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Review

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The role of microbiological analysis as a diagnostic tool of periodontitis: a clinical study

Znaczenie badania mikrobiologicznego w diagnostyce zapalenia przyzębia – badanie kliniczne

Agata Orzechowska[A, B, C, D, E], Bartłomiej Górski[C, D, E, F], Renata Górska[A, D]

[1] Department of Periodontology and Oral Diseases, Medical University of Warsaw, Poland

Summary

Aim: Oral bacteria take the form of biofilms, which – due to their complex structure – are characterized by high resistance to many external factors. The aim of the study was to analyze the composition of subgingival biofilm in pockets ≥7 mm in an effort to answer the question whether the presence of selected bacteria of the red complex and *Aggregatibacter actinomycetemcomitans* may determine the diagnosis in the context of the new classification of periodontal diseases.

Material/Methods: The study included two groups of patients: 30 individuals with periodontitis Grade B (Stage III or IV) and 30 subjects with periodontitis Grade C (Stage III or IV). Each patient had at least one PPD ≥ 7 mm. The study group consisted of 26 males and 34 females, mean age 43.8 ± 15.2 years. Selected periodontal parameters were evaluated as well as microbiological quantitative and qualitative analysis was performed regarding bacterial flora in the pockets for the presence of *Porphyromonas gingivalis*, *Treponema denticola* and *A. actinomycetemcomitans.*

Results: The total number of bacteria was higher in patients with periodontitis Grade B, as compared to periodontitis Grade C. The number of *P. gingivalis* was higher in patients with periodontitis Grade B at 157.4*10^3 versus 139.9*10^3 in Grade C patients (p = 0.033). There was no difference in the occurrence of *T. denticola* depending on the diagnosis (p = 0.228). The presence of *A. actinomycetemcomitans* was confirmed only in four patients with periodontitis Grade C (p = 0.186). A moderate correlation was observed between the occurrence of *P. gingivalis* and *T. denticola* in patients with periodontitis Grade B (r = 0.51, p = 0.004).

Conclusions: The composition of subgingival biofilm was very similar in patients with periodontitis Grade B and periodontitis Grade C. We may speculate that periododntitis grade may not be distinguished according to a simple analysis of subgingival plaque with respect to *P. gingivalis, T. denticola* and *A. actinomycetemcomitans.*

Keywords: **dysbiosis • correlation • periopathogens • periodontitis**

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Author's address: Agata Orzechowska DDS PhD, Department of Periodontology and Oral Diseases, Medical University of Warsaw, Miodowa St 18, 00-246 Warsaw; e-mail: agata.j.orzechowska@gmail.com

INTRODUCTION

Periodontitis belongs to complex diseases whose clinical characteristics depend on the interaction of environmental factors with many genes. Among the risk factors that increase the probability of periodontitis are unmodifiable factors (age, gender, race, genetic polymorphisms) and modifiable ones (periopathogens, general diseases, nicotinism, obesity, socioeconomic status, stress) [3]. Undoubtedly, the most important environmental factors are periopathogens in the dental biofilm. The interaction of many gene loci with periopatogens present in the oral cavity and with other environmental factors leads to the development of periodontitis.

The oral cavity is inhabited by more than 700 species of bacteria in the form of biofilms, i.e. multi-microbial communities attached to surfaces of dental tissues or dental materials [10]. Biofilms have a complex structure and due to their high metabolic order they are characterized by high resistance to many external factors. Immediately after tooth brushing formation of supragingival biofilm – called dental plaque – begins to form on the enamel surface. If dental plaque is not removed mechanically, it starts to colonize the subgingival area and triggers a gingival inflammatory reaction. With the deepening of the pocket, subgingival biofilm is formed on the cement surface that is even better organized than supragingival biofilm. Biofilm on the root surface consists of tightly adhering bacteria and matrix. In the periodontal pockets, in addition to the organized biofilm on root surfaces, planktonic phase is also present.

Various oral microbiota have been found associated with periodontitis. In 1998, Socransky et al. [40], on the basis of molecular identification of 13.261 subgingival biofilms obtained from 185 adults, selected 40 species of bacteria which were divided into seven complexes differing in the degree of pathogenicity towards periodontitis. Periopathogens occurring in periodontitis are located in the orange complex (predominant in periodontal pockets 4–6mm deep), and the most virulent ones (*P. gingivalis, T. denticola, T. forsythia*) in the red complex in periodontal pockets over 6 mm, and the dark green complex (*A. actinomycetemcomitans*). Periopathogens demonstrate pathogenicity only when the host is susceptible to colonization of the pockets, and subsequent clinical changes lead to the destruction of tooth supporting tissues. What occurs in the pockets is dysbiosis, i.e. a microbial shift from Gram-positive aerobic symbiont complex to Gram-negative anaerobic periopatogen complex [10]. A correlation was demonstrated between a large number (>10^5 of bacterial cells in the sample) of red complex bacteria and the severity of periodontitis [30]. However, the occurrence of specific periopatogens in the biofilm (e.g., highly leukotoxic strains of *A. actinomycetemcomitans*) was associated with the progression of the disease manifested by a very rapid connective tissue attachment loss [41].

The classification system introduced by the 1999 International Workshop on Classification of Periodontal Diseases differentiated between chronic periodontitis (ChP) and aggressive periodontitis (AgP) [4]. A typical feature of ChP was slow or moderate destruction of periodontal tissues, which correlated with suboptimal plaque control. On the other hand, AgP led to rapid loss of connective tissue attachment and to bone loss in healthy patients, and was characterized by familial aggregation. The amount of dental deposits was disproportionate to the severity of the disease. A later retrospective study concluded that patients wth AgP have a significantly faster linear pattern of progression than patients with ChP: 0.31 mm/year versus 0.20 mm/year, respectively [28]. However, as no objective clinical criteria existed, the differentiation between ChP and AgP suffered from subjective interpretation. Moreover, from the clinical point of view, both ChP and AgP had the same end results, such as disorientation of the attachment apparatus and bone loss. Current evidence does not support the distinction between ChP and AgP as two separate, pathophysiologically different diseases. Taking this information into account, the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions reiterated that case definitions of periodontitis should be based on a concoction of periodontitis stage and periodontits grade [45]. As staging relies on severity and extent of periodontitis, grading describes periodontitis progression rate and analysis of possible poor outcomes of treatment. The following grades have been distinguished: Grade A – slow rate of progression, Grade B – moderate or chronic rate of progression and Grade C – rapid or aggressive rate of progression.

The question of why the course and dynamics of periodontitis differ in individual patients has not been resolved. It has been suggested that genetic factors together with microorganisms affect the development of periodontitis in young patients, while factors associated with lifestyle become more decisive later in life [24]. There is a lack of consistency between the amounts of microbial deposits and the extent and progression rate of periodontitis. Therefore, the aim of the study was to analyze the composition of subgingival biofilm in pockets ≥7 mm in patients with periodontitis in order to answer the question whether the presence of red complex bacteria (*P. gingivalis, T. denticola*) and *A. actinomycetemcomitans* may determine the diagnosis.

MATERIAL AND METHODS

This study was carried out at the Department of Periodontology and Oral Diseases of Medical University of Warsaw, Poland, after receiving positive approval by the institutional review board (KB/171/2009). All clinical procedures were carried out in accordance with the Helsinki Declaration of 1975, as revised in Tokyo in 2004. Written informed consent forms was signed by the patients.

The study included 60 individuals with periodontitis stage III or IV. The first group consisted of 30 patients with periodontitis Grade B, and the other group included 30 subjects with periodontitis Grade C. The diagnosis was based on a clinical and radiological examination [45]. Periodontitis Stage III was diagnosed when: 1) interdental clinical attachment loss (CAL) ≥ 5mm; 2) radiographic bone loss extended to mid-third of root and beyond; 3) tooth loss due to periodontitis was ≤4 teeth; 4) probing pocket depth (PPD) ≥6 mm; 5) vertical bone loss ≥3 mm. Periodontitis Stage IV was diagnosed when: 1) interdental clinical attachment loss (CAL) ≥ 5mm; 2) radiographic bone loss extended to mid-third of root and beyond; 3) tooth loss due to periodontitis was ≥5 teeth 4) probing pocket depth (PPD) ≥6 mm; 5) vertical bone loss ≥3 mm.; 6) need for complex rehabilitation (due to, for example, less than 20 remaining teeth). Periodontitis grades were evaluated directly (radiographic bone loss) or indirectly (% bone loss/age). Periodontitis Grade B – moderate or chronic rate of progression- was diagnosed when: 1) radiographic bone loss <2 mm over 5 years; 2) % bone loss/age = 0.25 to 1.0. Radiographic bone loss was evaluated on dental radiograms as percentage of root length divided by the age of the subject. Periodontitis Grade C –rapid rate of progression was diagnosed when: 1) radiographic bone loss ≥2 mm over 5 years; 2) % bone loss/age > 1.0.

The criteria for inclusion in the study were the following: 1) diagnosed advanced, untreated periodontitis, and: 2) presence of at least one periodontal pocket at least 7mm deep in each patient. The criteria excluding the patient from the study were the following: 1) coexistence of a general disease that might affect the course of periodontal disease, 2) chronic intake of medications that can modify the course of periodontal disease (antibiotics, steroids, anti-inflammatory drugs, immunosuppressive drugs, antiepileptic drugs, calcium channel blockers) within 6 months preceding the study, 3) pregnancy, 4) nicotinism (both active and in the past), 5) current orthodontic treatment, 6) professional removal of deposits within 6 months preceding the study, 7) topical administration of chemotherapeutic agents with bactericidal and bacteriostatic action based on chlorhexidine within 6 months preceding the study.

The study consisted of a clinical and laboratory part. The clinical part included general and dental interviews as well as periodontal measurements. The periodontal examination was carried out by one calibrated examiner who used a graded periodontal probe (UNC probe 15 mm, Hu-Friedy, Chicago, USA) and included the assessment of: 1) dichotomous (yes/no) FMPI according to O'Leary et al. [27] on four tooth surfaces (i.e. distal, buccal, mesial, lingual). The index was determined by dividing the number of surfaces with plaque by the number of all tested surfaces; 2) dichotomous (yes/no) BoP index according to Ainamo and Bay [2]. Bleeding was evaluated at six points for each tooth (i.e. distobuccal, buccal, mesiobuccal, distolingual, lingual, mesiolingual). The index was determined by dividing the number of bleeding points by the number of all assessed points; 3) PPD was evaluated at six points for each tooth as a distance from the gingival margin to the bottom of the pocket; 4) CAL was assessed at six points of each tooth as a distance from the cementoenamel junction (CEJ) to the bottom of the pocket; 5) the number of teeth present in the oral cavity.

During the next stage, ready-made PET diagnostic kits from MIP Pharma (Icking, Germany) were used. One deepest PPD pocket ≥7 mm was selected for each patient (samples were not collected from PPD of third molars). After isolating the examined pockets from contact with saliva, a cotton pad was used to remove plaque from the gingival margin and then the area was dried. With sterile tweezers, one paper point was placed in the pocket. The points were placed in the pockets at full depth for 20 seconds, then packed into labeled tubes and sent in transport packaging to the manufacturer's laboratory.

The laboratory part of the study aimed at the quantitative and qualitative identification of bacteria in the submitted material. Using the polymerase chain reaction (Real Time PCR), the total number of bacteria in the sample was determined as well as the number of two species of the red complex – *P. gingivalis, T. denticola* and of the green complex – *A. actinomycetemcomitans.*

Statistical measurements were carried out with Statistica v. 13 (StatSoft Inc., Tulsa, USA). Any p values of less than 0.05 (p <0.05) were considered statistically significant. Data was expressed as mean ± standard deviation (SD). The Mann-Whitney test was used for statistical analyses of continuous variables. Relationships between periopathogens were evaluated using Spearman's rank correlation test. The strength of the correlations was established by R value: if it was >0.70, the correlation was considered as strong; if it was 0.40 to 0.70, it was a moderate correlation; and if it was <0.40, it was a weak correlation.

RESULTS

The study group consisted of 26 males and 34 females and the mean age of the subjects was 43.8 ± 15.2 years. The periodontitis Grade B group included 13 females and 17 males (among whom 19 subjects with periodontitis stage III and 11 with periodontitis stage IV), mean age 56.9 ± 8.9; the periodontitis Grade C group included 21 females and 9 males (among whom 29 subjects with periodontitis stage III and 1 with periodontitis stage

Table 1. Clinical characteristics of groups

Variable	Total (N = 60)	Periodontitis Grade B (n = 30)	Periodontitis Grade C (n = 30)	Comparison (Mann-Whitneya test)
Number of teeth	27	22	29	p < 0.001
Probing Pocket Depth (PPD) [mm]	4.0 ± 0.9	4.0 ± 0.7	4.1 ± 1.1	p = 0.728
Clinical Attachment Level (CAL) [mm]	4.1 ± 1.6	4.7 ± 1.3	3.4 ± 1.7	p <0.001
Full Mouth Plaque Score (FMPI) [%]	74,. ± 24.0	86.2 ± 16.4	61.9 ± 24.4	p <0.001
Bleeding on Probing (BoP) [%]	80.9 ± 19.8	81.5 ± 18.3	80.4 ± 21.5	p = 0.947

N/n – number of subjects; PPD – probing pocket depth; CAL – clinical attachment level; FMPI – full mouth plaque score; BoP – bleeding on probing

IV), with mean age of 30.7 ± 5.9. There were statistically significant differences in the assessed indices and periodontal parameters depending on the diagnosis; the characteristics of the group are presented in Table 1.

The total number of studied bacteria was higher in the case of periodontitis Grade B patients, as compared to those with Grade C (Table 2). The number of *P. gingivalis* was higher in patients with periodontitis Grade B and it equaled 157.4*10^3 versus 139.9*10^3 in the periodontitis Grade C group (p = 0.033). There was no difference in the occurrence of *T. denticola* depending on the diagnosis (p = 0.228). The presence of *A. actinomycetemcomitans* was confirmed only in four patients with periodontitis Grade C (p = 0.186).

A moderate correlation was found between the occurrence of *P. gingivalis* and *T. denticola* in patients with periodontitis Grade B (r = 0.51, p = 0.004) (Table 3 and Table 4).

DISCUSSION

The oral cavity is home to a plethora of different bacterial species, thus it is difficult to attribute particular functions to single pathogens. In their own work the authors evaluated the occurrence of three classic periopathogens: *P. gingivalis, T. denticola* and *A. actinomycetemcomitans* in deep periodontal pockets depending on periodontal diagnosis. The role of these periopathogens in the etiopathogenesis of periodontitis is beyond doubt.

P. gingivalis is able to secrete large amounts of *Porphyromonas* peptidylarginine deiminase (PPAD), an enzyme that converts peptidylarginine into citrulline residues. A recent study showed that PPAD neutralized human innate immune defenses at three specific levels: bacterial phagocytosis, being captured in neutrophil extracellular traps (NETs), and being killed by the lysozyme-derived cationic antimicrobial peptide LP9 [42].

Accordingly, PPAD seemed to be a new major agent of evasion associated with *P. gingivalis*. Some studies showed that with respect to alveolar bone loss induction, *P. gingivalis* and *Fusobacterium nucleatum* (*F. nucleatum*) synergize [32]. However, Ebbers et al. [15] compared single inoculations of mice with *P. gingivalis* to *F. nucleatum* to *A. actinomycetemcomitans* and observed the most severe bone

Table 2. Microbiological characteristics of study groups with respect to diagnosis (periodontitis grade B versus periodontitis grade C)

Variables	Total (N = 60)	Periodontitis Grade B (n = 30)	Periodontitis Grade C (n = 30)	Comparison (Mann-Whitney test)
Total number of bacteria	10612± 16240 * 10^3	13035 ± 18 366 * 10^3	8188 ± 13678 * 10^3	p = 0.395
Number of Porphyromonas gingivalis	148.6 ± 317.4 * 10^3	157.4 ± 298.0 *10^3	139.9 ± 340.7 * 10^3	p = 0.033
% Porphyromonas gingivalis	2.67 ± 9.02	1.33 ± 1.50	4.01 ± 12.63	p = 0.042
Number of Treponema denticola	38.3 ± 70.0 * 10^3	44.5 ± 72.5 * 10^3	32.0 ± 68.0 * 10^3	p = 0.228
% Treponema denticola	0.66 ± 1.16	0.74 ± 1.40	0.58 ± 0.87	p = 0.652
Number of Aggregatibacter actinomycetemcomitans	0.38± 2.23 * 10^3	0.00 ± 0.00	0.77 ± 3.13 * 10^3	p = 0.186
% Aggregatibacter actinomycetemcomitans	0.01 ± 0.06	0.00 ± 0.00	0.02 ± 0.09	p = 0.511

N/n – number of subjects

Table 3. Correlations between evaluated periopathogens in patients with periodontitis grade B – Spearman rank correlation test

	P. gingivalis	T. denticola	A. actinomycetemcomitans
P. gingivalis		r = 0.51 p = 0.004	–
T. denticola	r = 0.51 p = 0.004		–
A. actinomycetemcomitans	–	–	

P. gingivalis – Porphyromonas gingivalis; T. denticola – Treponema denticola; A. actinomycetemcomitans – Aggregatibacter actinomycetemcomitans

Table 4. Correlations between evaluated periopathogens in patients with periodontitis grade C – Spearman rank correlation test

	P. gingivalis	T. denticola	A. actinomycetemcomitans
P. gingivalis		r = 0.26 p = 0.171	r = 0.04 p = 0.821
T. denticola	r = 0.26 p = 0.171		r = 0.09 p = 0.649
A. actinomycetemcomitans	r = 0.04 p = 0.821	r = 0.09 p = 0.649	

P. gingivalis – Porphyromonas gingivalis; T. denticola – Treponema denticola; A. actinomycetemcomitans – Aggregatibacter actinomycetemcomitans

loss following inoculation with *P. gingivalis*. *A. actinomycetemcomitans* and *F. nucleatum* seemed to mediate protective effects. The authors concluded that genetic background must play a role in the observed phenomena.

T. denticola has a variety of virulence factors, such as the production of proteolytic enzymes (oligopeptidase, dentipain etc.). On the outer sheath of *T. denticola* several proteins, for example the major surface protein (Msp), dentilisin, peptide binding protein of oligopeptide ABC transporter (OppA) and newly found 95 kDa protein (TDE_1072) were detected. TDE_1072 might be a potential periplasmatic solute binding protein encoded by *dppA* that is commenced in the organization of peptide uptake system with proteins encoded by *dppB-dppF* [6]. These genes encode components of an ABC transporter for tri/di peptides. Of interest, it was reported that *T. denticola* encodes 4-times as many ABC transporters compared with other oral microbiota, albeit their functions are ambiguous [34].

A. actinomycetemcomitans produces leukotoxin, which binds to the lymphocyte function-associated antigen-1 (LFA-1), and induces β-hemolysis in red blood cells [8, 18]. This periopathogen has been grouped into six serotypes (a, b, c, d, e, f, g) on the basis of the polysaccharide antigen on the cell surface [19]. A systematic review by Brigido et al. [11] concluded that serotypes a, b and c are dominant worldwide, while serotypes d and e are rare. The distribution patterns of *A. actinomycetemcomitans* differ among subjects of various ethnic and geographic regions. Patients are usually infected by one serotype, as colonization of additional strains may prove difficult owing to competition between the resident strain and the invading strain. However, some studies found that more than 40% of samples showed a combination of serotypes [43]. Moreover, some strains of *A. actinomycetemcomitans* are seen as opportunistic pathogenes, whereas the specific JP2 clone has features of a true exogenous pathogen [23].

Identification of the microbial arrangement of different forms of periodontitis would be a milestone in providing clinical diagnosis and treatment planning. However, oral biofilms represent sophisticated and changing mixed-species communities, all of which hinder scientific attempts regarding this matter. Only advanced cases of the disease (Stage III and/or Stage IV) were qualified for this study, while the composition of subgingival biofilms was evaluated in relation to the dynamics of disease progression, distinguishing two groups: periodontitis Grade B and periodontitis Grade C. A PCR test showed a higher number of *P. gingivalis* in patients with periodontitis Grade B, whereas the presence of *A. actinomycetemcomitans* was confirmed only in four patients with periodontitis Grade C. No differences were observed in the biofilm composition for the presence of *T. denticola*. With regard to relationships in the occurrence of individual periopathogens, a moderate positive correlation was found between *P. gingivalis* and *T. denticola* in patients with periodontitis Grade B. As very strong discrepancies in microbiological compositions have not been found, the simple analysis of subgingival plaque does not seem to be very beneficial in differenting between clinical periodontal diagnoses (periodontitis Grade B or Grade C). We can only speculate that the emergence of deep periodontal pockets might constitute a perfect niche for various periopathogenes and promote distinct interplays between microbial species. However, the composition of a microbial consortium may be different between indi-

vidual cases, but not necessarily determine periodontal diagnosis. As we cannot justify our observations on a molecular level, we assume that periopathogenes temper the host immunologic system and lead to immune response that may promote either slower and chronic (Grade B) or faster and more aggressive (Grade C) pattern of tissue destruction and disease progression. Taking everything into account, periopathogenes might exert unique impact on both, alveolar bone loss and periodontal disease progression. What is more, the progression of periodontitis might concur with introduction of more intricate microbes [23].

Periopathogenes are capable of invading gingival epithelial cells and periodontal connective tissue, which may be an important catalyst of periodontitis bursts. Rajakaruna et al. [3] made use of immunohistochemistry to reveal the presence of *P. gingivalis* extracellulary, as aggregates or within bacterial plaque, and intracellulary in stromal inflammatory cells, squamous epithelium, and capillary endothelium of granulation tissue. The localization and density of *P. gingivaliswere* closely correlated with those of *T. forsythia*, and bacterial density was a factor causing cell-invasiveness and tissue-invasiveness of these periopathogenes. Increased levels of extracellular *T. forsythia* produces an environment that improves the colonization, proliferation and invasiveness of *P. gingivalis.* Cell-invasiveness of *P. gingivalis* was associated with cell molecules, for instance major fimbriae protein (FimA) and gingipain [36, 46]. Moreover, *P. gingivalis* invades deeper structures of connective tissues by way of a paracellular pathway by degrading epithelial cell-cell junction complexes [20]. However, intracellular *P. gingivalis* was only found in the presence of intracellular *T. forsythia*. On the other hand, *A. actinomycetemcomitans* enter epithelial cells by actin-dependent mechanisms, while host cell microtubules play a role in its spread and movement [25]. Zhu et al. [47] observed that both of the abovementioned microbiota up-regulated genes closely associated with the pathways of chemokine signaling, calcium signaling or cell cycle, such as IL6, CCL19, EDN 1, ADCY9 and BCL2, as well as down-regulated CCNB1, PLK1 and CCNA2. Furthermore, the co-regulated genes may be transcription regulated by three transcriptional factors: E12, NRSF and NRF1. *T. denticola* also has the ability to invade human gingival epithelial cells, fibroblasts, and keratinocytes, and the chymotrypsin-like protease dentilisin plays a vital part in this process by altering cellular tight-junctions [14]. This periopathogen invaded gingival keratinocyte HOK-16B cells by resisting endolysosomal degradation for many hours [37]. What is more, *T. denticola* invades deeper tissues through a paracellular route by degrading the tight junction proteins: zonula occludens-1 (ZO-1) and claudin-1, which might play a crucial role in the induction and progression of periodontitis [21].

The results of our study are reflected in the literature on the subject. Nibali et al. [26] evaluated the occurrence of *A. actinomycetemcomitans* and *P. gingivalis* in 267 patients with diagnosed periodontitis (ChP, n = 183, AgP n = 84). *A. actinomycetemcomitans* was present in 54% of patients with AgP and 48% of patients with ChP. The presence of *P. gingivalis* was slightly more frequently detected in ChP patients (67%) compared to patients with AgP (52%). In another study, 47.50% of samples tested positive for *A. actinomycetemcomitans* in AgP group, while in ChP group it was 42.50% [43]. All in all, the microbial profiles of periodontal diseases are very complex, with some differences among populations in various geographical areas. Some studies suggested that *P. gingivalis* may constitute a part of the subgingival normal flora in healthy periodontium and may act as a commensal opportunistic pathogen, playing a double role as normal flora and pathogen [5, 16]. What is more, it could be a prognostic factor for future ChP [5]. On the other hand, the presence of *A. actinomycetemcomitans* might be connected with a plethora of other microbes. This pathogen was present in low abundance before any periodontal destruction, present in healthy as well as diseased sides in susceptible subjects and decreased to very low levels after the disease occurred [17, 35]. Serotype b was the most commonly isolated serotype in AgP (both localized and generalized) and serotypes a and c in ChP, but the results were not consistent [22, 43]. The fact that serotype b was related to more severe disease in some populations but not in the others might indicate virulence differences within the serotype as well as in host susceptibility among various populations [9]. Taking everything into consideration, *A. actinomycetemcomitans* might be important in some, but not all, cases. It is of utmost importance to understand that periopathogenes may exist in periodontal tissues without causing periodontal attachment loss, which indicates the host response role and dissimilarities regarding pathogenic potential of oral microbiota [1].

It should be mentioned that viruses have been implicated in the etiology of periodontitis. Human cytomegalovirus (HCMV) was detected in 60% of crevicular fluid samples and in 60% of the patients with advanced periodontitis, Epstein-Barr virus (EBV) in 30%, herpes simplex virus in 20%, human papillomavirus in 17% and HIV in 7% (31). In a recent review, median values of 40%, 32% and 45% for the subgingival prevalence of human cytomegalovirus, Epstein-Barr virus and herpes simplex virus type I were determined, respectively, in ChP. In AgP, these values were 49%, 45% and 63%, respectively [38]. The influence of the role of viruses on the composition of periodontal pockets' microflora requires further research, but it should be assumed that their presence may regulate the occurring microbiological dysbiosis mechanisms. The presence of herpes viruses may provoke the release of cytokines and chemokines from the host's immunologically competent cells, providing an expedient environment for future colonization by periopathogenes [39]. A suggested vial-bacterial paradigm is consistent with the ability of *P. gingivalis* and *A. actinomycetemcomitans* to invade epithelial cells, which constitute the principal site for infection of HCMV [44]. A recent study showed a higher prevalence of *A. actinomycetemcomitans*, HCMV and *P. gingivalis* among AgP

patients than periodontally healthy controls [16]. An increased risk of AgP was the highest when *A. actinomycetemcomitans* was detected together with EBV-1 (OD 49.0, 95%CI [2.5, 948.7], p = 0.01) and HCMV (OD 39.1, 95%CI [2.0, 754.6], p = 0.02). All of the abovementioned support the coinfection role of *A. actinomycetemcomitans*, *P. gingivalis*, EBV-1 and HMCV in AgP. No correlations were found with respect to *T. denticola* and *T. forsythia.*

The question of which factors might be associated with the progression rate of periodontitis in some individuals remains unanswered. Many studies indicated the critical role of host-response aspects. It is highly unlikely that there are major immunological differences between the progression rate of distinct types of periodontal disease. Nevertheless, some discrepancies in neutrophilic polymorphonuclear leukocytes function with reference to chemotaxis, phagocytosis, superoxide production and adhesion were observed [13]. Neutrophils from the peripheral blood showed more frequent reduction of chemotactic accuracy and/or phagocytosis in AgP, as compared to ChP [7, 12]. Moreover, even subtle genetic variations may affect the amount of transcription and succeeding antimicrobial peptides production, thus determining host response and periodontitis character and progression rate [24]. Nevertheless, unhealthy lifestyle including cigarette smoking, poor nutrition and stress may also have a detrimental effect on host resistance and clinical picture.

There are limitations to our study that need to be addressed. Firstly, only three most clinically relevant periopathogens were tested on the basis of samples obtained from 60 patients. This sample is relatively small. In relation to the very diversified periodontal pocket flora, the observed correlations between populations of individual periopatogens constitute only a small part of all the relationships underlying the microbial shift. It seems very important to continue similar studies based on a larger number of assessed microbiota. Interesting observations could also be provided by verification extended to virological analysis. Secondly, *A. actinomycetemcomitans* serotypes were not determined, but considering the sample size, such an analysis would not bring additional benefits. Therefore, in future studies, the number of patients should be increased.

Our study does not support the thesis that the presence and number of specific bacteria may be related directly to the form of periodontitis. We hypothesize that the microbial arrangement is rather an effect of the emergence of deep periodontal pockets as well as individual inflammatory response to a biofilm starting point. Host resistance and susceptibility are determined by genetic and epigenetic risk factors as well as life style. However, it is still probable that, with the help of in-depth microbe analyses and more state-of-the-art molecular techniques, some specific microbes may be related to different types of periodontitis. Our study paves the way for future research elaborating the *in vivo* interplay of numerous periopathogenes in periodontal pockets.

CONCLUSIONS

In the present study we combined three major oral microbiota with clinical conditions. The subgingival biofilm composition was very similar in patients with periodontitis Grade B and periodontitis Grade C. We may speculate that periodontitis grade may not be distinguished according to the simple analysis of subgingival plaque with respect to *P. gingivalis*, *T. denticola* and *A. actinomycetemcomitans* alone. The results of own study confirm the conclusion of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions that grouped the forms of the disease previously recognized as chronic or aggressive under a single category periodontitis [29]. It was also found that based on modern knowledge, routine microbiological examination is not justified for diagnostic purposes.

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