Oral Microbiology Immunology 2004: 19: 314–321
Printed in Denmark. All rights reserved

Oral Microbiology and Immunology

Microbiological profile of early onset/aggressive periodontitis patients

J. J. Kamma[1], M. Nakou[2], R. Gmür[3], P. C. Baehni[4]

[1]Laboratory of Periodontology, Department of Periodontology, [2]Department of Periodontology, School of Dental Medicine, University of Athens, Greece; [3]Institute of Oral Microbiology and General Immunology, University of Zürich, [4]Department of Preventive Dental Medicine, School of Dental Medicine, Faculty of Medicine, University of Geneva, Switzerland

Kamma JJ, Nakou M, Gmür R, Baehni PC. Microbiological profile of early onset/aggressive periodontitis patients.
Oral Microbiol Immunol 2004; 19: 314–321 © Blackwell Munksgaard, 2004.

Objectives: The objectives of this study were to characterize the bacterial profile and to seek possible bacterial associations in the subgingival microbiota of early onset periodontitis/aggressive periodontitis patients by using two different techniques, culture and immunofluorescence.

Material and methods: The study group consisted of 66 systemically healthy individuals with evidence of early onset periodontitis – 41 females and 25 males aged 23–35 years (mean 31.1 ± 3.1 years). Bacterial samples were collected from the deepest site in each quadrant, resulting in a total of 264 sites with a mean probing pocket depth of 6.6 ± 1.5 mm. Samples were cultured anaerobically and in 10% CO_2 using selective and nonselective media, and isolates were characterized to species level. Indirect immunofluorescence using monoclonal antibodies was applied to detect *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia* (*Bacteroides forsythus, Tannerella forsythensis), Prevotella intermedia/Prevotella nigrescens, Campylobacter rectus, Peptostreptococcus micros* and *Actinomyces israelii*.

Results: 93.6% of sampled sites showed bleeding on probing and 23.5% were positive for suppuration. *P. intermedia/P. nigrescens, P. gingivalis*, and *C. rectus* were detected in 77.3–85.9% of samples using culture methods and in 85.6–91.3% using immunofluorescence. *P. micros* and *A. actinomycetemcomitans* were found, respectively, in 63.3% and 25.0% of all sites using culturing and in 58.7% and 27.7% sites using immunofluorescence. Significantly strong positive associations were observed between *T. forsythia* and *C. rectus* (odds ratio 109.46), and *T. forsythia* and *P. gingivalis* (odd ratio 90.26), whereas a negative association was seen between *P. intermedia/P. nigrescens* and *A. actinomycetemcomitans* (odds ratio 0.42). Coinfection by *P. gingivalis*, *T. forsythia*, *P. intermedia/P. nigrescens* and *C. rectus* was observed in 62.1% of the test sites, and in 89.4% of the studied subjects. The sensitivity of immunofluorescence for *T. forsythia, C. rectus, P. intermedia/P. nigrescens* and *P. gingivalis* was found to be very high (0.99–0.94) using culture as the reference detection method. The agreement between culture and immunofluorescence in detecting the presence or absence of the investigated species was 85.2–88.1% for *P. gingivalis, P. intermedia/P. nigrescens, C. rectus*, and *T. forsythia*, 75.9% for *A. actinomycetemcomitans* and 70.4% for *P. micros*.

Conclusions: The microbial profile of the early onset/aggressive periodontitis population was complex. The agreement between the two detection methods was very high.

Key words: aggressive periodontitis; bacterial associations; culture; early onset periodontitis; indirect immunofluorescence; microbiology; periodontal pathogens

Dr. Joanna J. Kamma, 6–8 Freattidos St., GR-185 37 Piraeus, Greece
Tel./fax: +30 210 45 25, 935;
e-mail: j.kamma@periodontology.gr
Accepted for publication May 25, 2004

Early onset periodontitis/aggressive periodontitis is a distinct form of periodontitis characterized by a relatively rapid loss of connective tissue attachment and alveolar bone in apparently healthy young adults. Disease onset may occur at any time between puberty and 35 years of age. Early onset periodontitis has been subdivided into three categories, prepubertal,

juvenile and rapidly progressive periodontitis, based primarily on the age of onset of clinically detectable attachment loss (5, 6, 9, 37, 52).

Over the years, several terms have been used to describe the wide distribution and rapid rate of destruction in otherwise healthy young individuals: 'severe periodontitis in young adults' (45), 'advanced destructive periodontitis' (16), 'postjuvenile periodontitis' (38) 'generalized juvenile periodontitis' (12), and 'rapidly progressive periodontitis' (50). Suzuki (62) described two subtypes of ''rapidly progressive periodontal disease'': type A, affecting young adults and teenagers (14–25 years) and type B, which is found in a slightly older subjects aged 26–35 years. A few years ago, a revised classification of periodontal diseases was introduced by an international workshop in which the term EOP was replaced by generalized aggressive periodontitis (65). It has been noted that there are features of this disease which can occur later in an individual's life, past the previously arbitrarily established age of 35 years (7, 65).

Pathogenic bacteria are the primary etiologic agents in the pathogenesis of early onset/aggressive periodontitis. *Actinobacillus actinomycetemcomitans* is strongly associated with progressing periodontitis in adolescent patients (8, 48, 67), although not in all subject populations (28, 44, 66). Between 21 and 40 years of age, the relative frequency of *A. actinomycetemcomitans*-associated periodontitis seems to decrease, with a concomitant increase in the prevalence of periodontitis cases associated with *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia (Bacteroides forsythus, Tannerella forsythensis), Dialister pneumosintes, Campylobacter rectus, Fusobacterium* species*, Selenomonas sputigena, Peptostreptococcus micros* and spirochetes (3, 13, 14, 30–34, 36, 42, 44, 47). The great majority of aggressive periodontitis involves gram-negative anaerobic rod species (3, 8, 13, 36, 47, 48, 53, 55).

Furthermore, several recent studies have shown the existence of different microbial associations in subgingival plaque (60, 61). Among these, the association of *T. forsythia, P. gingivalis* and *Treponema denticola* seems to be implicated in the progression of periodontal destruction (26, 51). In addition, human cytomegalovirus (HCMV) and Epstein–Barr virus 1 (EBV-1), identified by nested polymerase chain reaction (PCR), were frequently detected in disease-active sites of aggressive periodontitis (30). Slots & Contreras (57) hypothesized that active HCMV and other herpes virus infections may play a role in the initiation and progression of aggressive periodontitis.

Although bacteria and viruses are key etiologic factors in the development of periodontal disease, systemic, local, genetic and environmental susceptibility factors may also play a role.

The objectives of this study were to characterize the bacterial profile and to look for possible bacterial associations in the subgingival microbiota of early onset/aggressive periodontitis patients by blindly using two different methodologies, culture and immunofluorescence. Cultural methods have the advantage of being able to quantitatively detect a wide variety of species. The characterization of all isolates may allow the identification of unexpected or new species. However, taxa that are present in low proportions might be missed unless selective culture techniques are used, but such techniques are often too suppressive. Indirect immunofluorescence assays can bypass many of the restrictions of anaerobic cultures and have a better lower detection limit than nonselective cultures, but they are only applicable to preselected target species for which antibodies of known specificity must be available.

Material and methods

Subjects

The study group consisted of 66 patients with evidence of early onset/aggressive periodontitis, 25 men (age range 26–35 years) and 41 women (age range 23–35 years), with a mean age 31.1 ± 3.1 years. All subjects were selected from a private practice limited to periodontics in Piraeus, Greece.

The following criteria were used to select the patients:

- Otherwise healthy and without a history of systemic disease or pregnancy.
- Not on long-term medical therapy or under antibiotic treatment in the previous 6 months from the day of the first sampling, not using chlorhexidine mouthwash in the previous 4 weeks or any antiseptic mouthwash in the previous few days.
- The disease was untreated, with no history of subgingival debridement prior to referral for treatment.
- Under 35 years old and exhibiting severe periodontal destruction, clinical attachment loss exceeding 5 mm at 2–3 sites in more than 14 permanent teeth (at least three of them not being first molars and incisors), and radiographic evidence of advanced alveolar bone loss (9).
- The sites used in the experimental design were free of root surface caries or any subgingivally placed restorations.

The medical and dental history of each subject was obtained by interview. Smoking was measured by self-report.

Periodontal examination

At the time of enrollment, a complete periodontal examination of each patient was performed. Clinical examination included dichotomous measurements of plaque (49), bleeding upon probing (2) and suppuration (56). Probing pocket depth and attachment level (18) were measured to the nearest 1 mm using a Goldman/Fox Williams periodontal probe at six sites/tooth for all teeth present excluding third molars. The number of teeth present was also recorded. The deepest site with a probing pocket depth of >5 mm was then selected in each quadrant for microbial sampling.

Subgingival plaque samples were obtained 1 week later. For accuracy, clinical parameters were reexamined at the sampling sites immediately following microbial sampling. Statistical analysis was based on the second set of clinical measurements. All clinical measurements and microbial samplings were performed by one investigator (J.K.).

Radiographic measurements

A set of full mouth standardized intraoral radiographs was obtained from each subject. The level of alveolar bone was assessed by the Schei method (54). Measurements were made on the mesial and distal aspect of all teeth present.

Microbiological techniques

Sampling

Subgingival plaque samples were collected using the paper point technique (63) from the deepest pocket of each quadrant. Three paper points were inserted simultaneously into each selected periodontal pocket for 10 s and used for culture analysis. Immediately afterwards, one paper point was inserted into the same pocket for 10 s and used for immunofluorescence analyses. Culture and immunofluorescence analyses were performed blind in two independent laboratories.

Culture

The samples were placed in 0.9 ml of sterile reduced anaerobic transport fluid

(40) and transferred to the laboratory within 10 min. Samples were processed, cultured and quantitatively assessed for a broad range of microorganisms exactly as described in detail by Kamma et al. (31). Samples were dispersed and plated onto nonselective media including Enriched Trypticase Soy Agar supplemented with 4% defibrinated human blood, KVLB-2 (kanamycin 75 μg/ml − vancomycin 2 μg/ml − laked blood) as well as selective media including TSBV agar (tryptic-soy-serum-bacitracin 75 μg/ml − vancomycin 5 μg/ml), agar Wolinella agar (trypticase soy agar, vancomycin 9 μg/ml, ferrous sulfate 0.2 g/l, sodium thiosulfate 0.3 g/l, sodium fumarate 3 g/l, sodium formate 2 g/l), NAM agar (trypticase soy agar, enriched with 10 μg/ml N-acetylmuramic acid), Sabouraud® agar (Difco Laboratories, Detroit, MI) for the isolation of yeasts and MacConkey agar. Plates were incubated anaerobically (5% CO_2 + 10% H_2 + 85% N_2) and aerobically (10% CO_2 plus air) for 4–7 days.

The cloned isolates were characterized and identified to genus and species level, using micromethod kit systems. Identification was based on colony and cellular morphology, gram stain reaction, motility, respiration requirements, biochemical reactions, sugar fermentation pattern, enzymatic activity and additional tests where required.

Immunofluorescence

For immunofluorescence, paper points were immediately placed in 100 μl of 0.9% saline solution and stored frozen until further use. Samples were sent frozen to the Geneva laboratory and processed for indirect immunofluorescence exactly as described by Gmür & Guggenheim (21). Samples were dispersed by vortexing, distributed (6.6 μl/well) on a 15-well PTFE-coated glass slide (Milian, Geneva, Switzerland), air dried and fixed with methanol (2 min).

P. gingivalis, T. forsythia, P. intermedia/Prevotella nigrescens, Campylobacter rectus, P. micros, and *Actinomyces israelii* bacteria were labeled with previously characterized MAbs 61BG1.3 (24), 116BF1.2 (68), 37BI6.1/39BI1.1.2 (20), 212WR2 (68), 61BG1.3 (23), 326 PM2 (23), and 418AI1 (64), respectively. Bacteria of the species *A. actinomycetemcomitans* were labeled with a mixture of MAbs 150AA1.1, 141AA1, 138AA1.1, 330AA3.2, and 348AA4.4, each of which is characterized by specificity to one of the five serotypes a–e (23).

Stained cells were evaluated with a fluorescence microscope using a semi-quantitative 4-step scale:

1. + + + >250 positive cells per viewing field (corresponding to $>3 \times 10^6$ cells/ml of sample);
2. + + 80 cells/well < x > 250 cells/viewing field (between 1×10^4 and 3×10^6 cells/ml);
3. + 3 cells/well < x > 80 cells/well (between 300 and 1×10^4 cells/ml);
4. – less than 3 cells per well.

Thus the detection limit was 45 positive cells per sample. All immunofluorescence analyses were performed by the same person (A.C.).

Data analysis

For cultures, results were computed for each bacterial species by averaging the data per individual and then averaging these values among subjects.

Sensitivity was calculated as the number of positive samples for a given species in both the test (immunofluorescence) and reference (culture) methods divided by the number of positive and negative results in the reference method. Specificity was calculated as the number of negative results in both immunofluorescence and culture tests divided by the sum of negative and positive results with culture. Accuracy was estimated by the sum of true positives (sensitivity) and true negatives (specificity) divided by the total number of results (17).

Associations between pairs of microbial species were examined by evaluating pairs of bacteria for which both detection methods had yielded the same result (presence or absence). Strengths of associations were quantified by computing the odds ratio for the presence of one species in the presence or absence of the other (10).

Odds ratios >2.0 were considered to be indicative of positive associations and odds ratios <1.0 of negative associations.

Where a cell contained a zero count in the contingency table, the odds ratio became infinitive. In these cases we used the amended estimator described by Agresti (1):

$$OR = \frac{a + 0.5xd + 0.5}{b + 0.5xc + 0.5}$$

Results

Clinical data

The clinical parameters are presented in Table 1. Patients had an average of 27.2 teeth. The proportion of diseased sites was rather high, with a mean of 76.1 sites showing a probing pocket depth of >6 mm per subject. The mean probing pocket depth and clinical attachment level of diseased sites was 6.9 and 7.6 mm, respectively. Alveolar bone levels as assessed on radiographs showed a 45% loss. Thirty-three subjects (50%) were smokers with a mean consumption of 32 cigarettes/day.

Cultural data

A total of 24,786 isolates were recovered from the 264 samples included in the study. Of the 17,391 colonies picked, 893 (5.1%) did not yield isolates that survived through to identification. Thus, 16,498 isolates were identified to species level. Thirty-four different bacterial species were isolated from colonies occurring in proportions exceeding 1% of the total colony-forming units (predominant taxa). Other identified bacterial species, which composed less than 1% of the cultivable microbiota, included (in order of decreasing prevalence) *Veillonella atypica, Leptotrichia buccalis, Selenomonas noxia, Propionibacterium propionicus,* and *Bilophila wadsworthia*.

Table 1. Periodontal variables of early onset/aggressive periodontitis patients

Variables	Mean values ± SD
Number of teeth	27.2 ± 2.7
Plaque (%)	72.6 ± 8.3
Plaque (%)/sampling sites	94.3 ± 10.3
Bleeding on probing (%)	81.7 ± 7.4
Bleeding on probing (%)/sampling sites	93.6 ± 9.2
Suppuration (%)	33.2 ± 9.3
Suppuration (%)/sampling sites	23.5 ± 6.8
Mean number of sites with probing depth >6 mm	76.1 ± 4.2
Mean probing pocket depth (mm)/diseased sites*	6.9 ± 1.3
Mean clinical attachment level (mm)/diseased sites	7.6 ± 1.2
Mean probing pocket depth (mm)/sampling sites	6.6 ± 1.5
Mean clinical attachment level (mm)/sampling sites	6.5 ± 2.0
Bone loss (%)	45.0 ± 6.9

*Diseased sites: sites with probing depth >6 mm.

Table 2. Prevalence and relative proportions of bacterial species determined by culture in early onset/aggressive periodontitis patients

Bacterial species	% of sites $n = 264$	% of subjects $n = 66$	% of flora (range)
Most frequently detected bacterial species			
Prevotella intermedia	86	66	17.3 (7.1–23.2)
Tannerella forsythia	83	65	23.8 (8.6–33.8)
Porphyromonas gingivalis	80	59	27.3 (9.5–34.7)
Campylobacter rectus	77	64	13.7 (7.6–23.5)
Fusobacterium nucleatum	63	48	13.2 (5.7–21.9)
Peptostreptococcus micros	63	58	11.3 (2.9–15.3)
Actinomyces israelii	49	48	5.7 (1.3–7.8)
Streptococcus constellatus	47	32	4.2 (1.1–6.3)
Capnocytophaga ochracea	42	39	5.3 (1.8–9.1)
Streptococcus intermedius	39	39	4.9 (1.5–7.2)
Streptococcus oralis	36	23	4.5 (1.3–7.6)
Actinomyces naeslundii	34	29	4.8 (3.2–9.3)
Campylobacter concisus	34	52	9.4 (4.8–15.6)
Streptococcus sanguis	33	31	10.4 (4.3–23.3)
Veillonella parvula	30	29	5.2 (2.3–8.1)
Gemella morbillorum	29	29	4.1 (3.8–8.6)
Selenomonas sputigena	27	39	6.2 (2.7–15.6)
Campylobacter gracilis	26	39	7.5 (3.5–14.1)
Actinobacillus actinomycetemcomitans	25	33	5.9 (2.7–11.9)
Capnocytophaga sputigena	25	22	5.6 (1.7–10.3)
Eikenella corrodens	24	37	4.6 (1.8–8.6)
Least frequently detected bacterial species			
Escherichia coli	20	26	3.6 (1.6–7.2)
Prevotella denticola	19	25	7.6 (4.3–16.1)
Pseudoramibacter alactolyticus	17	21	6.3 (1.9–9.7)
Staphylococcus aureus	16	19	5.6 (3.4–10.2)
Fusobacterium varium	15	22	8.2 (2.7–14.6)
Capnocytophaga gingivalis	13	16	6.2 (4.1–12.7)
Candida albicans	11	13	3.5 (1.5–7.9)
Peptostreptococcus anaerobius	9	19	2.9 (1.9–6.6)
Prevotella loescheii	9	16	5.3 (2.8–14.2)
Aspergillus fumigatus	8	9	5.3 (1.2–9.4)
Eubacterium lentum	8	12	3.9 (1.1–6.5)
Prevotella oralis	4	5	3.9 (1.8–7.2)
Eubacterium aerofaciens	2	3	4.1 (1.2–5.3)

The prevalence and the relative proportion of the predominant bacterial species as identified by culture are reported in Table 2. Results are presented in descending order of detection frequency. *P. intermedia/P. nigrescens, T. forsythia, P. gingivalis, C. rectus, Fusobacterium nucleatum* and *P. micros* were the most frequently isolated taxa. *P. gingivalis, T. forsythia, P. intermedia/P. nigrescens, C. rectus, F. nucleatum, P. micros, Streptococcus sanguis, Campylobacter concisus, Prevotella denticola* and *Campylobacter gracilis* were the predominant species.

Comparison of detection by culture and immunofluorescence

Table 3 shows the agreement between culture and immunofluorescence in detecting the presence or absence of different species. The degree of correspondence between culture and immunofluorescence was high; agreement coefficients were between 85.2 and 88.1 for *P. gingivalis, P. intermedia/P. nigrescens*, *C. rectus,* and *T. forsythia,* and between 70.4 and 75.9 for *A. actinomycetemcomitans*, respectively.

Sensitivity and specificity scores for immunofluorescence assays, using culture as the reference assay, are presented in Table 4. Immunofluorescence sensitivity for detecting *T. forsythia, C. rectus, P. intermedia/P. nigrescens,* and *P. gingivalis* was found to be very high, with values between 0.94 and 0.99. Specificity was excellent for both *T. forsythia* (1.0) and *A. actinomycetemcomitans* (0.96), but much lower for *P. intermedia/P. nigrescens* (0.46). Accuracy was high for *P. gingivalis* (0.93), *A. actinomycetemcomitans* (0.91), and *P. intermedia/P. nigrescens* (0.90). Strong positive associations were found between *T. forsythia* and *C. rectus* and between *T. forsythia* and *P. gingivalis,* whereas a negative association was traced between *P. intermedia/P. nigrescens* and *A. actinomycetemcomitans* (Table 5). Associations between other bacterial species were much weaker.

The concurrent presence of three or more bacterial species was also investigated (Table 6). The trios of *P. gingivalis*, *T. forsythia* and *P. intermedia/P. nigrescens* or *P. gingivalis*, *T. forsythia* and *C. rectus* were found at 171 sites (64.8%). Coinfection with *P. gingivalis*, *T. forsythia*, *P. intermedia/P. nigrescens* and *C. rectus* occurred in 164 sites (62.1%). In contrast, the concurrent presence of *A. actinomycetemcomitans, P. gingivalis,* and *T. forsythia* was detected at only 18.6% of the test sites.

The prevalence of bacterial coinfection within patients is also presented in Table 6. Coinfection by *P. gingivalis*, *T. forsythia* and *P. intermedia/P. nigrescens* in all four samples was found in 21 patients, *P. gingivalis*, *T. forsythia* and *C. rectus* in 18 patients, and *P. gingivalis*, *T. forsythia*, *P. intermedia/P. nigrescens* together with *C. rectus* in 14 patients.

Discussion

Clinical features

The principal objective of this study was to characterize the microbial profile in a homogeneous group of young Greek adults who had been diagnosed with early onset/aggressive periodontitis. The term early onset/aggressive periodontitis has been suggested to describe a group of conditions characterized by rapid perio-

Table 3. Agreement between culture and immunofluorescence

Bacterial species	Frequency of detection with culture	Frequency of detection with immunofluorescence	Agreement between culture/immunofluorescence
P. intermedia/P. nigrescens	85.9	91.3	86.5
T. forsythia	83.3	96.2	85.2
P. gingivalis	80.3	81.4	88.1
C. rectus	77.3	85.6	85.3
P. micros	63.3	58.7	70.4
A. israelii	49.2	51.9	74.5
A. actinomycetemcomitans	25.0	27.7	75.9

Table 4. Sensitivity, specificity, accuracy of immunofluorescence

Bacterial species	Sensitivity*	Specificity*	Accuracy*
P. intermedia/P. nigrescens	0.95	0.46	0.90
T. forsythia	0.99	1.00	0.88
P. gingivalis	0.94	0.68	0.93
C. rectus	0.97	0.80	0.88
P. micros	0.80	0.62	0.76
A. israelii	0.87	0.77	0.83
A. actinomycetemcomitans	0.91	0.96	0.91

*Sensitivity, specificity, accuracy of immunofluorescence calculated with culture as the reference assay.

Table 5. Bacterial associations

Bacterial species	OR*
T. forsythia–C. *rectus*	109.46
T. forsythia–P. gingivalis	90.26
T. forsythia– micros	16.13
P. gingivalis. rectus	9.48
P. micros–C. rectus	5.45
P. intermedia/P. nigrescens–P. micros	4.83
P. intermedia/P. nigrescens–A. actinomycetemcomitans	0.42

*Odds ratio based on the presence or absence of species as determined by both assays (immunofluorescence and culture) using the amended estimator of Agresti (1).

dontal destruction and alveolar bone loss in young adults who are systemically healthy. Although age, according to the recent classification of aggressive periodontitis, is not the major classification criterion, the range of age in our group (23–35 years) is similar to that referred in other research groups studying early onset or aggressive periodontitis (13, 36, 47). With regard to the clinical characteristics, the periodontal destruction was generalized, with a mean probing depth of 6.9 mm among sites with >6 mm pocket depths. On average, 76.1% of the test subjects had pockets with >6 mm probing depth and 88.1% of the sites showed bleeding on probing. Similar figures have been reported by other authors for generalized early onset periodontitis (13, 47).

Prevalence of bacterial species

A total of 34 taxa including *P. gingivalis, P. intermedia/P. nigrescens, T. forsythia, F. nucleatum, C. rectus, C. concisus, S. sputigena,* and *P. micros* were identified by culture in the 66 subjects with early onset/aggressive periodontitis. These bacteria possess pathogenic potential (29, 58) and have been implicated in destructive forms of periodontal disease (15, 26, 27). They are often referred to as indicator microorganisms or key periodontal pathogens. The findings from the present study are in accordance with previous microbiological studies of early onset periodontitis (31, 32, 42, 45–47).

The most frequently detected bacterial entity was *P. intermedia/P. nigrescens* (present in 86% of all sites tested). For comparison, Albandar et al. (3), assessing a group of 148 early onset periodontitis subjects ranging in age from 19 to 25 years, detected *P. intermedia* with DNA probes in 82.4% of all their subjects. Similarly, Kuru et al. (36), studying 15 early onset periodontitis patients (age range 18–30 years), detected *P. intermedia/P. nigrescens* by culture in 80% of all sites. Recently, Darby et al. (13), studying a group of 24 patients with generalized early onset periodontitis, found *P. intermedia* in 79.2% of their subjects. In contrast, in 17 subjects with generalized early onset periodontitis, Mullally et al. (47) found only 58.8% to be *P. intermedia*-positive, using a PCR assay. The lower prevalence of *P. intermedia* in the latter study is probably explained by the fact that the PCR assay can differentiate *P. intermedia* and *P. nigrescens* from each other, in contrast to culture and immunofluorescence methods, which cannot. If one considers that Mullally et al. (47) found *P. nigrescens* in 82.4% of their subjects, the frequency of the combined detection of *P. intermedia/P. nigrescens* in our population is approximately the same.

T. forsythia was the second most frequently isolated species in our study, with a test site prevalence of 83% and a subject prevalence of 98.5%. This finding is very close to the 91.7% and 82.4% prevalence reported for the generalized early onset periodontitis populations investigated by Darby et al. (13) and Mullally et al. (47), respectively. The age distribution in both those studies was very similar to that of our study population (mean age 33.2 years and 30.3 years, respectively, compared to 31.1 years in our group).

P. gingivalis was also frequently detected in our early onset/aggressive periodontitis group (80% of the sites, 89.4% of the subjects). Again, this result is in good agreement with the 82.4% subject prevalence reported by Albandar et al. (3) for a similar study group, and with the 93.3% prevalence at all tests sites described by Kuru et al. (36). However, the present results contrast sharply with the 17.7% prevalence reported by Mullally et al. (47). The reason for this discrepancy is not known, but may be related to methodologic differences.

C. rectus was isolated in 96.9% of the patients and in 77% of the sites. This is comparable to 92.9% reported by Mullally et al. (47) and higher than the 71% score reported for the population assessed by Albandar et al. (3). It is also close to the prevalence at all test sites (63.3%) of the Kuru et al. (36) study.

Another frequently isolated bacterial species, *F. nucleatum*, was detected in 63% of sites in 72.7% (48/66) of the population. This prevalence is very close to that of Kuru et al. (36) who detected the

Table 6. Prevalence of bacterial coinfection within early onset/aggressive periodontitis patients

Bacterial species*	Patients $n = 66$	Total sites $n = 264$
Pg, Tf, Pi/Pn	59 (89.4%)	171 (64.8%)
4 sites	21	84
3 sites	13	39
2 sites	23	46
1 site	2	2
Pg, Tf, Cr	59 (89.4%)	171 (64.8%)
4 sites	18	72
3 sites	22	66
2 sites	14	28
1 site	5	5
Pg, Tf, Pi/Pn, Cr	59 (89.4%)	164 (62.1%)
4 sites	14	56
3 sites	21	63
2 sites	21	42
1 site	3	3
Pg, Tf, Pm	58 (87.9%)	114 (43.2%)
4 sites	12	48
3 sites	6	18
2 sites	8	16
1 site	32	32
Pg, Tf, Pi/Pn, Pm	58 (87.9%)	110 (41.71%)
4 sites	10	40
3 sites	5	15
2 sites	12	24
1 site	31	31
Aa, Pg, Tf	33 (50.0%)	49 (18.6%)
4 sites	3	12
3 sites	3	9
2 sites	1	2
1 site	26	26
More than 5 species	30 (45.5%)	82 (31.1%)
4 sites	13	52
3 sites	5	15
2 sites	3	6
1 site	9	9

Pg: *P. gingivalis.* Tf: *T. forsythia.* Pm: *P. micros.* Pi/Pn: *P. intermedia/P. nigrescens.* Aa: *A. actinomycetemcomitans.*

species in 76.7% of the sites of their early onset periodontitis population. In Albandar et al.'s study (3) 95.3% of the early onset periodontitis patients studied were positive for *F. nucleatum*.

In the present study population, a relatively low prevalence of *A. actinomycetemcomitans* was detected (25% of our observations) and in very low levels, constituting 5.9% of the total cultivable flora. This is in accordance with findings of van der Velden et al. (66) and Han et al. (28). The present data support the view that *A. actinomycetemcomitans* is a significant pathogen which is less prevalent in older early onset/aggressive periodontitis subjects.

A general comment can be made concerning the differences in the detection levels seen using the various methods (PCR, immunofluorescence, culture). It is very important to take into consideration the detectability level of each method when evaluating and comparing results from different studies. As noted, immunofluorescence identifies more *P. intermedia*-positive samples than DNA probes, and detection of *P. gingivalis, A. actinomycetemcomitans* compared to *F. nucleatum* differs greatly on blood agar. Therefore, the reported prevalence of the bacterial species in the studies is only an indication, as it may be affected by many parameters such as the methods used.

Comparison between culture and immunofluorescence techniques

Immunofluorescence resulted in elevated prevalence values compared with culture for all tested bacteria (Tables 3 and 4). Most likely this is related to the lower detection limit of the former technique, which has been reported to be between 200 and 4000 cells depending on the overall cell density of the test samples. These numbers correspond to density levels of between 0.001% and 0.06% of the total bacterial cell number (22).

The resolution of culture analyses is limited principally by three factors: the assessable portion of the sample, the maximum assessable colony density on a plate (which usually forces the investigator to work with appropriate sample dilutions) and the maximum number of colony selections that can be accepted for speciation. In this study one of 250 colonies per plate was recognized and speciated, yielding a detection limit of 0.4% of the total colony-forming units (predominant taxa). For immunofluorescence, the minimum detection limit was three positive cells per 6.66 μl of the original sample suspension, or 45 cells per sample.

Comparative analyses of subgingival plaque samples with culture and indirect immunofluorescence clearly document the usefulness of the fluorescence approach. Loesche et al. (41) compared a number of detection methods for periodontopathic bacteria in subgingival plaque samples. The authors employed culture, microscopic methods, DNA probes, ELISA and indirect immunofluorescence and defined as a true positive result consensus of at least three out of the four diagnostic procedures tested. In comparison with this 'gold standard', they demonstrated that the serial dilution anaerobic culture procedure was the poorest detection method.

Bonta et al. (11) detected the same prevalence of *A. actinomycetemcomitans* using immunofluorescence and culture techniques. In our study there was a 75.9% agreement between the two techniques for *A. actinomycetemcomitans*. In a juvenile periodontitis and rapidly progressive periodontitis population, Listgarten et al. (39) detected *A. actinomycetemcomitans* in 39.4% of the samples by culture and in 81.8% of the samples by immunofluorescence. The sensitivity of the method was 0.4 and the specificity 1.0. Slots et al. (59) reported a 27% prevalence of *A. actinomycetemcomitans* using culture and a 34% prevalence using immunofluorescence; the agreement between the two techniques was 51.5%. The sensitivity of immunofluorescence for detecting *A. actinomycetemcomitans* was 77% and the specificity 81%.

Agreement for the detection of *P. gingivalis* was very high at 88.1%. This result is closer to the study of Slots et al. (59) who found an 80.5% agreement between culture and immunofluorescence for *P. gingivalis* detection. The sensitivity of immunofluorescence for detecting *P. gingivalis* was 91% and the specificity 80%. In contrast, Zambon et al. (69) found *P. gingivalis* twice as frequently with immunofluorescence than with culture. Gmür (19) reported that culture consistently yielded lower numbers of *P. gingivalis* cells than did immunofluorescence. For *P. intermedia/P. nigrescens* the agreement was also high at 86.5%, which is in accordance with the findings of Gmür (19) who detected a remarkably similar number of cells by both techniques. The agreement for *T. forsythia* in the present study was 85.2%, whereas Gmür (19) found a greater discrepancy because culture failed to isolate it.

The high degree of agreement between immunofluorescence and culture techniques, with the high accuracy for all the bacterial species, ranging from 0.93 *P. gingivalis* to 0.76 *P. micros,* in combination with the ease of the procedure**,** are advantages of immunofluorescence. However, it is important to bear in mind the major advantage of the culture techniques in microbiological studies, that is, their capability to detect unexpected or new species.

Ecology and coinfection

Most microbial studies have been focused on individual bacterial species. However, subgingival biofilm is composed of microcolonies of bacterial cells. The association of different bacterial species within mixed biofilms is not random. The associations between specific species may be due to the nature of the available surfaces for colonization, to synergistic or antagonistic relationships between species, and to nutrient availability. These characteristics define the structure and properties of the biofilm.

Bacterial associations and complexes have been previously studied in the subgingival microbiota of adult periodontitis patients by Socransky et al. (60). *In vitro* biofilm models have shown cell-to-cell recognition, reporting each strain to have a defined set of coaggregation partners (35). One objective of the study was to examine possible associations in early onset/aggressive periodontitis between the identified bacterial species as assessed by both culture and immunofluorescence techniques. Our results indicate strong positive associations and the concurrent presence of the bacterial species *T. forsythia, C. rectus*, *P. gingivalis, P. micros,* and *P. intermedia/P. nigrescens.* These results in early onset/aggressive periodontitis subjects are in agreement with previous observations in adult periodontitis (60). In that study, Socransky et al. (60) described a tightly related complex in plaque, the so-called red complex, consisting of *T. forsythia, P. gingivalis,* and *T. denticola*. This community was most closely related to the orange complex, consisting of *Fusobacterium, Prevotella, Campylobacter* sp., *P. micros* and *Streptococcus constellatus*.

The relationship of *P. gingivalis* with *T. forsythia* and *T. denticola* has been recognized for some time. Gmür et al. (22) reported that *P. gingivalis* and *T. forsythia* were both detected in higher numbers in deeper periodontal pockets and that *P. gingivalis* was never detected in the

absence of *T. forsythia.* The same interesting observation was reported by Lotufo et al. (43). In a previous study done by our research group in patients with rapidly progressive periodontitis, we isolated these species together. *P. gingivalis* and *T. forsythia* are also risk indicators for loss of attachment (25). The two microorganisms may provide growth factors or attachment opportunities for each other or may thrive in the ecologic system associated with a periodontal inflammatory process.

A negative association was observed for *P. intermedia/P. nigrescens* and *A. actinomycetemcomitans* in the microbial community. This finding is in agreement with the observations of Mullally et al. (47) who detected *A. actinomycetemcomitans* in the presence of *P. gingivalis* but never in the presence of *P. intermedia/P. nigrescens* in the subgingival plaque of generalized early onset periodontitis.

P. gingivalis F. nucleatum, T. forsythia, and *P. intermedia/P. nigrescens* (4) showed a strong positive association in untreated adult periodontitis. Of particular interest was that the combination of *P. gingivalis, T. forsythia, P. intermedia/ P. nigrescens* and *C. rectus* was found in 62.1% of the sites and in all four sampled sites in 89.4% of the early onset/aggressive periodontitis patients. These bacterial species, by occurring in a mixed species biofilm, exchange genetic information, encourage the growth of pathogenic species and discourage the growth of beneficial ones, thereby determining the structure of the community.

The microbial profile of early onset/ aggressive periodontitis patients in this study included suspected or known pathogenic bacterial species, supporting earlier findings that the disease process in early onset/aggressive periodontitis is related to a complex microbiota. Furthermore, these results indicated the presence of specific bacterial associations between pathogenic species which may be of significance in the progression of disease in early onset/ aggressive periodontitis.

Acknowledgments

The authors wish to thank Anne Carrel and Efi Kontou for excellent technical assistance and Prof. P. Dellaportas for statistical analysis and helpful discussion.

References

1. Agresti A. Categorical data analysis. New York: John Wiley & Sons, 1990: 54.
2. Ainamo J, Bay I. Problems and proposals for recording gingivitis and plaque. Int Dent J 1975: **25**: 229–235.
3. Albandar JM, Brown LJ, Löe H. Putative periodontal pathogens in subgingival plaque of young adults with and without early-onset periodontitis. J Periodontol 1997: **68**: 973–981.
4. Ali RW, Skaug N, Nilsen R, Bakken V. Microbial associations of 4 putative periodontal pathogens in Sudanese adult periodontitis patients determined by DNA probes analysis. J Periodontol 1994: **65**: 1053–1057.
5. American Academy of Periodontology. Glossary of periodontal terms, 3rd edn. Chicago, Illinois: American Academy of Periodontology, 1992.
6. American Academy of Periodontology. Proceedings of the World Workshop in Clinical Periodontics, I/23-I/24. Chicago: American Association of Periodontology, 1989.
7. Armitage GC. Periodontal diagnoses and classification of periodontal diseases. Periodontol 2000 2004: **34**: 9–21.
8. Asikainen S. Occurrence of *Actinobacillus actinomycetemcomitans* and spirochetes in relation to age in localized juvenile periodontitis. J Periodontol 1986: **57**: 537–541.
9. Astemborski JA, Boughman JA, Myrick PO, Goodman SB, Wooten RK, Agarwal S, et al. Clinical and laboratory characterization of early onset periodontitis. J Periodontol 1989: **60**: 557–563.
10. Berry G, Armitage P. Statistical methods in medical research. Oxford: Blackwell Science, 1994.
11. Bonta Y, Zambon JJ, Genco R, Neiders ME. Rapid identification of periodontal pathogens in subgingival plaque. Comparison of indirect immunofluorescence microscopy with bacterial culture for detection of *Actinobacillus actinomycetemcomitans*. J Dent Res 1985: **64**: 793–798.
12. Burmeister JA, Best AM, Palcanis KG, Caine FA, Ranney RR. Localized juvenile periodontitis and generalized severe periodontitis: clinical findings. J Clin Periodontol 1984: **11**: 181–192.
13. Darby IB, Hodge PJ, Riggio MP, Kinane DF. Microbial comparison of smoker and non-smoker adult and early-onset periodontitis patients by polymerase chain reaction. J Clin Periodontol 2000: **27**: 417–424.
14. Duffau F, Bolivar I, Baehni PC. Presence of periodontal pathogens in a lifetime. Perio 2004: **1**: 67–73.
15. Dzink JL, Socransky SS, Haffajee AD. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J Clin Periodontol 1988: **15**: 316–323.
16. Ebersole JL, Taubman MA, Smith DJ, Frey DE. Human immune responses to oral microorganisms: patterns of systemic antibody levels to *Bacteroides* species. Infect Immun 1986: **51**: 507–513.
17. Gladen B. The use of jack-knife to estimate proportions from toxicological data in the presence of litter effects. J Am Stat Assoc 1979: **74**: 278–282.
18. Glavind L, Löe H. Errors in the clinical assessment of periodontal destruction. J Periodontal Res 1967: **2**: 180–184.
19. Gmür R. Applicability of monoclonal antibodies to quantitatively monitor subgingival plaque for specific bacteria. Oral Microbiol Immunol 1988: **3**: 187–191.
20. Gmür R, Guggenheim B. Antigenic heterogeneity of *Bacteroides intermedius* as recognized by monoclonal antibodies. Infect Immun 1983: **42**: 459–470.
21. Gmür R, Guggenheim B. Interdental supragingival plaque – a natural habitat of *Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus*, and *Prevotella nigrescens*. J Dent Res 1994: **73**: 1421–1428.
22. Gmür R, Strub JR, Guggenheim B. Prevalence of *Bacteroides forsythus* and *Bacteroides gingivalis* in subgingival plaque of prosthodontically treated patients on short recall. J Periodontal Res 1989: **24**: 113–120.
23. Gmür R, Thurnheer T. Monoclonal antibodies for the rapid identification in clinical samples of *Peptostreptococcus micros* and *Actinobacillus actinomycetemcomitans* serotypes a, d, and e. Med Microbiol Lett 1996: **5**: 335–349.
24. Gmür R, Werner-Felmayer G, Guggenheim B. Production and characterization of monoclonal antibodies specific for *Bacteroides gingivalis*. Oral Microbiol Immunol 1988: **3**: 181–186.
25. Grossi SG, Zambon JL, Hu AW, Koch G, Dunford RG, Machtei EE, et al. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. J Periodontol 1994: **65**: 260–267.
26. Haffajee AD, Cugini MA, Dibart S, Smith C, Kent RL Jr, Socransky SS. Clinical and microbiological features of subjects with adult periodontitis who responded poorly to scaling and root planing. J Clin Periodontol 1997: **24**: 767–776.
27. Haffajee AD, Cugini MA, Tanner A, Pollack RP, Smith C, Kent RL Jr, Socransky SS. Subgingival microbiota in healthy, well-maintained elder and periodontitis patients. J Clin Periodontol 1998: **25**: 346–353.
28. Han N, Xiao X, Zhang L, Ri X, Zhang J, Tong Y, et al. Bacteriological study of juvenile periodontitis in China. J Periodontal Res 1991: **26**: 409–414.
29. Holt SC, Bramanti TE. Factors in virulence expression and their role in periodontal disease pathogenesis. Crit Rev Oral Biol Med 1991: **2**: 177–281.
30. Kamma JJ, Contreras A, Slots J. Herpesviruses and periodontopathic bacteria in early-onset periodontitis. J Clin Periodontol 2001: **28**: 879–885.
31. Kamma JJ, Nakou M, Baehni PC. Clinical and microbiological characteristics of smokers with early onset periodontitis. J Periodontal Res 1999: **34**: 25–33.
32. Kamma JJ, Nakou M, Manti FA. Predominant microflora of severe moderate and minimal periodontal lesions in young adults with rapidly progressive periodontitis. J Periodontal Res 1995: **30**: 66–72.
33. Kamma JJ, Nakou M, Mitsis F. The clinical and microbiological effects of systemic ornidazole in sites with and without subgingival debridement in early onset perio-

dontitis patients. J Periodontol 2000: **71**: 1862–1873.
34. Kamma JJ, Nakou M, Persson RG. Association of early onset periodontitis microbiota with aspartate aminotransferase activity in gingival crevicular fluid. J Clin Periodontol 2001: **28**: 1096–1105.
35. Kolenbrander PE, Parrish KD, Andersen RN, Greenberg EP. Intergeneric coaggregation of oral Treponema spp. with Fusobacterium spp. & intrageneric coaggregation among Fusobacterium spp. Infect Immun 1995: **63**: 4584–4588.
36. Kuru B, Yilmaz S, Noyan U, Acar O, Kadir T. Microbiological features and crevicular fluid aspartate aminotransferase enzyme activity in early onset periodontitis patients. J Clin Periodontol 1999: **26**: 19–25.
37. Lang NP, Karring T. Proceedings of the 1st European Workshop on Periodontology. London: Quintessence Publishing Co., 1994.
38. Liljenberg B, Lindhe J. Juvenile periodontitis. Some microbiological, histopathological and clinical characteristics. J Clin Periodontol 1980: **7**: 48–61.
39. Listgarten MA, Wong MY, Lai CH. Detection of *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis* and *Bacteroides forsythus* in an *A. actinomycetemcomitans*-positive patient population. J Periodontol 1995: **66**: 158–164.
40. Loesche WJ, Hockett RM, Syed SA. The predominant cultivable flora of tooth surface plaque removal from institutionalised subjects. Arch Oral Biol 1972: **17**: 1311–1325.
41. Loesche WJ, Lopatin DE, Stoll J, van Poperin N, Hujoel PP. Comparison of various detection methods for periodontopathic bacteria: Can culture be considered the primary reference standard? J Clin Microbiol 1992: **30**: 418–426.
42. Loesche WJ, Syed SA, Schmidt E, Morrison EC. Bacterial profiles of subgingival plaques in periodontitis. J Periodontol 1985: **56**: 447–456.
43. Lotufo RFM, Flynn J, Chen C, Slots J. Molecular detection of *Bacteroides forsythus* in human periodontitis. Oral Microbiol Immunol 1994: **9**: 154–160.
44. Michalowicz BS, Ronderos M, Camara-Silva R, Contreras A, Slots J. Human herpesviruses and *Porphyromonas gingivalis* are associated with juvenile periodontitis. J Periodontol 2000: **71**: 981–988.
45. Moore WEC, Holdeman LV, Smibert RM, Hash DE, Burmeister JA, Ranney RR. Bacteriology of severe periodontitis in young adult humans. Infect Immun 1982: **38**: 1137–1148.
46. Moore WEC, Moore LVH. The bacteria of periodontal diseases. Periodontol 2000 1994: **5**: 66–78.
47. Mullally BH, Dace B, Shelburne CE, Wolff LF, Coulter WA. Prevalence of periodontal pathogens in localized and generalized forms of early onset periodontitis. J Periodontal Res 2000: **35**: 232–241.
48. Müller HP, Flores de Jacoby L. The composition of the subgingival microflora of young adults suffering from juvenile periodontitis. J Clin Periodontol 1985: **12**: 113–123.
49. O'Leary TJ, Drake RB, Naylor JE. The plaque control record. J Periodontol 1972: **43**: 38.
50. Page RC, Altman LC, Ebersole JL, Vandesteen GE, Dahlberg WH, Williams BL, et al. Rapidly progressive periodontitis. A distinct clinical condition. J Periodontol 1983: **54**: 197–209.
51. Papapanou PN, Baelum V, Luan WM, Madianos PN, Chen X, Fejerskov O, et al. Subgingival microbiota in adult Chinese: prevalence and relation to periodontal disease progression. J Periodontol 1997: **68**: 651–666.
52. Ranney R. Classification of periodontal diseases. Periodontol 2000 1993: **2**: 13–25.
53. Rodenburg JP, van Winkelhoff AJ, Winkel EG, Goene RJ, Abbas F, de Graaff J. Occurrence of *Bacteroides gingivalis, Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in severe periodontitis in relation to age and treatment history. J Clin Periodontol 1990: **17**: 392–399.
54. Schei O, Waerhaug J, Lovdal A, Arno A. Alveolar bone loss as related to oral hygiene and age. J Periodontol 1959: **30**: 7–16.
55. Schenkein HA, Van Dyke TE. Early onset periodontitis: systemic aspects of etiology and pathogenesis. Periodontol 2000 1994: **6**: 7–25.
56. Singh S, Cianciola L, Genco RJ. The suppurative index: An indicator of active periodontal disease. J Dent Res 1977: **56**: 200 (Abstr 593).
57. Slots J, Contreras A. Herpesviruses: a unifying causative factor in periodontitis? Oral Microbiol Immunol 2000: **15**: 277–280.
58. Slots J, Genco RJ. Microbial pathogenicity. Black-pigmented *Bacteroides* species, *Capnocytophaga* species and *Actinobacillus actinomycetemcomitans* in human periodontal disease: Virulence factors in colonization survival and tissue destruction. J Dent Res 1984: **63**: 412–421.
59. Slots J, Hafström C, Rosling B, Dahlén G. Detection of *Actinobacillus actinomycetemcomitans* and *Bacteroides gingivalis* in subgingival smears by the indirect fluorescent antibody technique. J Periodontal Res 1985: **20**: 613–620.
60. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998: **25**: 134–144.
61. Socransky SS, Haffajee AD, Dzink JL, Hillman JD. Associations between microbial species in subgingival plaque samples. Oral Microbiol Immunol 1988: **3**: 1–7.
62. Suzuki JB. Diagnosis and classification of the periodontal diseases. DCNA 1988: **32**: 195–216.
63. Tanner ACR, Goodson JM. Sampling of microorganisms associated with periodontal disease. Oral Microbiol Immunol 1986: **1**: 15–20.
64. Thurnheer T, Guggenheim B, Gmür R. Characterization of monoclonal antibodies for rapid identification of *Actinomyces naeslundii* in clinical samples. FEMS Microbiol Lett 1997: **150**: 255–262.
65. Tonetti M, Mombelli A. Early onset periodontitis. International Workshop for a classification of Periodontal Diseases, Oak Brook, Illinois. Ann Periodontol 1999: **4**: 39–52.
66. Van der Velden U, Abbas F, Van Steenbergen TJ, De Zoete OJ, Hesse M, De Ruyter C, et al. Prevalence of periodontal breakdown in adolescents and presence of *Actinobacillus actinomycetemcomitans* in subjects with attachment loss. J Periodontol 1989: **60**: 604–610.
67. van Winkelhoff AJ, Tijhof CJ, deGraaff J. Microbiological and clinical results of metronidazole plus amoxicillin therapy in *Actinobacillus actinomycetemcomitans*-associated periodontitis. J Periodontol 1992: **63**: 52–57.
68. Werner-Felmayer G, Guggenheim B, Gmür R. Production and characterization of monoclonal antibodies against *Bacteroides forsythus* and *Wolinella recta*. J Dent Res 1988: **67**: 548–553.
69. Zambon JJ, Reynolds HS, Chen P, Genco RJ. Rapid identification of periodontal pathogens in subgingival dental plaque. Comparison of indirect immunofluorescence microscopy with bacterial culture for detection of *Bacteroides gingivalis* J Periodontol 1985: **56** (Suppl.): 32–40.