, as periodontal pocket formation. Nevertheless, it cannot

be stated that the increased number of total bacteria in periodontitis patients has any pathogenic implications, as we quantified total number of bacteria in subgingival dental plaque, that also include microbiota not involved in periodontal tissue distraction. Comparable to our results, Nonnenmacher et al. [21, 22] detected statistically significant higher total number of bacteria in subgingival dental plaque in periodontitis patients compared to periodontally healthy patients, using sterile paper points. Likewise, the discrepancy in total bacteria between periodontitis patients and healthy subjects was found in a study of He et al. [23], but without statistical significance in supragingival plaque and saliva samples compared with healthy samples.

In most published data, a higher total number of bacteria were identified in patients than in healthy subjects, but in a study of Abiko et al. [24] total bacterial amount in both analyzed groups was similar. Data obtained in this study might be explained by the manner of collecting subgingival plaque samples, using sterile periodontal pocket probes. There have also been in reports in other studies that the applied technique of subgingival sampling may influence the number of microorganisms [25, 26]. Moreover, in a study by Abiko et al. [24] a higher prevalence of total obligate anaerobes in periodontitis subjects was registered.

As we have expected, the total number of bacteria in subgingival plaque samples varied among all the samples in the periodontitis group of patients, since each subject, and even each pocket, has unique microbiological profile. Nevertheless, the counted number of bacteria could also depend on the anatomy of each periodontal pocket and inhomogeneous distribution of bacteria in subgingival plaque within the pocket [27]. Interestingly, as published in the study by Socransky et al. [28], in periodontally healthy subjects, subgingival microbiological profile does not change with age.

The amount of microorganisms could be an important factor for the prognosis of a periodontal disease. It could be hypothesized that the increased total number of bacteria could lead to the progression of the disease. As a confirmation for this hypothesis, we found an increased rate of average gene copy number of total bacteria in patients with pocket deeper than 7 mm. This observation could be approved by noted coefficient of correlation with positive value. The clear association between deep pockets and high levels of *P. gingivalis* and *M. micros* was reported by Nonnenmacher et al. [23]. Moreover, these findings suggest that irreversible host tissue destruction occurs when bacterial level reaches a critical threshold, forming uncontrolled circulus vitiosus of the disease. Opposite to our data, in the study of Abiko et al. [24] the correlation between periodontal pocket depth and total bacterial amount was not registered, as subgingival sampling method and anatomy of each pocket may have affected the estimation of total bacterial load in the analyzed samples.

Taken in conjunction, our results indicate that the qRT-PCR protocol described in the present study might be suitable for the bacterial quantification in subgingival plaque samples taken from periodontitis patients and periodontally healthy subjects. Thus, in addition to ordering of

specific bacterial species related to periodontitis patients, the information on their quantity may be significant for disease prevention or treatment. As it has been shown in many studies, healthy and diseased subjects share the same population of oral bacteria, but to know the cut-off value for determining patients with higher risk of developing periodontitis is of great importance. Furthermore, many studies using a broad range of subgingival plaque samples, which evaluate different periodontopathogenic species based on their quantitative presence, would be useful for understanding the complex ecology observed in periodontitis and also might provide additional approaches for the diagnosis and therapy of this disease.

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CONCLUSION

Obtained results indicate the presence of a larger number of bacteria (higher gene copy number) in patients suffering from periodontitis compared with the control group of subjects, with clinically healthy periodontium.

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Процена броја укупних бактерија применом *real-time PCR* методе код пацијената са пародонтопатијом

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КРАТАК САДРЖАЈ

Увод Пародонтопатија се повезује са постојањем повећаног броја бактерија у пародонталном џепу.

Циљ рада Циљ рада је била квантификација укупних бактерија у узорцима субгингивалног денталног плака код особа оболелих од пародонтопатије.

Методе рада У 20 узорак субгингивалног плака испитаника са улцеронекрозном пародонтопатијом и 10 узорак особа са здравим пародонцијумом извршена је квантификација укупног броја бактерија коришћењем методе *qRT-PCR*. Квантификација бактерија је заснована на одређивању укупног броја генских копија за рРНК поређењем са ЦТ вредношћу стандарда.

Резултати Установљена је статистички значајна разлика у просечном броју генских копија укупних бактерија између испитаника са пародонтопатијом ($2,55 \times 10^7$) и испитаника контролне групе ($2,37 \times 10^6$), ($p=0,01$). Такође, тренд пораста броја генских копија укупних бактерија с повећањем дубине пародонталног џепа ($>7 \text{ mm}$) потврђен је позитивном вредношћу коефицијента корелације ($p=0,073$).

Закључак Процена укупног броја бактерија на основу броја генских копија за 16S рРНК може бити важан додатни параметар у дијагностиковању пародонтопатије.

Кључне речи: пародонтопатија; *qPCR*; укупне бактерије

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