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HETEROGENEITY OF VIRULENCE FACTORS AMONG *PORPHYROMONAS GINGIVALIS* CLINICAL ISOLATES FROM PATIENTS WITH CHRONIC GENERALIZED PERIODONTITIS

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Abstract. The development of chronic generalized periodontitis occurs due to a combination of a several causes, among which the leading role is assigned to periodontal pathogens, which include *P. gingivalis*. Among *P. gingivalis* virulence factors, the polysaccharide capsule, fimbria proteins, cysteine proteases, and hemagglutinins are of special importance. The study was aimed to investigate the prevalence of specific virulence genes and identify a virulent genotype among *P. gingivalis* isolates found in patients with severe chronic generalized periodontitis (CGP). 41 patients (27 women and 14 men, average age 43.9±1.5 years) were examined, of which main and control group consisted of 22 patients with severe CGP and 19 patients without inflammatory periodontal diseases, respectively. The PCR data allow to consider type II fimbria (FimA II), arginine-dependent type A protease (RghA) and lysine-dependent protease (Kgh) as specific markers for the detection of more virulent *P. gingivalis* strains. It was found that in St. Petersburg, the following *P. gingivalis* genotypes predominate among patients with severe CGP: *fimA* II:*kg*:*rghA*, *fimA* II:*kgh* and *fimA* II:*rghA*. In addition, it has been demonstrated that virulent genotypes are detected to a small extent in *P. gingivalis* isolates from healthy control group patients. The identification of *P. gingivalis* strains with a more prominent pathogenic potential and the detection of their virulent genotypes is of great practical importance, in the future allowing to develop advanced effective methods for disease prevention to be used in a personalized medicine strategy. The results obtained are also of high importance due to the recorded variability in the circulation of *P. gingivalis* strain genotypes in various worldwide regions.

Key words: *chronic generalized periodontitis, heterogeneity, virulent genotype, P. gingivalis, fimbriae, cysteine proteases.*

ГЕТЕРОГЕННОСТЬ ФАКТОРОВ ВИРУЛЕНТНОСТИ СРЕДИ КЛИНИЧЕСКИХ ИЗОЛЯТОВ *PORPHYROMONAS GINGIVALIS*, ВЫДЕЛЕННЫХ ОТ ПАЦИЕНТОВ С ХРОНИЧЕСКИМ ГЕНЕРАЛИЗОВАННЫМ ПАРОДОНТИТОМ

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Резюме. Развитие хронического генерализованного пародонтита обусловлено сочетанием целого ряда причин, среди которых ведущая роль отводится пародонтопатогенам, к которым относится *P. gingivalis*. Среди

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факторов вирулентности *P. gingivalis* особенно выделяют полисахаридную капсулу, белки фимбрий, цистеиновые протеазы, гемагглютинины. Цель исследования — изучение распространенности специфических генов вирулентности и определение вирулентного генотипа среди изолятов *P. gingivalis*, выявляемых у пациентов с хроническим генерализованным пародонтитом тяжелой степени (ХГП ТС). Было проведено обследование 41 пациента (27 женщин и 14 мужчин, средний возраст 43,9±1,5 лет), из которых основную группу составили 22 пациента с ХГП ТС и контрольную группу составили 19 пациентов без воспалительных заболеваний пародонта. Результаты, полученные с использованием ПЦР, позволяют рассматривать фимбрии II типа (FimA II), аргинин-зависимую протеазу А типа (RghA) и лизин-зависимую протеазу (Kgh) в качестве специфических маркеров для обнаружения более патогенных штаммов *P. gingivalis*. Установлено, что в Санкт-Петербурге среди пациентов с ХГП ТС преобладают следующие генотипы *P. gingivalis*: *fimA* II:*kgh*:*rghA*, *fimA* II:*kgh* и *fimA* II:*rghA*. Кроме того, было продемонстрировано, что вирулентные генотипы выявляются в незначительной степени в изолятах *P. gingivalis* от здоровых пациентов контрольной группы. Идентификация штаммов *P. gingivalis* с более выраженным патогенным потенциалом и обнаружение их вирулентных генотипов имеет важное практическое значение, позволяя в будущем разработать современные эффективные методы профилактики заболевания и использовать их в стратегии персонализированной медицины. Полученные результаты также представляют высокую значимость в связи зарегистрированной вариабельностью циркуляции генотипов штаммов *P. gingivalis* в различных регионах мира.

Ключевые слова: *хронический генерализованный пародонтит, гетерогенность, вирулентный генотип, P. gingivalis, фимбрии, цистеиновые протеазы.*

Introduction

Damaging effect of dental plaque plays a leading role in the development of inflammatory periodontal diseases. The today's science views dental plaque as a biofilm consisting of a structured bacterial community and their metabolic products [1, 17]. During the formation of dental plaque, the composition of microbiota takes a trend to change from the dominance of aerobic and facultative anaerobic forms to obligate anaerobic gram-negative rods and spiral-shaped bacteria [2, 15].

According to the "keystone pathogen hypothesis" theory *P. gingivalis* is considered to be a key periodontal pathogen [9, 10]. Well described the ability of *P. gingivalis* to have an impact on the innate immune system of the host organism resulting in dysbiotic changes in the composition of microbiota and, therefore, in an inflammatory response of periodontal tissues [11, 20]. Thus, *P. gingivalis* is rightfully considered a "keystone pathogen" agent and, even at low concentrations, it is capable of causing chronic periodontitis [9, 10, 12, 21]. Since *P. gingivalis* belongs to obligate anaerobes, the main habitat of this periodontal pathogen is the periodontal pocket. *P. gingivalis* is a secondary plaque colonizer and often forms colonies with *S. gordonii* and *P. intermedia* [12]. In addition, as Socransky "ecological theory" states, *P. gingivalis* forms a complex with *T. denticola* and *T. forsythia* [8]. This combination of microorganisms is detected in the most severe stages of chronic periodontitis. This "red complex" aggressively affects bone tissue and gum mucosa, forming deep periodontal pockets and causing severe destruction of jaw bone tissue [24, 28].

In recent years, it has become obvious that in the case of inflammatory periodontal diseases, there are factors that enhance some mechanisms of disease development making its course more severe. Differences in the course and disease development rate are due to the diversity of the composition and degree of periodontopathogen strains virulence [5, 23]. Scientists' views about the influence of genetic diversity of *P. gingivalis* strains on the development and course of inflammatory periodontal diseases are contradictory. It is believed that *P. gingivalis* strains differ in the degree of virulence in patients with different periodontitis currents, affecting the clinical course of the disease. Experimental and clinical studies have revealed differences in the pathogenic potential of *P. gingivalis* strains, dividing them into "invasive" and "non-invasive" strains [7, 21, 22]. On the other hand, there is an idea that the course of periodontitis is determined not by the diversity of strains of periodontal pathogens, but by the individual characteristics of the host organism reactivity [7]. Moreover, a number of studies have shown the availability of identical strains of *P. gingivalis* in patients with intact periodontium and with inflammatory periodontal diseases [26].

P. gingivalis is known to express many virulence factors: fimbriae, lipopolysaccharides, arginine- and lysine-dependent proteases, hemagglutinins and capsular polysaccharide, which genes are very diverse [3, 16, 27]. Fimbriae of *P. gingivalis* are classified into six genotypes — I, Ib, II, III, IV, V, that was demonstrated by a variation in the nucleotide sequence of the *fimA* gene encoding FimA (fimbryonic subunits) [6]. *P. gingivalis* with type I fimbriae has been shown to be associated with patients without inflammatory periodontal diseases [4]. On the other hand, there is an evidence that fimbriae of *P. gingivalis* with genotype II have greater adhesive ability and, therefore, greater virulence compared to other genotypes [6]. Pathogenic heterogeneity of *P. gingivalis* strains with type II fimbriae has been demon-

strated, that determines the proteolytic and invasive activity of this periodontopathogen [14]. At the same time, it is believed that there are no significant differences between *P. gingivalis* strains with different genotypes of fimbriae regarding virulence [26].

The issue of genetic heterogeneity of *P. gingivalis* and its virulence factors is being actively studied, and the scientific views about the role of those factors in the formation of a virulent genotype are contradictory. Therefore, the further need to examine the genetic heterogeneity of *P. gingivalis* in patients with inflammatory periodontal diseases makes this research relevant. To study genetic heterogeneity of fimbriae and cysteine proteases of *P. gingivalis* strains in patients with chronic generalized periodontitis, as well as deriving characteristics of the virulent genotype of *P. gingivalis* is very perspective. In addition, it is of interest to examine the prevalence of the virulent genotype among *P. gingivalis* strains isolated from patients without inflammatory periodontal diseases.

The purpose of the research: to study the prevalence of specific virulence genes and identify the virulent genotype among *P. gingivalis* strains detected in patients with severe chronic generalized periodontitis.

Materials and methods

A research was conducted on 41 patients (27 women and 14 men) aged 36 to 50 years (average age was 43.9±1.5 years). The patients were divided into two groups. The first group included 22 patients with severe chronic generalized periodontitis (CGPS). The control group consisted of 19 patients without inflammatory periodontal diseases (IPD). To form a control group, 69 patients without IPD were preliminarily examined and those patients in whom *P. gingivalis* was identified were selected.

In the course of research, a clinical examination of patients was performed, including collection of anamnesis, complaints, assessment of dental status, index assessment of the periodontal tissue condition (OHI-S index, Green, Vermillion, 1964; Silness-Loe index, Silness, Loe, 1964; CPITN index, WHO, 1978, Ainamo et al., 1982; PMA index, Parma S., 1960; BOP index, Ainamo, Bau, 1975), as well as microbiological examination of material from the periodontal sulcus or periodontal pockets of the examined patients using PCR diagnostics. X-ray examination included assessment of data obtained from GALILEOS cone-beam computed tomography (Sirona, Germany).

Material was collected from periodontal pockets in patients with CGPS (main group) and from the periodontal sulcus in healthy patients (control group) using sterile paper absorbents Absorbent Paper Points from Euronda, size No. 25. Paper absorbents were placed in the gingival sulcus or periodontal pockets for 7–10 seconds, after which they were immediately transferred into sterile sealed Eppendorf tubes and stored at –50°C. A special cooling device was used to transport the material to maintain storage conditions.

To isolate DNA, we used the Express-DNA-Bio kit in accordance with the instructions. The resulting DNA samples were stored at –20°C until polymerase chain reaction (PCR) was performed.

Primer 3 and OLIGO 4.0 computer programs was used to perform the design, analysis of oligonucleotide primers and identification of the primer melting temperature (Table 1). Primers to identify *fimA* I and IV genes were used from the publication of Takashi Yoshino et al. [25].

The data obtained in the course of the research were processed on a personal computer using the software system "Statistica for Windows" (v. 7.0). To visualize the results of the study, diagrams were constructed in Microsoft Excel.

Results

All patients of the main group complained of bleeding gums during brushing, tooth mobility, swelling and inflammation of the gums (Fig. 1). In 59.1% cases, patients in the main group complained of food getting between the teeth, as well as halitosis. Patients with CGPS in 72.73% cases marked the displacement of teeth resulting from a functional secondary traumatic occlusion and in 68.18% cases — itching and gum area burning caused by the inflammatory edema of periodontal tissue. 2 patients in the control group had complaints only about food

Table 1. Oligonucleotide primers

	Name	5' → 3'	T0ann.	DNA fragments size (bp)
1	**Gin1**	GTATATGCTCGACGAGGTGGAA	57.0	334
2	**Gin2**	ATTGTCCAGGGTAACTTCTTCG		
3	**Fim II1**	TGTTGCAGACAATAATCCTAC	51.0	250
4	**Fim II2**	CGATTACCAAGTAGCATTCTGA		
5	**Kgp1**	TCCACTTCTGACCACATCTCAA	56.0	397
6	**Kgp2**	AGCTTCCCGATAGTAATGAGCA		
7	**RgpA1**	AATCCCGGAACAACAACACTTT	56.0	331
8	**RgpA2**	TGAAGTTGGATGCATCGTTACC		

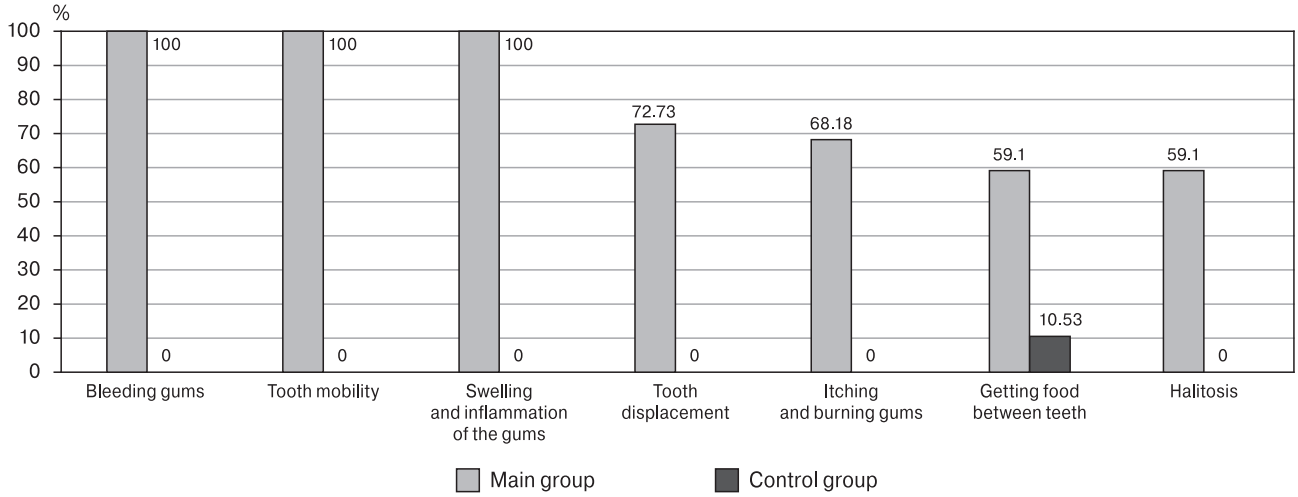

Figure 1. Complaints from patients in the main and control groups

getting between the teeth owing to lack of a contact point in the area of several tooth pairs in the maxilla and mandible.

Hyperemia of the marginal and attached gums, and exudation from periodontal pockets has been revealed in all patients of the main group. Gingival recession has been found in all patients with CGPS, the average value amounted to 1.96±0.07 mm. Lesions of the furcation in the area of the molars of the maxilla and mandible were detected in 15 patients (68.2% of cases). Tooth mobility has been revealed in all patients of the main group, most often grade 1–2.

The values of the OHI-S hygiene index in patients with CGPS in the main group were 4.77±0.12, in the control group — 0.44±0.04 (Fig. 2).

The obtained values of the OHI-S index indicate poor oral hygiene in patients of the main group that is typical for patients with CGPS who lack the correct skills to practice individual oral hygiene. Statistically significant differences have been found in the Silness-Loe index between patients of the main and control groups indicating a cause-and-effect correlation between the mineralized supra- and subgingival dental plaque and inflammation in the periodontal tissues ($p < 0.001$).

The PMA index and BOP bleeding index rates in patients of the main group indicate inflammatory phenomena in periodontal tissues. A distinct inflammatory-destructive process in periodontal tissues in patients of the main group was confirmed by the rates of the periodontal index. The rates of the CPITN index in patients with CGPS of the main group reached 3.97±0.23. High rates of the CPITN index for patients in the main group indicate the need for complex treatment, including surgical treatment of periodontal diseases.

The results obtained during cone-beam computed tomography in patients of the main group correspond to the clinical picture and the diagnosis of CGPS. In all patients of the main group, destruction of the compact lamina of the alveolar bone was revealed

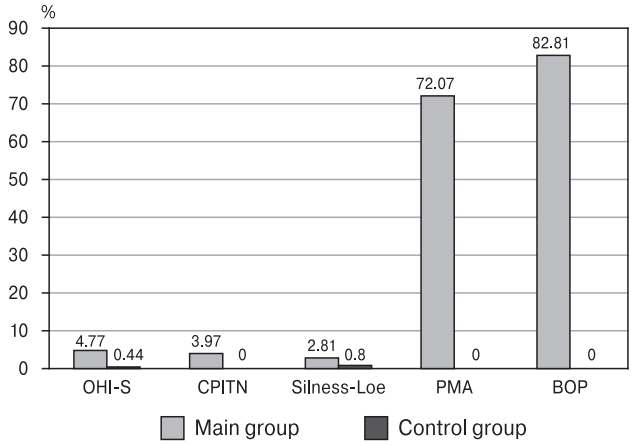

Figure 2. OHI-S, CPITN, Silness-Loe, PMA (%) и BOP (%) index rates in patients of the main and control groups

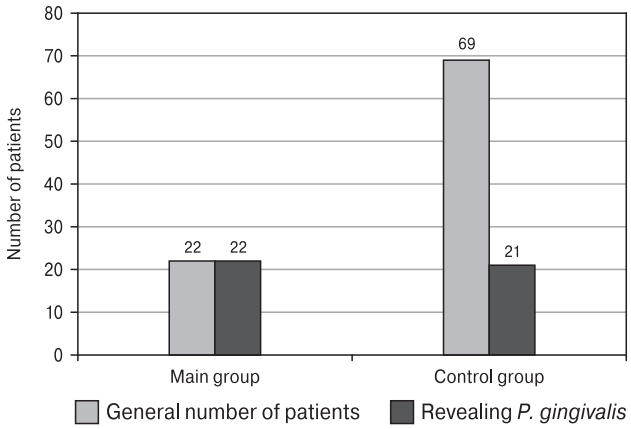

Figure 3. Frequency of *P. gingivalis* occurrence in periodontal pockets/gingival sulcus in patients of the main and control groups

along its entire length, and bone pockets were identified in the area of 15.2±3.7 teeth. The amount of bone tissue destruction "more than $^1/_2$ the length of the root" was identified in all patients with CGPS.

The contents of the periodontal pockets in patients of the main group and the subgingival sulcus in patients in the control group have been examined to reveal *P. gingivalis* using PCR screening (Fig. 3).

In the main group, the periodontopathogen *P. gingivalis* has been detected in all patients (100% cases). To form a control group, 69 patients without inflammatory periodontal diseases (inflammatory periodontal diseases) were previously examined, among whom *P. gingivalis* was detected in 21 patients (30.4% of cases).

22 *P. gingivalis* isolates of the main group and 19 isolates of the control group were examined by PCR to reveal genes encoding fimbriae types I, II, and IV (Fig. 4).

The results indicate the predominance of the *fimA* II genotype (40.9% of cases) among the *P. gingivalis* isolates of the main group. The *fimA* I genotype is detected quite often as well, in 22.7% cases, while the prevalence of the *fimA* IV genotype was insignificant (9.1% of cases). Thus, among *P. gingivalis* isolates from patients with CGPS, strains carrying fimbriae of types I and II predominantly circulate. Among the 19 *P. gingivalis* isolates of the control group, *fimA* II also dominates (31.6% cases), the prevalence of which is only by 22.7% lower compared to the prevalence rate of *fimA* II in the main group. The *fimA* I genotype in *P. gingivalis* isolates of the control group is found quite rarely (5.3% of cases), and the *fimA* IV genotype is not available at all.

The gene *(kgh)*, encoding a lysine-dependent protease, in *P. gingivalis* isolates of the main group is detected in 68.2% cases, while the gene (*rghA*), encoding an arginine-dependent protease type A, is detected in *P. gingivalis* isolates of all patients (100% cases) (Fig. 5).

When comparing the corresponding genes in control group *P. gingivalis* isolates, a significant reduction in the prevalence of *kgh* and *rghA* was revealed, to 26.3% and 47.4% cases, respectively.

Table 2 shows the prevalence of various genotypes of the studied virulence factors among *P. gingivalis* isolates of the main and control groups.

When considering three virulence factors, *P. gingivalis* isolates with genotype *fimA* II:*kgh:rghA* (27.3% cases) and *fimA* I:*kgh:rghA* (18.2% cases) dominate the main group. Both of those genotypes are present in almost half (45.5% cases) of *P. gingivalis* isolates of the main group that suggests a significant contribution of *P. gingivalis* with those genotypes in the development of chronic generalized periodontitis. In *P. gingivalis* isolates of the control group, genotypes *fimA* II:*kgh:rghA* and *fimA* I:*kgh:rghA* are found quite rare, in 5.3% cases. When considering different genotypes from two virulence factors

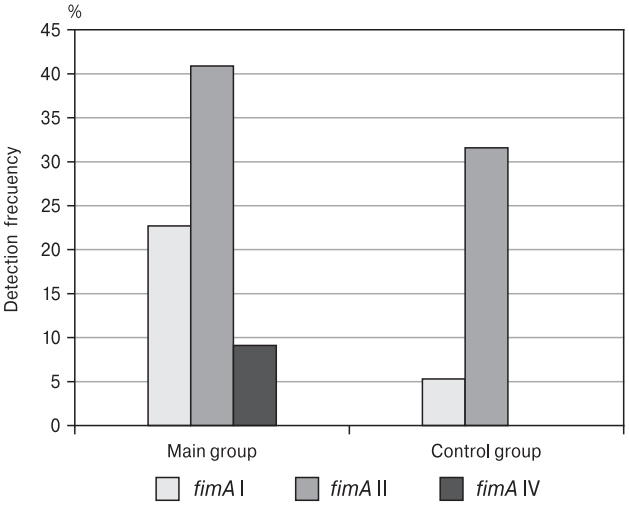

Figure 4. Prevalence of fimbriae types I, II and IV genes (*fimA* I, *fimA* II and *fimA* IV) among *P. gingivalis* isolates in patients of the main and control groups

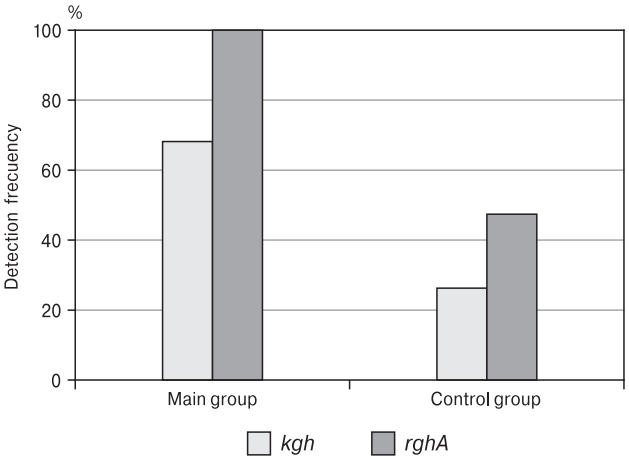

Figure 5. Prevalence of lysine-dependent protease *(kgh)* and arginine-dependent protease type A *(rghA)* genes among *P. gingivalis* isolates in patients of the main and control groups

Table 2. Distribution of fimbria types I, II and IV (*fimA* I, II, IV), lysine-dependent protease *(kgh)* and arginine-dependent protease type A *(rghA)* genotypes

Genotypes	Prevalence (%)	
	Main group	Control group
fimA I:kgh:rghA	18.2	5.3
fimA II:kgh:rghA	27.3	5.3
fimA IV:kgh:rghA	9.1	0
fimA I:kgh	18.2	5.3
fimA I:rghA	22.7	5.3
fimA II:kgh	27.3	5.3
fimA II: rghA	40.1	18.2
fimA IV:kgh	9.1	0
fimA IV:rghA	9.1	0

in *P. gingivalis* isolates of the control group, genotypes *fimA* II:*rghA* (40.1% cases), *fimA* II:*kgh* (27.3% cases) and *fimA* I:*rghA* (22.7% cases) predominate. In *P. gingivalis* isolates from the control group, the *fimA* II:*rghA* genotype also predominate (18.2% cases). The remaining genotypes listed above are found rare in *P. gingivalis* isolates from the control group, in 5.3% cases.

Discussion

Among the virulence factors of *P. gingivalis*, the polysaccharide capsule, fimbriae proteins, arginine- and lysine-dependent proteases, and hemagglutinins are particularly distinguished. In the work in question, the heterogeneity of genes encoding fimbriae types I, II and IV and arginine- and lysine-dependent proteases was studied in clinical isolates of *P. gingivalis* obtained from healthy patients and patients with CGPS. It has previously been demonstrated that *P. gingivalis* is isolated from healthy patients in 10–25% cases [13]. A higher percentage of *P. gingivalis* detection was reported in the dental plaque of healthy Japanese patients, up to 36.8% cases [4]. A study of samples obtained from the subgingival sulcus of 69 healthy patients in St. Petersburg demonstrates a high percentage of carriage of *P. gingivalis*, up to 30.4% cases. The prevalence of *P. gingivalis* in patients of the main group amounted to 100% cases that fully correlates with previous studies [13, 18]. Among *P. gingivalis* fimbriae, six genotypes of *fimA* have been described, of which genotypes II and IV have been characterized as the most common ones among *P. gingivalis* isolates received from patients with IPD (inflammatory periodontal disease) in Sweden and Japan [4, 25]. The remaining genotypes of fimbriae were detected quite rarely [25]. In agreement with previous studies, among the three types of arginine-dependent proteases, type A arginine-dependent protease (RghA) was found to be dominant. Among lysine-dependent Kgh proteases, both genotypes I and II occurred with approximately the same frequency [25].

When studying the prevalence of virulence factors in the main group, it has been demonstrated that genes encoding type II fimbriae (40.9% cases) and type A arginine-dependent protease (100% cases) were present in the first place by frequency of occurrence. The obtained data suggest a direct correlation between CGPS and *P. gingivalis* isolates carrying the *rghA* gene (r = 1.0). The lysine-dependent protease gene *kgh* is detected in 68.2% of cases. In *P. gingivalis* isolates from healthy patients, type II fimbriae and type A arginine-dependent protease were present in 31.6% and 47.4% of cases, respectively. Lysine-dependent protease Kgh is detected in 26.3% of cases. The presented results suggest that *P. gingivalis* strains producing fimbriae FimA II, as well as Kgh and RghA, have a more pronounced pathogenic potential. Thus, one can consider those virulence factors as markers for detecting more pathogenic strains of *P. gingivalis*.

Comparing data on the prevalence of *P. gingivalis* virulence factors in the St. Petersburg with previously published data for Sweden, it's safe to say that type II fimbriae are most often found in *P. gingivalis* isolates from patients with IPD. However, it is worth noting that *P. gingivalis* isolates with type II fimbriae are recorded more often in Swedish patients (71% cases) compared to patients from St. Petersburg (40.9% cases). Differences in *P. gingivalis* isolates from St. Petersburg are also revealed when identifying type I and IV fimbriae. In *P. gingivalis* isolates from Swedish patients, the second most common finding was *P. gingivalis* with type IV fimbriae (16.1% cases). In the St. Petersburg, *P. gingivalis* with type IV fimbriae from patients with IPD are rarely found (9.1% cases); moreover, *P. gingivalis* isolates with type IV fimbriae from healthy patients are not recorded at all. On the other hand, in St. Petersburg, isolates of *P. gingivalis* with type I fimbriae are more often detected from patients with IPD, in 22.7% cases. The relatively frequent detection of *P. gingivalis* isolates with type I fimbriae (19.6% cases) from Brazilian patients with IPD has been previously published [19]. In comparison, type I fimbriae was rarely detected in *P. gingivalis* isolates from Swedish patients (4.8% of cases). In addition, all *P. gingivalis* isolates from St. Petersburg patients with IPD carry the gene for arginine-dependent protease type A, while in *P. gingivalis* isolates from Swedish patients with IPD the gene for the mentioned protease is found less frequently, in 75.8% cases. In summary, a study of clinical isolates of *P. gingivalis* from patients with IPD demonstrates significant regional differences in the prevalence of individual *P. gingivalis* virulence factors. The findings highlight the significance of such studies for specific regions.

When analyzing genotypic variants for the studied virulence factors of *P. gingivalis* in the main group, *fimA* II:*kgh*:*rghA* (27.3% cases), *fimA* II:*kgh* (27.3%) and *fimA* II:*rghA* (40.1% cases) are most common. Those results allow taking into consideration the above-mentioned gene combinations as virulent genotypes of *P. gingivalis*. It is important to mention that virulent genotypes were found in *P. gingivalis* isolates from healthy control patients (*fimA* II:*kgh*:*rghA* and *fimA* II:*kgh* — in 5.3% cases; *fimA* II:*rghA* — in 18.2% cases).

Identification of *P. gingivalis* strains with more distinct pathogenic potential and detection of their virulent genotypes is of great practical importance. Using obtained clinical and microbiological data, an in-depth research of the etiology of IPD, development and implementation of up-to-date efficient methods to prevent the disease are possible. In addition, the detection of virulent genotype strains among control group patients allows predicting that some healthy patients have an increased susceptibil-

ity to developing periodontitis. Keeping that knowledge in mind the dentist will be able to prevent inflammatory periodontal diseases in advance, develop individual oral hygiene for the patient and give appropriate recommendations.

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