ORIGINAL ARTICLE

# Microbiological characteristics of subgingival microbiota in adult periodontitis, localized juvenile periodontitis and rapidly progressive periodontitis subjects

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**Objective** To describe the prevalence of the cultivable subgingival microbiota in periodontal diseases and to draw attention to the polymicrobial nature of periodontic infections.

**Methods** The study population consisted of 95 patients, 51 females and 44 males, aged 14–62 years. Twenty-nine patients exhibited adult periodontitis (AP), six localized juvenile periodontitis (LJP), and 60 rapidly progressive periodontitis (RPP). Two to four pooled bacterial samples were obtained from each patient. Samples were collected with sterile paper points from the deepest periodontal pockets. The samples were cultured under anaerobic and microaerophilic conditions using selective and non-selective media. Isolates were characterized to species level by conventional biochemical tests and by a commercial rapid test system.

**Results** *Prevotella intermedia* and *Capnocytophaga* spp. were the most frequently detected microorganisms in all diagnostic groups. *Porphyromonas gingivalis* and *Peptostreptococcus micros* were found more frequently in AP and RPP patients, while *Actinobacillus actinomycetemcomitans* and *Eikenella corrodens* were associated with AP, LJP and RPP patients. The other bacterial species, including *Actinomyces* spp., *Streptococcus* spp. and *Eubacterium* spp., were detected at different levels in the three disease groups.

**Conclusions** The data show the complexity of the subgingival microbiota associated with different periodontal disease groups, indicating that the detection frequency and levels of recovery of some periodontal pathogens are different in teeth affected by different forms of periodontal disease.

**Keywords** Anaerobic bacteria, periodontal diseases, subgingival plaque

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## INTRODUCTION

Periodontitis may be defined as a mixed infection affecting individual or multiple sites within the oral cavity and leading to the loss of the supporting periodontal tissues. Gingivitis is an infectious inflammatory process limited to the gingiva. Periodontal diseases are generally chronic in nature and can persist in the absence of treatment [1–3]. These diseases result from the exposure of the periodontium to dental plaques that accumulate on the teeth to form bacterial masses at or below the gingival margin.

Periodontal destruction probably results from the action of various toxic products released from specific pathogenic subgingival plaque bacteria, as well as from the host responses elicited against plaque bacteria and their products. The inflammatory response may result in gingival ulceration around the tooth which can allow intact bacterial cells or their products into the systemic circulation. These infections may thus influence overall health and the course of some systemic diseases.

The bacterial etiology of periodontal disease is complex, with a variety of organisms responsible for the initiation and progression of disease. Although over 400 different bacterial species have been detected in the oral cavity [4], only a limited number have been implicated as periodontal pathogens. Many of these organisms may also be present in periodontally healthy individuals and can exist in commensal harmony with the host.

The microorganisms of the dental plaque have been shown to be capable of initiating the mechanisms of destruction of the periodontal tissues, while their effective control has been shown to be the most appropriate means of arresting the progression of periodontal diseases. The nature of these pathogenic agents varies among different disease entities, as well as among patients

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and even different disease sites within a patient. Certain groups of Gram-negative bacteria have been found consistently in periodontal lesions [5,6]. Among them, *Porphyromonas gingivalis, Prevotella intermedia, Campylobacter rectus, Bacteroides* spp., *Selenomonas* spp. and spirochetes have been associated with adult or refractory periodontitis [7,8]. Furthermore, *Actinobacillus actinomycetemcomitans, Capnocytophaga* spp., *Prevotella intermedia and Eikenella corrodens* have been associated with early-onset periodontitis [9–11]. However, some anaerobic, Gram-positive microorganisms such as *Peptostreptococcus micros* and certain *Eubacterium* species have only recently been implicated in destructive periodontal diseases [8,12].

The purpose of this investigation was to register the prevalence of different bacteria that constitute the subgingival microbiota in different disease groups by means of anaerobic culture procedures and to obtain information on the microbial etiology of these polymicrobial infections.

## MATERIALS AND METHODS

The study is based on data obtained from a series of subgingival microbial samples collected at the Department of Periodontology and processed at the Institute for Medical Microbiology of a German university hospital over a 2-year period.

Ninety-five patients aged 14–62 years, who had been referred to the Department of Periodontology for diagnosis and treatment of periodontitis, underwent microbiological examination. Of these, six subjects presented with a clinical diagnosis of localized juvenile periodontitis (LJP), 29 subjects with adult periodontitis (AP) and 60 subjects with rapidly progressive periodontitis (RPP).

### Microbiological sampling

Prior to the clinical examination, the subgingival microbial samples were collected from the deepest pockets. The sampling area was isolated with cotton rolls, carefully cleaned with sterile cotton pellets, and then air dried. For single sites, two sterile paper points (Antaeos, Munich, Germany) were inserted to the bottom of the pocket for a 20-s period and then transferred into a reduced transport fluid (RTF) medium with 25% glucose [13]. Being a liquid, this transport medium offers the advantage of dilution of the microorganisms, maintaining at the same time the viability of the wide variety of facultative anaerobes and obligate anaerobes. In addition, this medium allows the samples to be frozen for further examination. For pooled samples, at least one paper point per site from up to four sites was collected.

### Microbiological procedure

The samples were processed within 24 h. For isolation of anaerobes present in the specimen, the samples were plated on non-selective Brucella agar plates (Becton Dickinson, Heidelberg, Germany) enriched with 5% horse blood, 0.5% hemolysed blood and 5 mg/L of menadione. Kanamycin–vancomycin-laked blood (KVLB, Becton Dickinson, Heidelberg, Germany) agar plates were used for selective recovery of obligately anaerobic Gram-negative rods. Columbia agar with 5% sheep blood and standard chocolate agar were used for the cultivation of microaerophilic microorganisms. Trypticase soy agar plates supplemented with horse serum, bacitracin and vancomycin (TSBV) were used for selective recovery of *Actinobacillus actinomycetemcomitans* [14].

BBL and KVLB agar plates were incubated for 7 days at 36 °C under anaerobic conditions (BBL GasPak System, Becton Dickinson Microbiology Systems, Cockeysville, Md, USA). The TSBV, Columbia agar and chocolate agar plates were incubated at 36 °C under microaerophilic conditions (BBL CampyPak plus, Becton Dickinson Microbiology Systems, Cockeysville, Md, USA).

After 7 days of incubation, colonies with differing characteristics were subjected to various tests. One to three colonies of each selected type were isolated and purified for further identification based on cell morphology, Gram's stain reaction and indole production. Catalase activity was tested by adding a few drops of $H_2O_2$ to the isolated colonies. Some additional tests for the identification of anaerobic microorganisms based on enzymatic activities were performed using the BD Crystal ANR ID Kit (Becton Dickinson, Heidelberg, Germany).

Small round and star-shaped colonies adhering strongly to TSBV agar, being catalase, galactose, maltose and xylose positive [15], were identified as *Actinobacillus actinomycetemcomitans.* Gram-positive facultative cocci forming chains were classified as streptococci and were identified with the BBL Crystal Positive ID Kit (Becton Dickinson, Heidelberg, Germany).

## RESULTS

Table 1 shows the demographic characteristics of the subject groups who provided samples for this study: 29 patients (mean age 47.1 years) exhibited AP, six (mean age 25.1 years) LJP, and 60 (mean age 39.3 years) RPP. The frequency of detection and the number of isolates of the various microorganisms monitored are shown in Tables 2 and 3.

**Table 1** Patient demographics

| Group | Age (years) | Females | Males |
|---|---|---|---|
| AP | 48.4 | 17 | 12 |
| LJP | 23.3 | 4 | 2 |
| RPP | 39.3 | 30 | 30 |

AP, adult periodontitis; LJP, localized juvenile periodontitis; RPP, rapidly progressive periodontitis.

**Table 2** Detection frequency of bacterial morphotypes

| | Anaerobes | | | | Microaerophilics | |
|---|---|---|---|---|---|---|
| | Gram$^-$ rods | Gram$^+$ rods | Gram$^-$ cocci | Gram$^+$ cocci | Gram$^-$ rods | Gram$^+$ cocci |
| AP | 25 | 7 | 2 | 8 | 29 | 7 |
| LJP | 13 | 1 | – | 3 | 8 | 5 |
| RPP | 82 | 24 | – | 32 | 90 | 14 |

AP, adult periodontitis; LJP, localized juvenile periodontitis; RPP, rapidly progressive periodontitis.

**Table 3** Number of isolates and frequency of detection of the predominant cultivable microbiota

| | AP *n* (%) | LJP *n* (%) | RPP *n* (%) |
|---|---|---|---|
| Anaerobes | | | |
| Gram-negative rods | | | |
| *Bacteroides capillosus* | 1 (1.3) | – | 1 (0.4) |
| *Bacteroides gracilis* | – | 1 (2.9) | – |
| *Bacteroides ureolyticus* | – | – | 2 (0.8) |
| *Fusobacterium nucleatum* | 1 (1.3) | 1 (2.9) | 6 (2.5) |
| *Fusobacterium russi* | – | – | 2 (0.8) |
| *Fusobacterium varium* | – | – | 1 (0.4) |
| *Leptotrichia buccalis* | – | – | 1 (0.4) |
| *Porphyromonas asaccharolytica* | – | – | 2 (0.8) |
| *Porphyromonas endodontalis* | 1 (1.3) | 2 (5.9) | 3 (1.3) |
| *Porphyromonas gingivalis* | 5 (6.4) | – | 14 (5.9) |
| *Prerotella buccae* | 2 (2.6) | 2 (5.9) | 3 (1.3) |
| *Prerotella buccalis* | 1 (1.3) | 1 (2.9) | 1 (0.4) |
| *Prerotella corporis* | 3 (3.8) | – | 3 (1.3) |
| *Prerotella denticola* | – | – | 3 (1.3) |
| *Prerotella disiens* | 3 (3.8) | 2 (5.9) | 4 (1.7) |
| *Prerotella intermedia* | 8 (10.2) | 4 (11.8) | 24 (10.1) |
| *Prerotella melaninogenica* | – | – | 2 (0.8) |
| *Prerotella oralis* | 1 (1.3) | – | 5 (2.1) |
| *Prerotella oris* | 1 (1.3 | – | –) |
| *Prerotella veroralis* | – | –1 (0.4) | |
| *Tissierella preacuta* | – | – | 5 (2.1) |
| Gram-positive rods | | | |
| *Actinomyces israelii* | – | – | 2 (0.8) |
| *Actinomyces meyeri* | – | 1 (2.9) | 3 (1.3) |
| *Actinomyces naeslundii* | 2 (2.6) | – | 7 (2.9) |
| *Actinomyces pyogenes* | – | – | 1 (0.4) |
| *Actinomyces viscosus* | – | – | 1 (0.4) |
| *Clostridium glycolicum* | 1 (1.3) | – | 1 (0.4) |
| *Clostridium limosum* | – | – | 1 (0.4) |
| *Clostridium ramosum* | – | – | 1 (0.4) |
| *Eubacterium* spp. | 1 (1.3) | – | 4 (1.7) |
| *Eubacterium aerofaciens* | 1 (1.3) | – | – |
| *Lactobacillus* spp. | – | – | 2 (0.8) |
| *Propiortibacterium propionicus* | – | – | 1 (0.4) |
| Gram-negative cocci | | | |
| *Veillonella* | 2 (2.6) | – | – |
| Gram-positive cocci | | | |
| *Peptostreptococcus anaerobius* | – | 1 (2.9) | 7 (2.9) |
| *Peptostreptococcus magnus* | 4 (5.1) | 1 (2.9) | 3 (1.3) |
| *Peptostreptococcus micros* | 4 (5.1) | 1 (2.9) | 15 (6.3) |
| *Peptostreptococcus prevotii* | 2 (2.6) | – | 6 (2.5) |
| *Peptostreptococcus tetradius* | – | – | 1 (0.4) |
| Microaerophilics | | | |
| Gram-negative rods | | | |
| *Actinobacillus actinomycetemcomitans* | 7 (9.0) | 4 (13.3) | 12 (5.0) |
| *Capnocytophaga* | 21 (27.0) | 7 (20.6) | 60 (25.3) |
| *Eikenella corrodens* | 1 (1.3) | 1 (2.9) | 9 (3.8) |
| *Haemophilus aprophilus* | – | – | 5 (2.1) |
| *Haemophilus parainfluenzae* | – | – | 3 (1.3) |
| *Kingella* spp. | – | – | 1 (0.4) |
| Gram-positive cocci | | | |
| *Streptococcus constellatus* | 1 (1.3) | 2 (5.9) | 1 (0.4) |
| *Streptococcus intermedius* | 5 (6.4) | 2 (5.9) | 10 (4.2) |
| *Streptococcus milleri* | 1 (1.3) | – | 1 (0.4) |
| *Streptococcus mutans* | – | – | 1 (0.4) |
| *Streptococcus salivarius* | – | 1 (2.9) | – |

AP, adult periodontitis; LJP, localized juvenile periodontitis; RPP, rapidly progressive periodontitis.

Anaerobic Gram-negative rods and microaerophilic Gram-negative rods were the most frequently detected bacterial categories in the samples from all groups (Table 2). *Capnocytophaga* spp. *and Prevotella intermedia* were the predominant species cultivated in all diagnostic groups (Table 3). *Porphyromonas gingivalis* was equally recovered from AP and from RPP samples (6.4% and 5.9%, respectively). *Fusobacterium nucleatum* was detected at higher frequency in both RPP (2.5%) and LJP (2.9%) than in AP (1.3%) samples.

*Peptostreptococcus micros* had a detection rate of 6.3% in the RPP group, in contrast to 5.1% in the AP group and 2.9% in the LJP group. The other species of *Peptostreptococcus* were less frequently detected in the LJP and RPP groups, as shown in Table 3.

*Actinobacillus actinomycetemcomitans* was detected in AP, LJP and RPP samples (9%, 11.8% and 5%, respectively). *Eikenella corrodens* was also present in the three group samples (AP = 1.3%, LJP = 2.9% and RPP = 3.8%), while

microaerophilic Gram-negative rods such as *Haemophilus aprophilus, Haemophilus parainfluenzae and Kingella* spp. were detected only in the RPP group (2.1%, 1.3% and 0.4%, respectively).

*Streptococcus intermedius* was present in samples from all diagnostic groups (AP = 6.4%, LJP = 5.9%, RPP = 4.2%), as well as *Streptococcus constellatus* (AP = 1.3%, LJP = 5.9%, RPP = 0.4%), *Streptococcus milleri* (AP = 1.3%, RPP = 0.4%) and *Streptococcus salivarius* (LJP = 2.9%). In the anaerobic Gram-positive rods group, *Actinomyces naeslundi* was the most frequently detected microorganism (AP = 2.6%, RPP = 2.9%), followed by *Eubacterium* spp. and *Actinomyces meyeri* (Table 3).

## DISCUSSION

Results from this evaluation confirm the complexity of the microbial composition associated with distinct forms of periodontitis, with analysis of the subgingival microbiota having shown 50 different bacterial species. All groups demonstrated a high detection level of anaerobic Gram-negative bacilli; these bacterial morphotypes tend to be associated with diseased states [16].

*Prevotella intermedia* and *Capnocytophaga* spp. were identified in the highest proportions in all three groups. This was in accordance with a study [17] where *Prevotella intermedia* was shown to be the most prevalent species in a group of young adults suffering from periodontitis. Von Troil-Linden et al [18] found that *Prevotella intermedia* was significantly elevated in saliva samples from subjects with advanced periodontitis compared with samples from subjects with initial or no periodontitis. In most articles, no distinction was made between the newly proposed species *Prevotella nigrescens* (formerly *Prevotella intermedia* genotype 2) and *Prevotella intermedia* (in the strict sense). However, as demonstrated by Conrads et al [19], *Prevotella nigrescens* could be considered a marker bacterium for physiologically healthy conditions in plaque as found in nearly all samples from periodontally healthy individuals (children), in contrast to *Prevotella intermedia*, which was not detected. Within *Capnocytophaga* spp., the differentiation between *Capnocytophaga ochracea, Capnocytophaga sputigena and Capnocytophaga gingivalis* could not be carried out. Genco et al [20] named only *Capnocytophaga* species as prominent bacteria in subgingival samples of patients suffering from juvenile periodontitis.

*Porphyromonas gingivalis* is one of the most strongly active organisms associated with adult periodontitis and also seems to play a role in the pathogenesis of other forms of periodontitis [21]. Our findings showed a low prevalence of this periodontopathogen in patients suffering from AP and RPP. These results are in accordance with the study by Riggio et al [22], who detected *Porphyromonas gingivalis* with a prevalence of 11% using conventional culture methods but recorded a recovery prevalence of 24% when using the PCR method. Some other studies [23] have found a higher prevalence of this microorganism, suggesting that the differences reported are due to the differing methods applied.

*Actinobacillus actinomycetemcomitans* and *Eikenella corrodens* were found in patients suffering from AP, LJP and RPP. There is extensive evidence associating *Actinobacillus actinomycetemcomitans* with LJP [24,25], and in some studies *Eikenella corrodens* and *Actinobacillus actinomycetemcomitans* have been found together in some lesions of LJP [26,27].

Streptococci were also present. These are considered beneficial to the host and, when colonizing the pocket in high numbers, may retard the disease process [24]. *Actinomyces naeslundii* and *Actinomyces israelii* were elevated in the development of experimental gingivitis [28,29], but gingival inflammation was considered to be associated with increased Gram-negative rather than with Gram-positive rod species [4].

*Peptostreptococcus micros* is considered to be a pathogen in the etiology of mixed anaerobic infections, including periodontitis [6,30]. It is isolated more often and in increased percentages from patients with periodontitis, especially in disease-active subjects [6,31,32]. Our results are in accordance with previous observations [33–35] suggesting that *Peptostreptococcus micros* might be associated with advanced forms of periodontitis. The periodontopathogens *Bacteroides forsythus* and *Campylobacter rectus* have been associated with progressive lesions in studies of advanced periodontal loss [4,6]. Indeed, the failure to identify these two microorganisms was probably due to the difficulties in cultivating them.

The data of the present investigation suggest that there is great heterogeneity in the subgingival microbiota among subjects classified into different disease groups. Cultural methods for recovery of bacteria are still standard in microbiology. However, as many bacteria in the oral cavity cannot be cultured, it is likely that these still uncharacterized bacteria might play a role in the initiation and progression of periodontal disease.

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