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EXPERIMENTAL WORKS

Quantitative Analysis of the Microbiota of Periodontal Pockets and Saliva by Real-Time PCR before and after Treatment of Periodontitis

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Abstract—Work has been done on standardization of detection and quantification of periodontopathogenic microorganisms in the clinical material (contents of periodontal pockets and saliva) using real-time PCR. To optimize the conditions for the analysis, a method for obtaining clinical samples of known volume was developed and a calibration sample to obtain reliable results in the diagnosis of periodontitis was designed.

Keywords: periodontitis, real-time PCR, early diagnosis, evaluation of treatment effectiveness

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INTRODUCTION

Inflammatory periodontal diseases have a high prevalence among the population and represent a serious problem, especially among adults, and are showing an upward trend in incidence, reaching 98% of cases [2, 4]. The formation of a dental plaque as a multilayer microbial biofilm with the participation of the periodontopathogenic microorganisms *Porphyromonas gingivalis* and *Treponema denticola*, as well as representatives of the resident microflora of the oral cavity *Streptococcus sobrinus*, *Streptococcus salivarius*, *Streptococcus oralis*, *Streptococcus mutans*, *Streptococcus mitis*, and *Streptococcus sanguis*, is the most important trigger factor in initiating a destructive process [14, 15]. The activity of these microorganisms promotes initiation of an infectious process characterized by loss of collagen fibers and their connection with cement of the tooth, migration of the apical epithelium, deepening of the periodontal pockets, and resorption of the alveolar bone [6, 9, 12, 13].

At present, real-time PCR is widely used in scientific and practical studies in the field of infectious disease diagnostics. The use of this method makes it possible to accurately analyze the qualitative and quantitative composition of the microbiota of periodontal pockets, identifying the DNA of the microorganisms of interest in a complex mixture of nucleic acids. Quantitative assessment of the ratio of periodontopathogens in the material under study is an important diagnostic tool, as the ratio of pathogenic and opportunistic microbiocenosis representatives of subbiotopes of the oral cavity varies significantly with the development of the disease. At present, there are no precise data on the ratio of oral microorganisms in periodontitis, which is associated with the complexity of identification and quantification of anaerobic and facultative-anaerobic microbiota. The solution of this problem is topical, since the obtained quantitative data make it possible to carry out dynamic monitoring of a patient's condition, select the most suitable etiotropic therapy, and subsequently evaluate its effectiveness.

The purpose of this study was to evaluate the change in the qualitative and quantitative composition of the microbiota of periodontal pockets and saliva in patients with chronic generalized periodontitis using real-time PCR.

MATERIAL AND METHODS

Clinical specimens. The study included 173 patients (62 males and 111 females) aged between 29 and 74, who made up the observation group. Of these, 98 (56.6%) had turned for help for the first time, while 75 (43.4%) individuals had previously been treated and visited a dentist at least once a year. According to the history of the disease, the duration of the disease had been from several months to 15 years. In terms of severity, 137 (79.2%) patients had moderate periodontitis and 36 (20.8%) patients had severe periodontitis.

Patients received a standard dental examination with determination of the form and degree of damage to periodontal tissues, after which one of the following treatment regimens was prescribed: (1) daily single administration of the antibiotic, (2) single therapeutic

impact by ultrasound using the Vector device (Durr Dental, Germany) on the surface of the dentogingival pockets and root, or (3) complex therapy, including ultrasound and antibiotic therapy. The treatment was carried out for 10 days.

The comparison group consisted of 65 almost healthy patients (25 males and 40 females) without concomitant pathology, after prophylactic sanitation of the oral cavity.

The material for molecular genetic research consisted in the contents of periodontal pockets of teeth and the oral fluid. Contents of periodontal pocketы were taken with a sterile paper endodontic point (size no. 25), which was injected with forceps into the deepest areas of the periodontal pocket for 10 s and then placed in a sterile plastic Eppendorf tube (1.5 mL) containing 1 mL of physiological saline. Sampling was carried out in duplicate for each patient. The specimens were stored and transported to the laboratory at +4°C for 2 h. Transportation of batches of samples to the laboratory was carried out in thermocontainers with refrigerant. A molecular-genetic study of patients was carried out twice: before and after 10 days of treatment according to the described scheme.

Positive control samples. Positive control samples were obtained by inserting the 16S rRNA regions of the periodontopathogenic microorganisms *Porphyromonas gingivalis*, *Treponema denticola*, *Streptococcus oralis*, *Streptococcus sanguis*, and *Streptococcus sobrinus* into the pAL-TA vector (Evrogen, Moscow), followed by transformation and development of the plasmid in *E. coli* XL1–Blue.

Determination of DNA concentration. The concentration of nucleic acids in the control materials was determined spectrophotometrically on a QUBIT fluorometer (Invitrogen, United States) using the Quant-iT DNA HS commercial set of reagents (Invitrogen, United States).

Designing a calibration sample. The calibration sample was constructed using as a DNA template the pAL-TA plasmid (3.0 kb) inserted with a section of the 16S rRNA gene of *Streptococcus sobrinus* (235 bp), resulting in the pAL-TAStrSob16S plasmid. Cloning was carried out according to T. Maniatis et al. [5]. The purity and concentration of the DNA preparation were determined by spectrophotometry using a QUBIT fluorimeter. The concentration of double-stranded DNA was 2.48 μg/mL (7.08×10^{11} DNA copies/mL).

Extraction of bacterial DNA. Bacterial DNA was isolated from 50 μL of clinical material (contents of the periodontal pocket and oral fluid) using Chelex100 ion-exchange resin.

PCR amplification. Real-time PCR was carried out using selected and tested pairs of species-specific primers to DNA sections of *Porphyromonas gingivalis*, *Treponema denticola*, *Streptococcus oralis*, *Streptococcus sanguis*, and *Streptococcus sobrinus* [7] and the SYBR Green I PCR-Mix reaction mixture (SINTOL). PCR was carried out using a CFX96 Touch REAL TIME detection amplifier (Bio-Rad, United States). The results were recorded using the Bio-Rad CFX Manager software. The instrument was calibrated with three dilutions of calibration samples prepared by serial dilution of the sample with pAL-TAStrSob16S plasmid of known concentration. The amplification reaction was carried out in 25 μL of a mixture containing 10 μL 2.5× PCR-Mix SYBR Green I reaction mixture, 9 μL ddH_2O, 2 μL each of the primer pair, and 2 μL total DNA. The amplification mode: initial denaturation at 95°C for 5 min; 35 cycles, including denaturation at 95°C for 10 s, annealing of primers at 59°C for 25 s, and elongation at 72°C for 30 s; and terminal elongation at 72°C for 30 s. Additionally, electrophoresis of PCR products was carried out in a 1.7% agarose gel in the presence of ethidium bromide.

RESULTS AND DISCUSSION

Determination of volume of the fluid absorbed by a paper endodontic point (size no. 25). Collection of the contents of periodontal pockets using sterile paper endodontic points (size no. 25) is the best way to obtain material for molecular-genetic research in periodontitis patients due to the excellent absorbent capacity of the points, as well as the possibility of collecting clinical material in a certain volume and excluding the traumatization of periodontal tissue.

Quantitative evaluation of the absorbency of the sterile endodontic point was carried out by measuring the sorption of distilled water, 0.9% aqueous sodium chloride (NaCl), and oral fluid at three different exposures: 5, 10, and 15 s. For this, using a glass graduated capillary (up to 100 μL) and a stopwatch, the volume of water absorbed by the point immersed in water for 5, 10, or 15 s was measured. The paper point was placed on a dry flat base with the test side to a capillary containing 100 μL distilled water, which was then placed at the lower end onto the test tip of the point, tightly pressing to prevent fluid leakage. The experiment was repeated in three series and in 20 repetitions for each exposure separately. After the exposure time, the volume of absorbed liquid was determined by the decrease in the volume of water in the capillary.

The average volumes of distilled water (1.045 ± 0.171 μL), 0.9% aqueous NaCl solution (1.065 ± 0.162 μL), and oral fluid (1.05 ± 0.175 μL) absorbed by the point were practically identical (Table 1). Obtained at different exposures, these volumes indicate that in 10 s the point absorbs 1 μL of liquid contents of the periodontal pocket. This amount of clinical material is sufficient for PCR analysis.

Qualitative estimation of the content of periodontopathogenic microorganisms in clinical samples. The study of the content of periodontal pockets in patients with chronic generalized periodontitis by PCR revealed a high incidence of all periodontopathogens, espe-

Table 1. The volume of fluid absorbed by endodontic paper points (size no. 25) depending on exposure (μL)

Type of fluid	Distilled water			0.9% aqueous NaCl			Oral fluid		
	1st group $M \pm \sigma$ ($n = 20$)	2nd group $M \pm \sigma$ ($n = 20$)	3rd group $M \pm \sigma$ ($n = 20$)	1st group $M \pm \sigma$ ($n = 20$)	2nd group $M \pm \sigma$ ($n = 20$)	3rd group $M \pm \sigma$ ($n = 20$)	1st group $M \pm \sigma$ ($n = 20$)	2nd group $M \pm \sigma$ ($n = 20$)	3rd group $M \pm \sigma$ ($n = 20$)
Exposure time, s	5	10	15	5	10	15	5	10	15
Average volume of fluid, μL	0.50 ± 0.11	1.045 ± 0.171*, **	1.145 ± 0.089	0.495 ± 0.116	1.065 ± 0.162*, **	1.165 ± 0.085	0.52 ± 0.132	1.05 ± 0.175*, **	1.175 ± 0.088

M is the mean, σ is the standard deviation.
* The difference with the value in the first group is significant ($p < 0.01$).
** The difference with the value in the third group is significant ($p < 0.01$).

Table 2. The frequency of PCR detection of periodontopathogenic and opportunistic bacteria in the contents of periodontal pockets and saliva in patients before treatment

Bacterial species	Observation group ($n = 173$)		Comparison group ($n = 65$)	
	PPC	saliva	PPC	saliva
	abs, %	abs, %	abs, %	abs, %
Porphyromonas gingivalis	83 (47.9)*	68 (39.3)	18 (27.7)	17 (26.2)
Treponema denticola	67 (38.7)*	54 (31.2)*	16 (24.6)	11 (16.9)
Streptococcus oralis	47 (27.2)	36 (20.8)	27 (41.5)	23 (35.4)
Streptococcus sanguis	81 (46.8)	104 (60.1)	31 (47.7)	26 (40.0)
Streptococcus sobrinus	125 (72.3)*	106 (61.3)*	29 (44.6)	27 (41.5)

* The difference with values in the comparison group is significant ($p < 0.001$).

cially the species *Streptococcus sobrinus* (72.3%), *Porphyromonas gingivalis* (47.9%), and *Streptococcus sanguis* (46.8%) (Table 2). Relative to the comparison group, the frequency of occurrence of these bacteria in the contents of periodontal pockets in patients with periodontitis was significantly higher in the species *S. sobrinus* (by 27.7%, $\chi^2 = 20.05$, $p < 0.001$), *Porphyromonas gingivalis* (by 20.2%, $\chi^2 = 16.08$, $p < 0.001$), and *T. denticola* (by 14.1%, $\chi^2 = 7.01$, $p < 0.001$).

Relatively similar data on the content of these microorganisms were obtained by molecular-genetic examination of saliva samples. The most common were *Streptococcus sobrinus* (61.3%), *Streptococcus sanguis* (30.1%), and *Porphyromonas gingivalis* (39.3%). However, the occurrence of periodontopathogenic bacteria in the saliva of patients in the observation group was significantly higher than in the comparison group only for *S. sobrinus* (by 19.8%, $\chi^2 = 17.3$, $p < 0.001$) and *T. denticola* (by 14.3%, $\chi^2 = 24.9$, $p < 0.001$).

Quantitative estimation of the content of periodontopathogenic microorganisms in clinical samples. Studies of the quantitative content of bacteria in volume-aligned clinical samples were determined by real-time PCR in a device calibrated by three dilutions of pAL-TAStrSob16S recombinant plasmid of known concentration, which allowed determining the absolute amount of the pathogenic gene-equivalent DNA in the sample (GE/sample). As clinical signs of the disease became more severe, there was a tendency toward an increase in the species composition and quantitative content of bacteria in the contents of periodontal pockets and saliva. Quantitative estimation of the content of periodontopathogenic microorganisms in the groups of patients with chronic generalized periodontitis with different treatment regimens made it possible to establish a relationship between the disruption of the balance of the periodontopathogenic and opportunistic microflora of the oral cavity and the progression of infectious inflammatory processes in the periodontal tissues.

The group of patients who had undergone systemic antibiotic therapy was observed to have a statistically significant decrease in the concentration of periodontopathogens *Porphyromonas gingivalis* in the contents of periodontal pockets (median, $10^{3.36}$ GE/sample) and saliva ($10^{4.36}$ GE/sample), as well as *Treponema denticola* ($10^{4.71}$ GE/sample) in the contents of periodontal pockets (Table 3). According to the literature data, these species of periodontopathogenic microorganisms exert the most pronounced damaging effect on periodontal tissues. It was found that the increased concentration of periodontopathogen *Porphyromonas gingivalis* in microbiocenoses of periodontal pockets correlates with the severity of the disease due to the

Table 3. The absolute amount of pathogenic and opportunistic bacteria in the contents of periodontal pockets and saliva in patients with chronic generalized periodontitis after systemic antibiotic therapy (GE/sample), $N = 55$

Bacteria	Contents of periodontal pockets		Saliva	
	before treatment	after 10 days	before treatment	after 10 days
	Me ($P_{0.05}$, $P_{0.95}$), Lg GE/sample			
Porphyromonas gingivalis	7.13 (4.05; 10.27)	3.36 (4.08; 8.46)*	9.05 (4.92; 14.3)	4.36 (2.54; 6.78)*
Treponema denticola	6.48 (3.11; 12.3)	4.71 (2.01; 8.16)*	9.26 (3.14; 13.47)	5.73 (3.47; 7.89)
Streptococcus oralis	9.84 (2.82; 12.13)	7.91 (2.56; 10.32)	9.81 (5.1; 14.01)	7.89 (4.12; 11.04)
Streptococcus sanguis	9.43 (3.57; 14.8)	7.11 (3.11; 8.67)	9.51 (4.72; 11.23)	7.03 (3.14; 10.3)
Streptococcus sobrinus	11.14 (4.18; 14.7)	7.53 (3.03; 9.52)	11.28 (4.67; 15.3)	7.41 (3.47; 10.27)

Me, median; $P_{0.05}$, $P_{0.95}$, interquartile range (5th–95th percentiles).
* Significance of differences in indices during treatment ($p < 0.001$).

Table 4. The absolute amount of pathogenic and opportunistic bacteria in the contents of periodontal pockets and saliva in patients with chronic generalized periodontitis after treatment with the Vector device (GE/sample), $N = 47$

Bacteria	Contents of periodontal pockets		Saliva	
	before treatment	after 10 days	before treatment	after 10 days
	Me ($P_{0.05}$, $P_{0.95}$), Lg GE/sample			
Porphyromonas gingivalis	9.2 (4.45; 13.04)	2.04 (1.82; 6.47)*	9.73 (5.12; 14.1)	2.15 (1.47; 5.78)*
Treponema denticola	10.89 (5.23; 12.81)	3.46 (2.57; 6.14)*	10.83 (5.14; 14.32)	3.59 (1.98; 8.47)*
Streptococcus oralis	9.94 (7.44; 15.86)	6.97 (4.7; 10.21)*	10.85 (7.41; 15.89)	7.83 (3.45; 9.04)*
Streptococcus sanguis	9.83 (6.57; 16.32)	7.52 (4.65; 9.74)	9.74 (7.47; 14.58)	7.62 (4.21; 9.86)*
Streptococcus sobrinus	10.18 (8.56; 15.32)	7.26 (4.36; 9.87)*	10.37 (7.69; 16.02)	8.18 (4.32; 10.5)

Me, median; $P_{0.05}$, $P_{0.95}$, interquartile range (5th–95th percentiles).
* Significance of differences in indices during treatment ($p < 0.001$).

increased adhesive, invasive, and toxic properties of the microorganism [3, 8, 9, 11].

The study of the microbiocenosis of periodontal pockets and saliva in the group of patients treated with ultrasound using the Vector device showed a significant decrease in the absolute number of several periodontopathogens: *Porphyromonas gingivalis* both in the contents of periodontal pockets (median, $10^{2.04}$ GE/sample) and saliva ($10^{2.15}$ GE/sample), *Treponema denticola* in the contents of periodontal pockets ($10^{3.46}$ GE/sample) and saliva ($10^{3.59}$ GE/sample), *Streptococcus oralis* in the contents of periodontal pockets ($10^{6.97}$ GE/sample) and saliva ($10^{7.83}$ GE/sample), *Streptococcus sanguis* only in saliva ($10^{7.62}$ GE/sample), and *Streptococcus sobrinus* only in the contents of periodontal pockets ($10^{7.26}$ GE/sample) (Table 4). The analysis of the clinical effectiveness of the Vector device showed that, at the end of therapy, the patients with chronic generalized periodontitis showed an improvement in the condition of periodontal tissues, which was characterized by the absence of such symptoms as soreness and bleeding of gums and detachment of periodontal pockets. The data obtained are consistent with several studies showing the high clinical effectiveness of using the Vector device in the treatment of patients with inflammatory periodontal diseases [1, 7, 10].

The inclusion of antibiotic therapy in ultrasound treatment of patients with chronic generalized moderate and severe periodontitis not only significantly reduced the overall bacterial load on periodontal tissue, but also significantly reduced the frequency of detection and quantitative content of all the studied periodontopathogens in the periodontal pockets and saliva (Table 5).

The end of therapy was marked with shorter periods of the reduction of inflammatory processes in the gum and the achievement of stable remission. Consequently, therapeutic ultrasound treatment using the Vector device on the surface of the root and dentogingival pockets in combination with antibiotic therapy leads to eradication of, or a significant decrease, in the absolute number of periodontopathogenic microorganisms, as well as restoration of the physiological function of periodontium.

Thus, we have carried out work on standardization of detection and quantitative determination of periodontopathogenic microorganisms in the clinical material (contents of periodontal pockets and saliva) by real-time PCR. To optimize the conditions for the

Table 5. The absolute amount of pathogenic and opportunistic bacteria in the contents of periodontal pockets and saliva in patients with chronic generalized periodontitis after using the combined treatment, Vector + antibiotic therapy (GE/sample), $N = 71$

Bacteria	Contents of periodontal pockets		Saliva	
	before treatment	after 10 days	before treatment	after 10 days
	Me ($P_{0.05}$, $P_{0.95}$), Lg GE/sample			
Porphyromonas gingivalis	9.02 (4.23; 15.02)	4.76 (2.14; 7.47)*	9.03 (7.45; 14.63)	4.0 (2.31; 7.04)*
Treponema denticola	10.4 (5.47; 14.29)	4.68 (3.04; 8.14)*	10.62 (5.13; 15.21)	4.92 (2.65; 8.01)
Streptococcus oralis	13.15 (8.1; 16.48)	7.99 (5.47; 9.64)*	13.38 (7.45; 15.68)	7.23 (3.54; 8.67)*
Streptococcus sanguis	14.92 (8.64; 17.01)	6.46 (3.78; 8.48)*	14.15 (8.09; 15.64)	6.63 (4.15; 8.57)*
Streptococcus sobrinus	13.92 (7.12; 17.14)	5.45 (2.31; 7.08)*	13.18 (7.35; 16.45)	5.64 (3.56; 8.45)

Me, median; $P_{0.05}$, $P_{0.95}$, interquartile range (5th–95th percentiles).
* Significance of differences in indices during treatment ($p < 0.001$).

analysis, a method for obtaining clinical samples of known volume has been developed and a calibration sample to obtain reliable results in the diagnosis of periodontitis has been designed. In the future, this method will make it possible to diagnose the disease at early stages, as well as to assess the dynamics of the development of the infectious process in the tissues of periodontium.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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