Identification of Bacteria Involved in Periodontal Disease Using Molecular Biology Techniques

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Periodontal disease is progressive and episodic in nature, with tissue destruction resulting from the host response to bacterial antigens and irritants.This study aimed to evaluate the genotype of a certain group of pathogen agents. In order to to this we used a total of 45 patients with PAG included in the epidemiological analyzes with a mean age of 34.7 Four samples were collected from the subgingival plaque from each patient, a total of 224 samples of dental plaques were investigated. All samples were tested for the presence of Tannerella forsythensis, Porphyromonas gingivalis and Treponema denticola. We found that patients had a large number of pockets colonized by Tannerella forsythensis gingivalis (88.6%) Porphyromonas gingivalis (59%) and Aggregatibacter actinomycetemcomitans (25%). The data of this study support the evidence Tannerella forsythensis as a periodontopatogen and confirmed a strong association between Porphyromonas gingivalis and Tannerella forsythensis in aggressive periodontitis and support previous findings that generalized aggressive periodontitis are associated with more complex microbiota.

Keywords: periodontal disease, biofilm, DNA hybridization

The oral cavity can be considered as an ecosystem comprising a plurality of microbial species performing antagonistic activites [1,2]. As long as this antagonism is kept in a stable balance, periodontal health and oral tissues remain in a state of normal health [3]. When this balance disappears and certain bacteria increase virulence or tissue host defense power decreases, the disease is triggered [4,5]. To a certain extent, the chronic and aggressive periodontitis, share many clinical features, but the specific details of their common characteristics are not necessarily identical in both forms of the disease [6]. Thus far, it was determined that both are complex infections that occur in susceptible hosts caused by biofilms that form on the surfaces of the teeth [7,8]. The ability to form biofilms, considered recently the attribute only for a few species is now seen as an attribute for almost all microorganisms [9,10]. In both cases, biofilms include disease-producing microorganisms which are components of indigenous oral microbiota [11]. The supragingival biofilm forms a reservoir for periodontal bacteria and the development of subgingival biofilm [12]. Mature subgingival biofilm is dynamic, well organized and structured as a solid mass with fluid-filled channels within it; protects bacteria in its depth with diffusion barriers; and enables the migration and colonization of periodontal bacteria at adjacent periodontal sites and in periodontal tissues themselves [13,14].

Aggressive periodontitis, a destructive and rarely occurring periodontal disease, is characterized by the following: rapid loss of bone attachment and bone destruction in a healthy clinical patient, increased amount of microbial deposits regardless of disease severity and family aggregation of diseased individuals. It usually occurs in the first decades of life, and the affection has been classified into two types: localized and generalized [15-18].

In the generalized forms of Generalized Aggressive Periodontitis (PAG), it is important to know whether the specific condition is associated with *A.actinomycetem-comitans* or other periodontal pathogens such as *P.gingivalis*, or is a combination of several pathogenic microorganisms. This information is needed to complete conventional therapy with antimicrobial therapy [19].

Clinical studies have also indicated that the success of localized aggressive periodontitis (LAP) treatment depends on the removal of *A. actinomycetemcomitans*, and that the removal of this microorganism by conventional periodontal procedures is difficult [20].

Since *A. actinomycetemcomitans and P. gingivalis* can be transmitted from patients with periodontitis to family members, microbial testing of spouses, children, or siblings of patients with GAP may be indicated for the early detection of susceptible diseases [21].

A variety of methods have been developed and applied for the detection and identification of microorganisms. Bacterial culture has long been considered the *gold standard*, although these techniques are extremely delicate and require experienced staff and strict quality assurance procedures. Many organisms will not grow on the currently available culture media [22].

Recent studies show that besides genetic influences, environmental factors can affect the clinical expression of PAG. In a large study, smoking was a risk factor for patients with generalized PAG . Smokers with PAG had their teeth more affected and an average loss of attachment greater than non-smokers with PAG [23].

Exposure to cigarette smoke seems to add a significant risk to the severity and prevalence of this disease, and the mechanism is not fully understood. Research shows that serum levels of IgG, IgG2 as well as anti-Aggregatibacter actinomycetemcomitans levels for the same group are significantly reduced in PAG smokers [24].

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The aim of this study was to evaluate the genotype of the following periodontal pathogens agents: *Agreggatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythensis, Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum, Veillonella parvula, Capnocytophaga ochracea and Fusobacterium spp.* in groups of patients with aggressive periodontitis PAG and patients with chronic periodontitis PC.

Experimental part
Material and methods
The study was conducted at Innsbruck Medical University - Department of molecular mechanisms of infectious diseases, analysis and microbiological investigations were conducted at the Department of Hygiene and Medical Microbiology. Patients were from the clinic of Dentistry and Surgery UMF Zahnmedizen of Tyrol Innsbruck - Department of Oral Prevention - periodontology. A total of 45 patients with PAG were included in the epidemiological analyzes. All patients were previously untreated and monitored between 2007-2012.

I GROUP-patients with PAG
Patients were diagnosed according to clinical and radiographic signs from 5 years ago. They were treated and the revaluation has found periodontal disease with aggressive persistence, given their age and history of periodontal disease. Mean age of patients was 34.7 years.
Exclusion criteria:
- patients with chronic periodontitis (PC)
- patients receiving the anti-inflammatory treatment in the last 6 months
- patients receiving antimicrobial therapy in the last 6 months
Four samples were collected from the subgingival plaque from each patient before any therapy and from the deepest pockets [PD 4 mm], who bled on probing, preferably one from each quadrant. In addition, control samples were collected from clinically unaffected sites. After removal of the plaque with a curette, in the periodontal pockets were placed three sterile paper cones [ISO 35; Becht, Offenburg, Germany]. After 10 s the paper cones were removed and placed in 1 ml of reduced transport fluid [RTF], transferred to the laboratory and processed immediately.
From the PAG a total of 224 samples of dental plaques were investigated, 180 of periodontal pockets and 44 healthy control sites]. The average depth of the bag of sample sites was 8.7 ± 1.8 mm and the average depth of monitoring sites was 2.7 ± 0.8 mm.

II GROUP-patients with PC
The second group used for comparison study consisted of 21 patients with PC.
Inclusion criteria:
- age ≥ 35 years
- the presence of at least 20 teeth
- no history of severe periodontitis
- no site with PD> 5 mm

Exclusion criteria:
- presence of gingivitis to severe parodontitis,
- presence of systemic chronic diseases
- patients receiving the anti-inflammatory or antimicrobial therapy in the last 6 months
All samples were tested for the presence of *T. forsythensis, P. gingivalis and T. denticola.* The tube containing the cone paper in the conveyor VMGA III was centrifuged for 1 min. After removal of the cone paper, each sample was collected by centrifugation at 10,000 rpm for 5 min. The resulting sample was suspended in 200 mL of sterile distilled water, incubated at 56 °C for 30 min, boiled at 100° C for 10 min, centrifuged at 10,000 rpm for 5 minutes. 5μL of supernatant of each sample was used as the basis of PCR. 50 mL of the product of the PCR reaction containing 5 mL of the sample, 5 mL 10 x PCR buffer solution of ammonium sulfate, 1.25 units Taq DNA polymerase, 0.2 mM of each dNTP, 1.0 mM of each primer and 1.5 mM MgCl2 for *T. forsythia, P.gingivalis and T. denticola.*
The thermal profile first PCR *T. forsythia, P. gingivalis* and *T. denticola* included an initial period of heating at 95° C for 2 min followed by 36 cycles of 95° C for 30 s, 60° C for 1 min, 72° C for 1 min and a final extension at 72° C for 2 min.
The amplified PCR products were analyzed by electrophoresis in 1% [vt /v] agarose gel at 4V / cm in triacetate-EDTA buffer. The gel was stained with 0.01 mg / mL ethidium bromide and photographed under ultraviolet light of 300nm. PCR amplification was repeated at least two times for each sample.
The prevalence of the three microorganisms was determined by hybridizing the pathogenesis of bacterial PCR products using primers of oligonucleotides (table 1).
Dot-blot hybridization of dental plaque from the patient were carried out sequentially with each DNA probe hybridization in these conditions. Negative and positive controls were included in each test run. The membranes were washed with stripping buffer [10 mL] for 2 x 15 min at 37 °C to remove the probe and rinsed in 2 x SSC [10 mL]. Identical membranes were used for all experiments hybridisation probes reported. Dot-blot hybridization of the amplified dental plaque was used to detect small amounts of highly pathogenic bacteria in periodontal patients. PCR allows the detection of at least 100 bacterial cells in a sample. The detection limit of the PCR-amplification and hybridization were subsequently reported to be approximately 20 CFU / mL using a pure culture.
When we compared the sites of colonization and control periodontal pockets of patients with PAG, we chose an arbitrary periodontal pocket and a shallow site per patient were analyzed and evaluated using chi-square test. The assess of the prevalence of species in periodontal pockets in patients PAG compared to sites of chronic periodontitis patients was performed for four sites on the subject and using chi-square test. As only one site of control has been available for each patient with PAG comparison of the bacteria in the presence of similar shallow sites with the PC it has been done very arbitrary single site.

BACTERIA	Sample sequence[5'-3']	T[°C]	Nr. of access
T.forsythia	CGTATCTCATTTTATTCCCCTGTA	61	X73962, LI6495
P. gingivalis	TACTCGTATCGCCCGTATTC	57	X73964, L16492
T. denticola	CGACTTGCATGBTTAARAC	56	33521

Tabel 1
SPECIFIC SAMPLES [PRIMER] OLIGONUCLEOTIDE USED FOR HYBRIDIZATION

Analysis of the presence of bacteria at different depths of the pockets was performed with data from only 23 patients PAG using chi-square test.

Mann-Whitney two test samples of independent non-parametric test was used to test the null hypothesis that the bacteria on the patient, i.e. the number of negative sites per patient, is the same in both groups of subjects.

Four pockets were analyzed per patient with PAG and four sites on the subject of the lot with PC. The relationship between two species of bacteria, for example the frequency of co-existence, was made by considering the odds ratio [OR] calculations are as suggested by Socransky.

Results and discussions

We have analyzed a total of 224 subgingival samples from 45 patients with PAG and 84 samples from 21 patients with PC.

The frequency was evaluated for the detection of bacteria in the periodontal lesions and healthy control sites in 44 patients with PAG (fig. 1).

Fig. 1. Pacients with PAG and PC

Patients had a large number of pockets colonized by *T. forsythensis gingivalis* (88.6%) *P. gingivalis* (59%) and *A.actinomycetemcomitans* (25%). Comparison of positive pockets and control sites showed a highly significant difference (p<0.001) for *P.gingivalis* and *T. forsythensis.* These specie were more frequently detected in diseased sites as compared to clinically healthy sites.

The colonization of sites without clinical signs of disease is still remarkable. *T. forsythensis* could be identified in 34%, *P.gingivalis* in 22.7% and *T.forsythensis* in 35% of control sites (fig. 2).
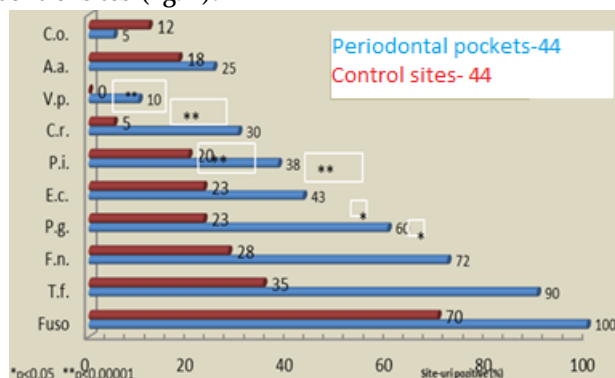

Fig. 2. Bacterial colonization of periodontal pockets compared to control sites

More detailed information about differences in the prevalence of species between the two groups could be obtained by comparing the number of positive pockets in patients with PAG and positive sites of patients with PC (fig. 3).

From each subject were included in the calculations four protected sites (from PAG patients only from pockets and for patients with PC from sulcus also) and pronounced discrepancies were observed between groups.

Comparison of the shallow sites (1-3 mm PD) in patients with PAG and PC do not have shown significant differences for most species (fig. 4).

T. forsythensis occurred in 95.5% of patients with PAG and 85.7% of patients with PC so there is no significant difference between the two groups. The organism seems
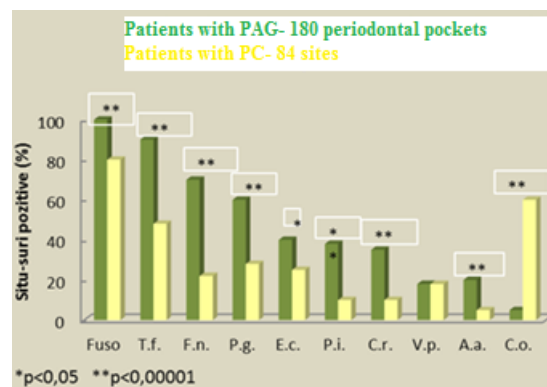

Fig. 3. Comparative analysis of bacterial positive sites between patients with aggressive periodontitis and chronic periodontitis

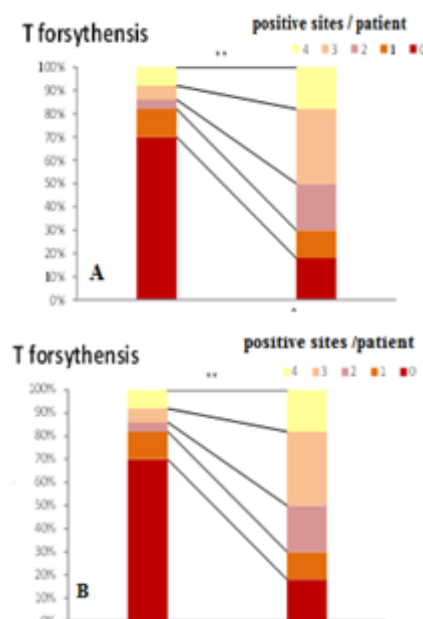to be a common colonizer even in healthy individuals (fig 4).

Fig. 4. Tannerella forsythensis frequency in patients with PAG (A) and PC (B)

The bars depict the percentage of subjects with 0, 1, 2, 3 or 4 site positive for *T. forsythensis* in the PAG group [N = 45] and in PC group (n = 21). The significance of differences between groups were determined using the Mann-Whitney test. *T.forsythensis* level was higher in patients with PAG. Number of patients with 4 positive pockets was 73.3, 53.3 and 51.1%, respectively.The charging for these species was significantly lower in group PC. However, 37.7% of patients PAG showed no *P.gingivalis* colonization in any sample.

Evidence for the association of *T. forsythensis* with severe periodontal diseases are based primarily on epidemiological data and assess the correlation between the species and the presence of clinical conditions. The data of this study support the evidence *T.forsythensis* as a periodontopatogen. The species was found significantly more often in pockets of patients with PAG than in control sites.

P.gingivalis is the most extensively studied specie. It was commonly associated with severe periodontitis. Quantitive culture as approach can be considered appropriate for detecting *P.gingivalis* as Conrads shown by comparative analyses using DNA hybridization.

The prevalence of *P.gingivalis* in the group of generalized aggressive periodontitis was advantageous elevated to 63.6% in this study [table 2].The species was significantly more frequently detected in the periodontal pocket than in the control sites (59.1 and 22.7%, respectively) (fig. 5).
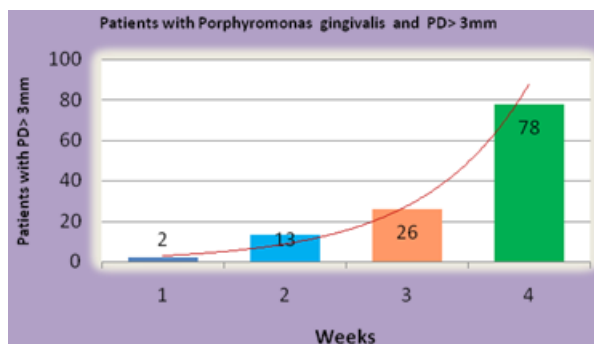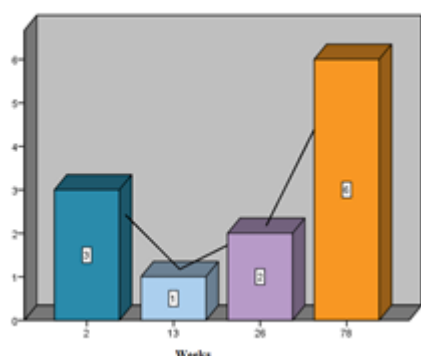
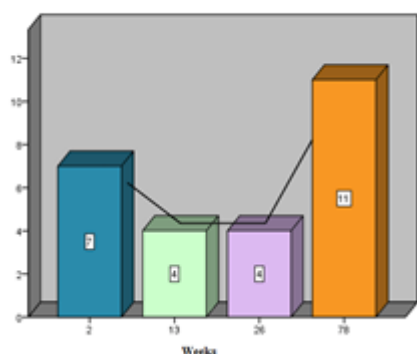Fig. 5.The number of patients with *P.gingivalis* at a depth of the gingival sulcus > 3mm

Fig. 6.The number of patients with *P.gingivalis* at the depth of gingival sulcus > 4mm

The prevalence was significantly lower than in the population group with PC than in group with PAG. *P.gingivalis*, therefore, may be associated to the development of aggressive periodontitis.The current study identifies particular *T.forsythensis* more frequently as a member of the microflora of healthy sites than in the past as suspected based on investigations by culture.

This contradicts the assumption that the mere presence of *T.forsythensis* can be taken as an indicator of active periodontal destruction. *T.forsythensis* was detected in 85.7% of people with PC.

P.gingivalis prevalence was as high as 89.4%, and 80% of the sample pockets were positive. *P.gingivalis* has also been strongly associated with chronic periodontitis and recurrent periodontitis. Several researchers have found a significant correlation between *P.gingivalis* and proportions of attachment loss. There are conflicting data on the presence of *P.gingivalis* in periodontal health conditions. *P.gingivalis* detection frequent with *T.forsythensis* in active periodontal pockets / deep supporting evidence that certain consortia are of great importance in disease progression.This epidemiological study confirmed a strong association between *P. gingivalis-T.forsythensis* in aggressive periodontitis.

In the current study, a relatively high prevalence of *P.gingivalis* [62%] was observed in the PC group without a significant difference in the group with PAG. However, only 32% of sites in PC group were positive, showing rare *P.gingivalis* colonization in the control group.The species was rare in shallowsites.

A.actinomycetemcomitans is considered to be a major etiologic factor in the localized form of aggressive periodontitis (PGA) [9]. The role of *A.actinomycetem-comitans* in aggressive generalized periodontitis is still unclear (fig. 8). The study presented here showed a low prevalence of this species (36.4%) in PAG patients (table 2).

It can not be excluded that some of the PAG patients had previously the localized form of aggressive periodontitis. Apparently, *A.actinomycetemcomitans* (perhaps special strains) may be considered relevant for aggressive periodontal disease in young adults. However the evidence is not as strong as the localized form.

Microbiological tests for detecting suspected periodontal pathogens are not diagnostic criteria for periodontal disease, periodontitis as a consequence of opportunistic

Table 2
DETECTION FREQUENCY OF *A.ACTINOMYCETEMCOMITANS* IN PATIENTS WITH PAG COMPARATIVE WITH PC. HOWEVER, IN 11% OF PATIENTS *A.ACTINOMYCETEMCOMITANS* WERE PRESENT IN ALL SITES.

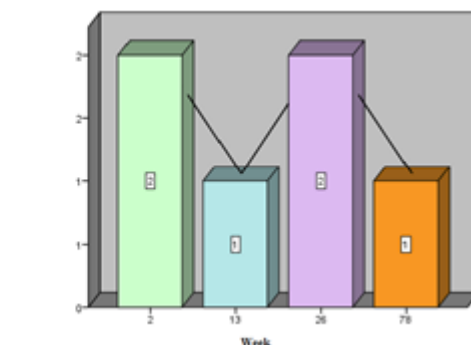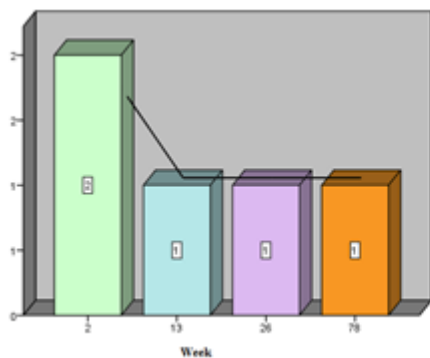| Detection frequency	Week	Detection Nr.	Detection Nr.	Detection Nr.	Detection Nr.
		PC< 3mm	PC>3mm	PAG<4mm	PAG>4mm
Porphyromonas gingivalis [n = 29]	2	1	1	3	7
	13	2	2	1	4
	26	1	5	2	4
	78	8	8	6	11
Aggregatibacter actinomycetemcomitans[n = 29]	2	0	0	2	2
	13	1	2	1	1
	26	1	2	1	2
	78	1	1	1	1

Fig. 7. Comparative analysis of *A.actinomycetemcomitans* presents in patients with PAG
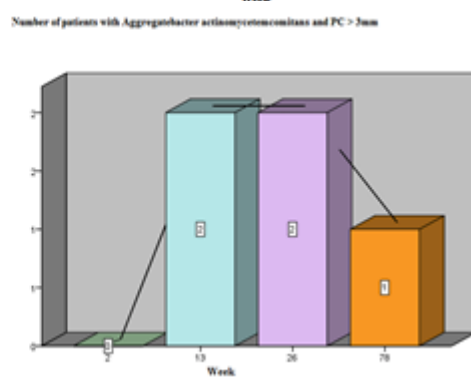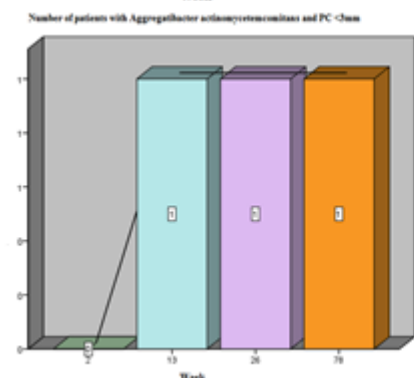

Fig. 8. Comparative analysis of *A.actinomycetemcomitans* presents in patients with PC

infections caused by microorganisms belonging to the resident microflora. Obviously, the influence of host immunity amend the measure of the clinical outcome at a high level [11]. Patients with an altered inflammatory response may be less able to tolerate the presence of specific organisms. People with low apparent risk of developing periodontal disease may have a protective destructive established so-called beneficial subgingival flora. It is already recognized and demonstrated in this study that for this multifocal periodontal risk model also contributes the bacterial aggression, smoking, age, diabetes, socio-economic and genetic factors[12]. In the initial phase, pathogenic microbiota appears to play an important role rather than single periodontal pathogens.

Microbiological diagnosis may be useful at different stages of the treatment plan, as part of the initial diagnosis and re-evaluation during the maintenance phase. The need of microbiological information before therapy depends on the overall treatment strategy.

Understanding the relationship between microbial activities and host response is crucial to prevention or therapeutic measures. Previous studies have suggested a positive association between different etiological pathogens and the occurrence of periodontal disease. However, epidemiological studies have not yet revealed any permanent correlation between different bacteriological parameters that lead to a diagnosis of aggressive periodontitis.

The purpose of this investigation was to study the association of pathogenic agents suggested to cause aggressive generalized periodontitis and associated with periodontal status in subjects with chronic periodontitis using molecular biological methods.The methods used in this study presents qualitative data. No attempt was made to quantify it. In general, the assessment of the quantitative aspects is complicated by the fact that the amounts of bacteria depend on the method of sampling, the number of samples for a subject, the criteria for selection site, the method used to calculate based on the sample volume and dilution of the sample and count how is the average value determined -physical vs. mathematical .

There have been designed and assessed oligonucleotide probes for detecting bacteria associated with periodontitis and the results of the study showed frequent *T.forsythensis* and *P. gingivalis* colonization, in patients with aggressive periodontitis. However, there were individual variations evidence.

These microorganisms may be predominantly identified in periodontal pockets, but were significantly less frequent in subjects with PC and control sites.

T.forsythensis and *P.gingivalis* as suspected pathogens, may therefore be considered key bacteria in patients with aggressive periodontitis. *A.actinomycetemcomitans* could be detected only in a few patients, being an important bacteria for generalized aggressive periodontitis etiopathogenesis.

Conclusions

Presently, no definite answer can be given to the question whether the statement either aggressive etiologic agents (involving infection with microbiota highly virulent) or a high level of individual susceptibility to periodontal disease, or a specific combination of both factors is determinative in aggressive periodontitis pathogenesis. The results support previous findings that generalized aggressive periodontitis are associated with more complex microbiota.

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